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PhD Thesis

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Investigations into the use of human embryonal carcinoma stem cells as a model to study dopaminergic neurogenesis

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Abstract:

Parkinson's disease in reality arises as a result of a complex series of events, however it is strongly linked to the loss of a specific cellular population of midbrain dopaminergic neurons making it a candidate for stem cell based research. Stem cells can be cultured *in vitro* and via asymmetric cell division possess the capacity for both self renewal and the production of differentiated derivatives. The use of specific molecules and culture conditions can be applied to promote the differentiation of them towards particular cellular fates, in turn facilitating the possibility of producing enriched populations of cells displaying characteristics of a certain phenotype of interest.

There has been much research focussed on the *in vitro* generation of dopaminergic neurons from various stem cell types. In this work the Tera2.cl.SP12 embryonal carcinoma stem cell line was the primary vehicle investigated for its ability to produce cells that were reflective of a dopaminergic phenotype. Retinoic acid was found to be able to up regulate the expression of a range of dopaminergic markers in the Tera2.cl.SP12 cell line over time. However it was clear that lowered oxygen culture, a method known to promote the production of neurons reflective of a dopaminergic phenotype in mesencephalic cultures, had no effect on the dopaminergic differentiation capacity of the embryonal carcinoma stem cells.

The glycoprotein Wnt1 when applied to Tera2.cl.SP12 cultures in concert with retinoic acid was shown to increase the number and percentage of cells positive for the neuronal marker Beta III tubulin approximately 1.5 fold. This was accompanied by a concomitant rise in the mRNA expression of this marker, thus suggesting that the use of Wnt1 may be a means to produce cultures derived from embryonal carcinoma cells that are more neuronal, based on marker expression data. Other established methods to achieve dopaminergic differentiation such as suspension culture, stromal cell co-culture and the application of Sonic hedgehog and Fibroblast growth factor 8 are also able to induce a degree of neuronal and dopaminergic marker expression in Tera2.cl.SP12 cultures. Overall these results suggest that the Tera2.cl.SP12 cell line might be one vehicle for the study of dopaminergic neurogenesis *in vitro*, in particular when Wnt1 and retinoic acid are used as a means to favourably enrich the population of cells displaying neuronal characteristics.

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On a personal level I would also like to thank my late grandparents for nurturing my mother into a truly rounded individual who has provided me with monumental support from day one and who has given me every opportunity and chance to succeed in life, it is safe to say because of your love and care, 'I won't die wondering,' and for this I am infinitely grateful. I must also give mention to Red House School, Norton, and to those other family members and close friends who are in the main too numerous to acknowledge individually, with the exception of Mike Cooke, Steve Hardy, and Gareth Hinchliffe with whom I have shared much laughter and many memorable moments.

Declaration:

I declare that the work in this thesis is, except where acknowledged as being carried out in collaboration, entirely my own. None of the material has been used in any other documentation submitted for award of an alternative degree. This thesis has been amended from its original form as per the requirements of my examiners.

Table of Contents:

Section Title	Page Number
Abstract	ii
Acknowledgements	iii
Declaration	iii
Table of Contents	iv
List of Tables	vi
List of Figures	vii
Abbreviations used in this Thesis	xi
Chapter 1 Literature Review	1
• 1.1 Introduction	2
• 1.2 Overview of Parkinson's disease	3
• 1.3 Stem cells as a tool to study neurogenesis	8
• 1.4 In vitro methods to generate dopaminergic neurons	12
Chapter 2 Materials and Methods	52
• 2.1 Cell Culture	53
• 2.2 Intracellular staining for flow cytometry	55
• 2.3 Cell surface antigen detection flow cytometry	57
• 2.4 Immunocytochemistry	59
• 2.5 Western Blotting Protocol	60
• 2.6 MTS assay	63
• 2.7 RNA isolation using Tri-Reagent®	64
• 2.8 Reverse transcription and real time polymerase chain reaction	66
• 2.9 Real time polymerase chain reaction (Lilly)	71
• 2.10 Statistical analysis	72
Chapter 3 Characterisation of the Tera2.cl.SP12 human EC cell system as a model to study dopaminergic neurogenesis, in the presence of retinoic acid and in a lowered oxygen culture environment	76
• 3.1 Introduction	77
• 3.2 Cell culture methods	82
• 3.3 Results	83
• 3.4 Discussion	112
Chapter 4 The effects of Wnt1 on the dopaminergic differentiation of the Tera2.cl.SP12 cell line	117
• 4.1 Introduction	118
• 4.2 Cell culture methods	125
• 4.3 Results	126
• 4.4 Discussion	168
Chapter 5 The effects of suspension culture, Sonic hedgehog, Fibroblast growth factor 8 and ascorbic acid, on the acquisition of a dopaminergic phenotype by human embryonal carcinoma stem cells	175

<ul style="list-style-type: none"> • 5.1 Introduction • 5.2 Cell culture methods • 5.3 Results • 5.4 Discussion 	176 180 184 221
Chapter 6 The use of a mesodermal cell co-culture method to make a comparison of the dopaminergic differentiation potential of the human EC cell systems Tera2.cl.SP12 and Ntera2.D1	227
<ul style="list-style-type: none"> • 6.1 Introduction • 6.2 Cell culture methods • 6.3 Results • 6.4 Discussion 	228 232 236 262
Chapter 7 Final Discussion	267
Summary of Key Findings	274
Bibliography	276
Appendices <ul style="list-style-type: none"> • Appendix A • Appendix B • Appendix C • Appendix D • Appendix E 	293 294 298 299 300

List of Tables:

Table Number	Page Number
Table 2.1: Primary antibody dilutions for flow cytometry	56
Table 2.2: Secondary antibody dilutions for flow cytometry	56
Table 2.3: Primary antibody dilutions for live cell flow cytometry	58
Table 2.4: Secondary antibody dilutions for live cell flow cytometry	58
Table 2.5: Primary antibody dilutions for Western Blotting	62
Table 2.6: Primer Sequences	69
Table 6.1: Quantification of cDNA	243

List of Figures:

Figure Number	Page Number
Figure 1.1: Potential Interactions of α -Synuclein with environmental toxins	4
Figure 1.2: Stem cells from which neurons may be derived	9
Figure 1.3: Dopaminergic development in the mouse embryo	14
Figure 1.4: The temporal effects of Shh and FGF8 on the acquisition of a dopaminergic phenotype	18
Figure 1.5: A summary of the elements of the stromal derived inducing activity	25
Figure 1.6: The roles of Wnt family members in specifying a dopaminergic fate	32
Figure 1.7: The variation in oxygen pressure within a spherical cellular aggregate	35
Figure 1.8: Factors and Signals involved in the specification of a dopaminergic fate	42
Figure 2.1: Characteristic EC cell morphology	54
Figure 2.2: The different passages of Tera2.cl.SP12 cells used in this project	54
Figure 2.3: Dissociation Curve and Agarose gel image for GAPDH	67
Figure 2.4 Example T-test output	73
Figure 2.5: Example one way anova output	74
Figure 2.6: Example two way anova output	75
Figure 3.1: Characterisation of stem cell differentiation	84
Figure 3.2: Nurr1 mRNA Expression in Tera2.cl.SP12 cells and their derivatives	86
Figure 3.3: Otx2 mRNA Expression in Tera2.cl.SP12 cells and their derivatives	88
Figure 3.4: Sox1 mRNA Expression in Tera2.cl.SP12 cells and their derivatives	89
Figure 3.5: The effect of oxygen pressure and duration of R.A. exposure on the expression of tyrosine hydroxylase mRNA in Tera2.cl.SP12 cultures	91
Figure 3.6: The effect of oxygen pressure and duration of R.A. exposure on the relative expression of D2 mRNA in Tera2.cl.SP12 cells	92
Figure 3.7: Morphological Images	93
Figure 3.8: Nurr1 protein expression in Tera2.cl.SP12 cells and their derivatives	96
Figure 3.9: TH protein expression in Tera2.cl.SP12 cells and their derivatives	98
Figure 3.10: Tuj1 protein expression in Tera2.cl.SP12 cells and their derivatives	100

Figure 3.11: Summary mean fluorescence intensity flow cytometry data	101
Figure 3.12: The effect of oxygen on TH protein expression in Tera2.cl.SP12 cells	103
Figure 3.13: The effect of oxygen on Tuj1 protein expression in Tera2.cl.SP12 cells	104
Figure 3.14: Assessment of TH expression via Western Blotting	105
Figure 3.15: Assessment of Nurr1 expression via Western Blotting	106
Figure 3.16: Immunostaining of Sub-Confluent Undifferentiated Tera2.cl.SP12 EC cell cultures	109
Figure 3.17: Immunostaining of Confluent Undifferentiated Tera2.cl.SP12 EC cell cultures	110
Figure 3.18: Immunostaining of 4 week R.A. differentiated EC cells	111
Figure 3.19: Midbrain dopaminergic neuron development	113
Figure 3.20: Investigating the potential effects of Wnt1 on the dopaminergic differentiation potential of Tera2.cl.SP12 human EC stem cells	116
Figure 4.1: Wnt Signalling Pathways	119
Figure 4.2: Possible Signalling Pathways Implicated in Dopaminergic Neurogenesis	121
Figure 4.3: Factors & Signals Involved in The Specification of a Dopaminergic Neuronal Fate from Neuroepithelial Stem Cells	122
Figure 4.4: Testing for an effect due to the exogenously applied Wnt1	127
Figure 4.5: Proliferation of Tera2.cl.SP12 cultures treated with R.A. and Wnt1	129
Figure 4.6: The effects of Wnt1 and R.A. on Frizzled 8 mRNA expression	131
Figure 4.7: The effects of Wnt1 and R.A. on Beta-catenin mRNA expression	132
Figure 4.8: The effects of Wnt1 and R.A. on Nurr1 mRNA expression	134
Figure 4.9: Nurr1 protein expression in EC cells treated with Wnt1 and R.A.	135
Figure 4.10: The effects of Wnt1 and R.A. on Otx2 mRNA expression	138
Figure 4.11: The effects of Wnt1 and R.A. on Lmx1a mRNA expression	139
Figure 4.12: The effects of Wnt1 and R.A. on Msx1 mRNA expression	140
Figure 4.13: The effects of Wnt1 and R.A. on Ngn2 mRNA expression	140
Figure 4.14: The effects of Wnt1 and R.A. on Nkx 6-1 mRNA expression	141
Figure 4.15: The effects of Wnt1 and R.A. on Beta III Tubulin mRNA expression	143
Figure 4.16: The effects of Wnt1 and R.A. on TH MRNA expression	144
Figure 4.17: The effects of Wnt1 and R.A. on AADC mRNA expression	145
Figure 4.18: The effects of Wnt1 and R.A. on DAT mRNA expression	147
Figure 4.19: The effects of Wnt1 and R.A. on D1 mRNA expression	148

Figure 4.20: The effects of Wnt1 and R.A. on D2 mRNA expression	149
Figure 4.21: Tuj1 staining of Tera2.cl.SP12 cultures treated with R.A., Wnt1 or a combination of both reagents for 7 days	152
Figure 4.22: Tuj1 staining of Tera2.cl.SP12 cultures treated with R.A., Wnt1 or a combination of both reagents for 14 days	154
Figure 4.23: Immunostaining of Tera2.cl.SP12 cultures treated with Wnt1 for 21 days	156
Figure 4.24: Immunostaining of Tera2.cl.SP12 cultures treated with retinoic acid for 21 days	158
Figure 4.25: Immunostaining of Tera2.cl.SP12 cultures treated with Wnt1 plus retinoic acid for 21 days	160
Figure 4.26: TH protein expression in EC cells treated with Wnt1 and R.A.	163
Figure 4.27: Nestin protein expression in EC cells treated with Wnt1 and R.A.	164
Figure 4.28: Cell counts in Tera2.cl.SP12 cultures treated with R.A., Wnt1 or both reagents for 3 weeks	166
Figure 4.29: The number and % of neurons in Tera2.cl.SP12 cultures treated with R.A., Wnt1 or both reagents for 3 weeks	167
Figure 4.30: The effects of Wnt1 and R.A. on Sox1 mRNA expression	171
Figure 5.1 – Shh/FGF8/A.A./R.A. Differentiation Protocol	182
Figure 5.2 – Shh/FGF8/A.A. +/- R.A. Differentiation Protocol	183
Figure 5.3: Nestin, Sox1 & Tuj1 expression in suspension & adherent cultures	187
Figure 5.4: TH, D2 & Nkx 6-1 expression in suspension & adherent cultures	188
Figure 5.5: Lmx1a, Msx1 & Ngn2 expression in suspension & adherent cultures	189
Figure 5.6: Transporter/receptor expression in suspension & adherent cultures	190
Figure 5.7: Morphological Images of cells growing in the presence of a combination of Shh/FGF8/R.A. & A.A.	192
Figure 5.8: Tuj1 immunostaining of Shh/FGF8/R.A. & A.A. treated Tera2.cl.SP12 cultures at 17 days	193
Figure 5.9: Beta III Tubulin mRNA expression in Tera2.cl.SP12 cultures treated with a combination of Shh, FGF8, R.A. & A.A.	196
Figure 5.10: TH mRNA expression in Tera2.cl.SP12 cultures treated with a combination of Shh, FGF8, R.A. & A.A.	197
Figure 5.11: AADC mRNA expression in Tera2.cl.SP12 cultures treated with a combination of Shh, FGF8, R.A. & A.A.	198
Figure 5.12: D1 mRNA expression in Tera2.cl.SP12 cultures treated with a combination of Shh, FGF8, R.A. & A.A.	199
Figure 5.13: D2 mRNA expression in Tera2.cl.SP12 cultures treated with a combination of Shh, FGF8, R.A. & A.A.	200
Figure 5.14: Lmx1a mRNA expression in Tera2.cl.SP12 cultures treated with a combination of Shh, FGF8, R.A. & A.A.	203

Figure 5.15: Msx1 mRNA expression in Tera2.cl.SP12 cultures treated with a combination of Shh, FGF8, R.A. & A.A.	204
Figure 5.16: Ngn2 mRNA expression in Tera2.cl.SP12 cultures treated with a combination of Shh, FGF8, R.A. & A.A.	205
Figure 5.17: Nkx 6-1 mRNA expression in Tera2.cl.SP12 cultures treated with a combination of Shh, FGF8, R.A. & A.A.	206
Figure 5.18: AADC & D2 expression in the plus and minus R.A. conditions	208
Figure 5.19: TH & Tuj1 expression in the plus and minus R.A. conditions	209
Figure 5.20: Lmx1a & Msx1 expression in the plus and minus R.A. conditions	210
Figure 5.21: Ngn2 & Nkx 6-1 expression in the plus and minus R.A. conditions	211
Figure 5.22: Flow cytometry analysis of MG63 cells	215
Figure 5.23: Beta III tubulin & TH protein expression in the plus & minus R.A. conditions	218
Figure 5.24: A summary of the trends in MRNA & protein expression in EC cells treated with a dopaminergic differentiation cocktail	220
Figure 6.1: Schematic of co-culture studies	235
Figure 6.2: D2 & AADC mRNA expression in EC/Stromal cell co-cultures	239
Figure 6.3: TH & Tuj1 mRNA expression in EC/Stromal cell co-cultures	240
Figure 6.4: Lmx1a & Msx1 mRNA expression in EC/Stromal cell co-cultures	241
Figure 6.5: Ngn2 & Nkx 6-1 mRNA expression in EC/Stromal cell co-cultures	242
Figure 6.6: TH & Tuj1 protein expression in EC/Stromal cell co-cultures	245
Figure 6.7: Staining of Ntera2 EC cells for TH & Tuj1	249
Figure 6.8: Tuj1 staining in EC/Stromal cell co-cultures	250
Figure 6.9: TH staining in EC/Stromal cell co-cultures	252
Figure 6.10: The morphology of R.A. treated EC/Stromal cell co-cultures	255
Figure 6.11: Tuj1 staining in R.A. treated EC/Stromal cell co-cultures	256
Figure 6.12: TH staining in R.A. treated EC/Stromal cell co-cultures	257
Figure 6.13: Nestin staining in R.A. treated EC/Stromal cell co-cultures	258
Figure 6.14: TH & Tuj1 expression in R.A. treated EC/Stromal cell co-cultures	260
Figure 6.15: D2 & Lmx1a expression in R.A. treated EC/Stromal cell co-cultures	261
Figure 7.1: The advantages and disadvantages of the Tera2.cl.SP12 cell line as a model to study dopaminergic differentiation <i>in vitro</i>	273

Abbreviations used in this Thesis:

A.A.	Ascorbic acid
AADC	Dopa decarboxylase (otherwise known as aromatic acid decarboxylase)
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary deoxyribonucleic acid
CO ₂	Carbon dioxide
cRET	Glial cell line derived neurotrophic factor receptor cRET
Ct	Cycle threshold
D1	Dopamine receptor 1
D2	Dopamine receptor 2
DAT	Dopamine transporter
DOPAC	3,4-dihydroxy phenyl acetic acid
E	Embryonic day
EC	Embryonal carcinoma
EDTA	Ethylene diamine tetra acetic acid
En1	Engrailed 1
FACS	Fluorescence activated cell sorting
FGF8	Fibroblast growth factor 8
FGFR4	Fibroblast growth factor receptor 4
FITC	Fluorescein isothiocyanate
Fzd 8	Frizzled 8
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GSK	Glycogen synthase kinase
GTP	Guanosine triphosphate
HBSS	Hanks Balanced Salts Solution
HCl	Hydrochloric acid
hNT	Human Ntera2 derived neurons
HPLC	High performance liquid chromatography
Lmx1a	LIM homeobox transcription factor, alpha
LRP	Low density lipoprotein related receptor protein
LRRK2	Leucine rich repeat kinase 2
MgCl ₂	Magnesium chloride
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mRNA	Messenger Ribonucleic acid
MSC	Mesenchymal stem cell
Msx1	Msh homeobox 1
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
Ng2	Neurogenin2
Nkx 2-2	Homeodomain protein Nkx 2-2
Nkx 6-1	Nk6 homeobox 1
Nm	Nanometre
Nurr1	Nuclear receptor related 1
O ₂	Oxygen

Oct4	POU class 5 homeobox 1
Otx2	Orthodenticle homeobox 2
P	Post natal day
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PES	Phenazine ethosulfate
PINK-1	PTEN induced putative kinase-1
R.A.	Retinoic acid
ROC	Ras of complex proteins
RT	Reverse transcription
S.D.	Standard deviation
Std. Dev.	Standard deviation
S.E.M.	Standard error of the mean
SDIA	Stromal derived inducing activity
Shh	Sonic Hedgehog
Sig.	Significance
SLC23A1	Solute carrier family 23 (nucleobase transporters) member 1
Sox 1/2	SRY (Sex Determining Region Y)-box 1/2
SSEA-3	Stage specific embryonic antigen 3
TBP	TATA box binding protein
TCF/LEF	T-cell factor/Lymphoid enhancer elements
TH	Tyrosine hydroxylase
Tuj1	Beta III tubulin
U	Units

Chapter 1

Literature Review

1.1 Introduction

The primary aim of this thesis is to try to assess the potential of the human embryonal carcinoma stem cell line Tera2.cl.SP12 as a model for the study of dopaminergic differentiation *in vitro*. This cellular system in itself is of value mainly as a tool for basic research that may help extend our knowledge of the processes specifying a dopaminergic fate and potentially if deemed viable, it may be suitable for applied usage in the context of toxicological and pharmacological screening assays. However the study of dopaminergic neurogenesis is of broader significance due to the substantial loss of midbrain dopaminergic neurons from the substantia nigra pars compacta that are associated with and recognised as one of the major traits of Parkinson's disease. It is the direct relevance to this condition that drives the body of research focussed upon the generation of dopaminergic neurons *in vitro*. As such the introduction to this work starts with a brief appraisal and background to Parkinson's disease, prior to considering the role of stem cells as a research tool particularly in relation to neurogenesis, before finally concentrating in greater depth on the current understanding of dopaminergic differentiation *in vitro* as this essentially forms the heart of this disquisition.

1.2 Overview of Parkinson's disease

The hallmark of Parkinson's disease is the loss of dopaminergic neurons from the substantia nigra pars compacta and the concomitant depigmentation of it (Devine & Lewis, 2008). The condition affects roughly 100 – 180/100,000 of the population, although estimates vary significantly, and becomes increasingly prevalent with the onset of old age. It presents a considerable economic burden of around £6 billion a year in the United Kingdom alone (Findley et al., 2003) which is only likely to increase given the demographic trend towards an increasingly elderly population.

However the condition is not just linked to the dopaminergic system of the substantia nigra pars compacta, with the earliest changes taking place in the medulla oblongata/pontine tegmentum as well as the olfactory bulb (Braak et al., 2006). In the initial steps, Braak stages 1 and 2 sufferers do not display any symptoms, subsequent to this though in Braak stages 3 and 4 the more well known areas such as the substantia nigra pars compacta become implicated, along with sections of the midbrain and basal forebrain, prior to the neocortex displaying pathological changes (Davie, 2008).

At present Parkinson's disease is typically thought of as being idiopathic, in other words it may arise without a known cause (Di Monte et al., 2002); however there may be genetic elements and environmental exposures that increase the risk, on balance though it is likely to occur as a result of a range of factors, such as ageing, exposure to toxins in the environment and potential genetic susceptibility (Samii et al., 2004).

Alongside loss of dopaminergic neurons another key pathological trait seen in many cases of Parkinson's disease is the detection of α -synuclein rich Lewy bodies (Spillantini et al., 1998). It has been proposed that exposure to toxins could affect not only the structure of α -synuclein but also its ability to bind ubiquitin and be processed by the 26S proteasome (Figure 1.1). In turn this may further enhance its aggregation and subsequently add to the impairment of the process of abnormal protein degradation (Di Monte et al., 2002). In work by Uversky et al., 2001 it was demonstrated that when certain pesticides were incubated in the presence of α -

synuclein its rate of fibrillation *in vitro* was accelerated. Like wise similar findings were presented in work carried out *in vivo* (Manning-Bog et al., 2002). These studies give support to the concept that environmental exposure to particular toxins may have a direct role in the pathogenesis of Parkinson's disease via a mechanism involving α -synuclein (Di Monte et al., 2002).

Figure 1.1: Potential Interactions of α -Synuclein with environmental toxins
(redrawn from Di Monte et al, 2002)

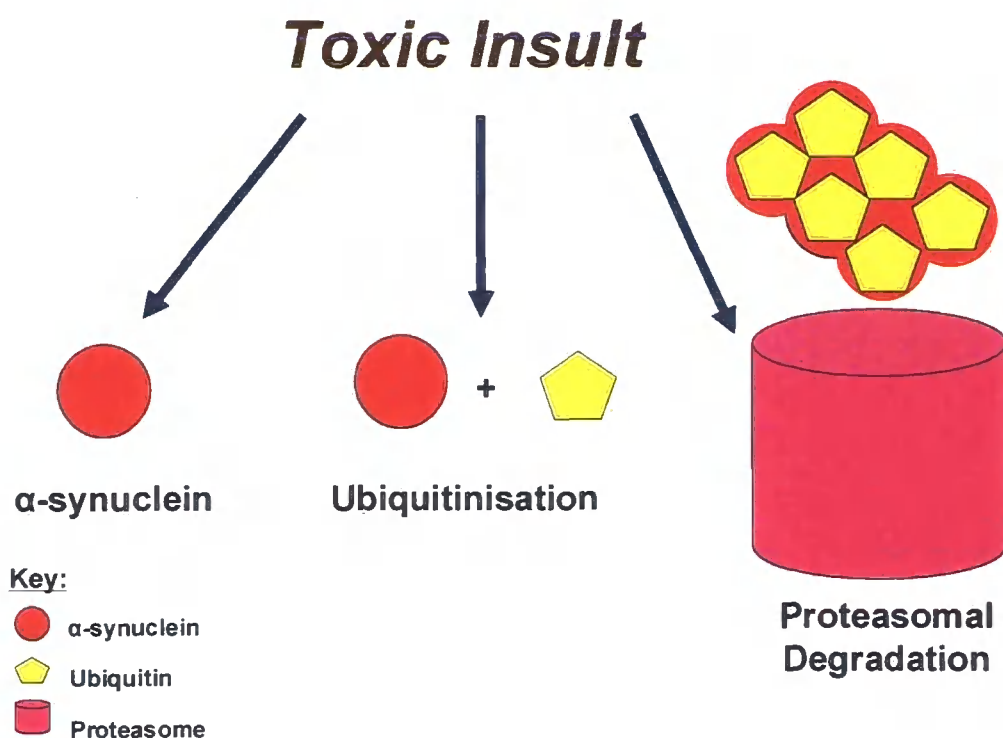


Figure 1.1: α -synuclein could be directly damaged by a toxic insult; such an event could alter the covalent binding of it with ubiquitin; and in turn hamper its degradation by the 26S proteasome (Di Monte et al., 2002).

However this molecule may not only play a role in forms of Parkinson's disease that may be attributable to environmental exposures, as the gene encoding it was the first site to be identified as possessing a mutation in it (an alanine to threonine substitution) that could cause the condition (Polymeropoulos et al., 1997). Two additional point

mutations of this gene, along with gene duplications and triplications give rise to a rare, autosomal dominant type of Parkinson's disease (Kruger et al., 1998; Zarranz et al., 2004; Singleton et al., 2003; Nishioka et al., 2006). It is of interest that sufferers with duplications of the gene display a later age of onset and less severe phenotype than their counterparts possessing triplications, suggesting that disease severity may be linked to α -synuclein expression (Nishioka et al., 2006; Eriksen et al., 2005; Savitt et al., 2006).

The importance of this phosphoprotein is further underlined by the observation that polymorphisms in the promoter for α -synuclein are accompanied by an increase in the risk of developing Parkinson's disease (Pals et al., 2004; Hadjigeorgiou et al., 2005; Tan et al., 2004). In addition α -synuclein null mice display resistance to the toxin MPTP that is often used to induce Parkinsonism in models of the disease (Dauer et al., 2002). This relates neatly to the concept defined earlier whereby exposure to certain toxins may via a mechanism involving α -synuclein lead to the pathogenesis of Parkinson's disease.

There have though been mutations identified such as those in the protein known as Parkin that give rise to a form of Parkinsonism independent of Lewy body formation in juvenile cases, indicating that this protein may play a role in the development of such structures (reviewed in Davie, 2008). Parkin is an E3 ligase that is involved in the attachment of ubiquitin molecules to target proteins that are as such marked for degradation by the proteasome (Zhang et al., 2000). One protein that Parkin may add ubiquitin to is the α -synuclein interacting protein Synphilin-1 in turn facilitating the production of Lewy bodies (Chung et al., 2001). Thus supporting the above concept that Parkin may be involved in the process of Lewy body formation which would explain why mutations in it that often lead to the early onset forms of Parkinson's disease usually, with rare exception, display an absence of such structures (Mori et al., 1998; Farrer et al., 2001; Sasaki et al., 2004; Savitt et al., 2006).

Despite most cases of Parkinson's disease being sporadic in nature other mutations in single genes have been observed that may enhance our understanding of the mechanisms responsible for the condition. Mutations in the DJ-1 gene are somewhat akin to those in Parkin and give rise to an autosomal recessive, early onset form of the

disease (Bonifati et al., 2003). The function of DJ-1 protein is not precisely defined at present but it can prevent via its interaction with α -synuclein, when in a specific oxidised state, the formation of fibrils (Zhou et al., 2006). Thus suggesting that it acts as a redox sensitive chaperone that can shield cells exposed to oxidative stress from the potentially detrimental effects of α -synuclein misfolding (Savitt et al., 2006).

In a similar fashion to DJ-1, mutations in PINK-1 result in an autosomal recessive, early onset form of Parkinson's disease (Valente et al., 2004). Both factors are also present in the mitochondria; given that abnormalities, in Complex 1 of the oxidative phosphorylation enzyme pathway are a regular trait of sporadic forms of the disease (Abou-Sleiman et al., 2006) and have been described in other complexes (Schapira, 2008), as well as the susceptibility of neurons in the substantia nigra pars compacta to oxidative damage, it would appear that malfunction of this organelle may have a role in the pathogenesis of Parkinson's disease (Savitt et al., 2006; Davie, 2008).

One final set of mutations of relevance were identified in the gene for leucine rich repeat kinase 2 (LRRK2) (Zimprich et al., 2004; Paisan-Ruiz et al., 2004). These are the most frequent genetic cause of Parkinson's disease with one mutation involving a glycine to serine substitution being responsible for between 2 and 40%, depending on the study population, of all cases of this condition (Bonifati, 2006; Devine & Lewis, 2008). At present the role of LRRK2 is unknown but work in rat neurons suggests it may have a function in regulating the morphology of neuritic processes (Macleod et al., 2006). In addition when a mutant form of it is expressed in SH-SY5Y cells the result is a shortening of neurites that is linked to increased autophagic vacuole content (Plowey et al., 2008). It may also interact with Parkin *in vitro* (Smith et al., 2005), as well as possessing a ROC (Ras of complex proteins) domain that can similarly relate to β -tubulin a component of microtubules and Moesin (Gandhi et al., 2008; Jaleel et al., 2007). These findings together suggest that LRRK2 may interact with pathways that could themselves form part of the mechanism of the onset of Parkinson's disease (Devine & Lewis, 2008).

After this brief consideration of the possible causes of Parkinson's disease the question is raised of what therapeutic interventions have been used to try and ameliorate the symptoms of it? The initial link between Parkinson's disease and

dopamine deficiency lead to early efforts to rectify this, via the use of the dopamine precursor levodopa (Cotzias et al., 1969). This was then utilised in conjunction with a peripheral decarboxylase inhibitor, for example carbidopa or benserazide (Rinne & Sonninen, 1973; Rinne et al., 1972) that facilitated the entry of more of it into the brain (Savitt et al., 2006). In a comparable fashion catechol-o-methyl transferase inhibitors that act to elongate the half life of levodopa and dopamine were found to augment the effects of levodopa administration (Ericsson, 1971; Myllyla et al., 1993; Roberts et al., 1993).

In contrast to trying to increase the availability of dopamine precursors, efforts were also made to curb the decrease in endogenous levels of the neurotransmitter. Monoamine oxidase type B inhibitors such as Selegiline act in such a manner (Chrisp et al., 1991). Elsewhere other efforts have focussed on producing dopamine agonists that bypass the need for synthesis of this compound, by acting directly on postsynaptic dopamine receptors (Gopinathan et al., 1981; Calne et al., 1974).

Before the arrival of levodopa treatment, surgical therapies had also been used to reduce the tremor and rigidity seen in patients with the disease, but their usage became less common as administration of the drug became more widespread (Brophy, 1998). However more recently still there has been a resurgence towards surgical based strategies, founded on the use of deep brain stimulation (Benabid et al., 2000; Stefani et al., 2007).

Although all of the treatments outlined are of value their capacity to provide a satisfactory quality of living for Parkinson's disease sufferers tends to diminish with time. In addition they are unable to address the problem of the continual degeneration evident in both the dopaminergic and non dopaminergic systems. As such a significant amount of recent research has concentrated on identifying the reasons underlying the loss of dopaminergic cells. In addition to investigating the potential for neuroprotective, restorative or possibly cell replacement based strategies (Savitt et al., 2006). These approaches may well involve the use of stem cells which are the focus of the next section of this Chapter.

1.3 Stem cells as a tool to study neurogenesis

There are a wide variety of stem cell types that may be of use both as research tools to further our understanding of development or as potential sources of cells for use in therapeutic strategies such as cell transplantation that may help in the treatment of certain diseases. Like wise they may also provide a supply of cells for *in vitro* assays to screen for example novel compounds for a specific activity that may help in drug based remedies for known conditions. Individual areas of research tend to have associated with them, varieties of stem cell that are particularly pertinent for use in the context of the topic being studied. This thesis is primarily focussed on the use of embryonal carcinoma stem cells as a model to study the formation of dopaminergic neurons. However there are additional cell types that are often generally utilised for the study of neurogenesis and these are discussed briefly along with embryonal carcinoma cells in this Section.

When studying neurogenesis it is unsurprising to find multipotent neural stem cells that exist in both the developing as well as the adult mammalian brain are often used. Broadly speaking these cell types are derived from the nervous system and/or can generate neural tissue, possess some degree of ability for self renewal and can give rise to cells that differ from them via asymmetric cell division. It is also possible to derive such cells from other more primitive cell types that are capable of generating neural stem cells as well as those of alternative tissues (Figure 1.2) (Gage, 2000). The distinction between multipotent stem cells and neural progenitors as identified in Figure 1.2 is still somewhat hazy as there are instances where cells described as neural progenitors are obtained from; for example embryonic brain that are proliferated post isolation, prior to differentiation in a manner analogous to that utilised for neural stem cells (Gage, 2000). It is always advisable therefore to try and assess any effects on such cells relative to the specific developmental stage they were isolated at and in relation to the species from which they were obtained.

Figure 1.2: Stem cells from which neurons may be derived (based on Gage, 2000)

Potential Stem Cells with Neural Capability

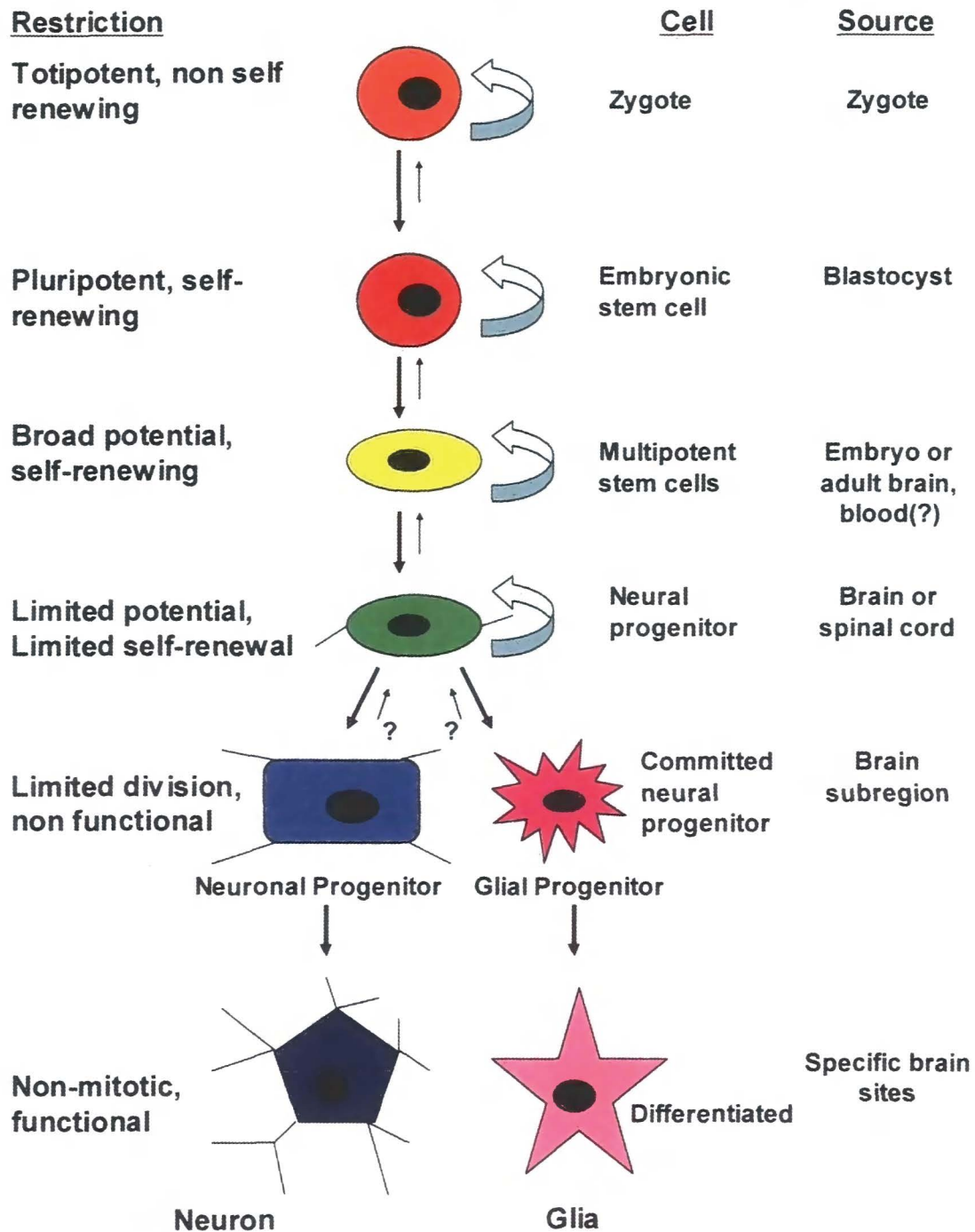


Figure 1.2: A hierarchy of mammalian stem cells that may give rise to neurons. The least restricted cells are at the top with the degree of restriction increasing going down the page. The small arrows pointing up the page allow for the potential of stem cells to dedifferentiate (Gage, 2000).

Mesenchymal stem cells are another type of multipotent stem cell that have been evaluated as a source from which neurons may be generated *in vitro*. However their utilisation is less widespread and would require them to possess the capacity to trans differentiate given their mesodermal origins. This process has been brought into question and is an area of some controversy (Hardy et al., 2008), which perhaps accounts for their limited employment in this context.

Also in widespread usage for the study of neurogenesis *in vitro* are the pluripotent embryonic stem cells derived from the blastocyst inner cell mass, which have now been isolated from a number of species such as mouse (Evans & Kaufman, 1981) and human (Thomson et al., 1998). Embryonal carcinoma stem cells arise from germ cell tumours and given their resemblance to the pluripotent embryonic stem cells of the inner cell mass are considered an apposite model system to them. Both systems possess certain advantages and these often dictate the choice of cell decided upon for use. There are a number of human embryonal carcinoma cell types such as the Ntera2.D1 (Andrews et al., 1984) and Tera2.cl.SP12 cell lines (Przyborski, 2001) that can grow independently of feeder layers associated with embryonic stem cell culture, that provide a possible model of human development. This was seen as an advantage of such cells, however there are now protocols that facilitate the culture of human embryonic stem cells in the absence of feeder layers (Amit & Itskovitz Eldor, 2006), which promote the embryonic stem cell cause. In the context of the embryonal carcinoma versus embryonic stem cell dichotomy, embryonal carcinoma cells were also seen as a more ethical option, although once again new methods were developed for the extraction of human embryonic stem cells that left the embryo from which they were derived intact (Klimanskaya et al., 2006). In turn promoting still further the case for using embryonic stem cells, which also possess a greater more diversified differentiation capacity and are not of cancerous origin, which is an undeniable drawback of embryonal carcinoma cells that limits their potential usage particularly in regard to transplantation based approaches. However embryonal carcinoma cells are robust, easy to manipulate and relatively inexpensive to culture and are thus attractive *in vitro* tools. In addition they can be rapidly expanded to give large numbers of cells and indeed given their more restricted differentiation capability, might be better viewed as a source of cells with a strong propensity for neural differentiation. To this end early indications using the Tera2.cl.SP12 line obtained via immunomagnetic

sorting (Przyborski, 2001) were that it may possess a greater disposition than its counterpart Ntera2 for such neural development (Przyborski et al., 2000; Przyborski et al., 2004).

Both embryonal carcinoma and embryonic stem cell models may be valuable tools for studying neurogenesis, embryonic stem cells appear more regularly in the literature but when embryonal carcinoma cells are used they tend to react similarly to their embryonic stem cell counterparts in response to many dopaminergic phenotype inducing agents in some of the widely used protocols aimed at generating dopaminergic neurons *in vitro* (Schwartz et al., 2005; Ravindran & Rao, 2006). It is such procedures that are focussed on in the final section of this Chapter as they unite the use of stem cells with the desire to produce dopaminergic neurons for application in developing improved therapies for Parkinson's disease.

1.4 In Vitro Methods to Generate Dopaminergic Neurons

Introduction

Dopamine although originally thought to merely be a precursor in the synthesis of noradrenaline and adrenaline was after the discovery of it in the mammalian brain (Montagu, 1957) shown to be a significant catecholaminergic neurotransmitter of the vertebrate central nervous system. Many populations of dopaminergic cells exist for example in the retina, olfactory bulb, retrorubral field and those of the ventral tegmental area that are implicated in obsessive compulsive disorder and schizophrenia (Lin & Rosenthal, 2003). These mesencephalic ventral tegmental area dopaminergic neurons that form the mesolimbic system are one of the two most significant groups of this neuronal subtype. The other being the nigrostriatal dopaminergic neurons of the substantia nigra pars compacta.

The discovery of dopamine deficiency in the substantia nigra and corpus striatum of brains from Parkinson's disease patients (Ehringer & Hornykiewicz, 1960) and subsequent link between the striatum and substantia nigra lead to the understanding that loss of the dopamine producing cells there, lead to a loss of dopaminergic innervation to the corpus striatum and thus a deficit of the chemical was observed in this location (Poirier & Sourkes, 1965). Parkinson's disease can therefore be linked to a specific brain region and to a specific abnormality in one neurotransmitter. Although the disease is in fact more complex with many stages, of which the loss of midbrain substantia nigra dopaminergic neurons is just one, it does make the condition a suitable target for cell based therapies, if an enriched population of the phenotype of interest can be produced. Though the primary focus of such strategies tends to be on cell replacement, there are many obstacles to this. For example survival of grafted cells, the location of the graft which is generally into the striatum not the substantia nigra itself, loss of transplanted cells due to immune rejection or toxic factors that have already caused loss of the native dopaminergic neurons having the same effect on their grafted counterparts (Svendsen, 2008). There is also the possibility of aberrant cells that could give rise to teratomas being transplanted, and the process of transplantation itself may physically damage the relatively delicate

neuronal cells, in particular any neuritic extensions. Some of these hurdles may be overcome through studies in cell based systems utilising the variety of currently available and developing technologies. However *in vitro* methods also have potential not only as basic tools for furthering our understanding of the developmental mechanisms of specific cellular phenotypes, but may provide a range of models for pharmacological screening. In addition such approaches may afford an understanding that facilitates the design of novel compounds that can stimulate endogenous repair mechanisms in the brain. This is exemplified by work using neural stem cells from the sub ventricular zone of adult rats where dopamine D3 receptors are known to be expressed. When cultures of these cells were treated with a dopamine D3 receptor selective agonist 7-hydroxy-dipropylaminotetralin there was an increase in cell number (Coronas et al., 2004), which gives support to the concept that it may be possible to influence the inherent ability of certain cellular populations in the brain to assist in the self repair of this organ.

The demographic trend towards an increasingly elderly population in much of society, issues with current methods for treating Parkinson's disease particularly in the later stages, the specificity of the neuronal subtype affected and the array of potential therapeutic options they may offer have lead to much investigation into the possible methods to generate dopaminergic neurons *in vitro*, it is these that form the main basis of this section. However a brief description of the work on the developmental processes that specify midbrain dopaminergic neurons *in vivo* is first given, as this has provided the foundation for many of the *in vitro* studies.

Specification of Mesencephalic Dopaminergic Precursors

A myriad of intricate interactions between a host of transcription factors and a cascade of signalling events give rise to the induction of midbrain dopaminergic neurons. Principally though they form from interacting signals provided by two different organising centres, Sonic hedgehog arising from the floor plate and Fibroblast growth factor 8 arising from the isthmic organiser, which acts as a boundary between the mid and hindbrain. Sonic hedgehog is the primary soluble signalling molecule along the dorsoventral axis and can induce the formation of dopaminergic neurons in explants from the fore and midbrain (Hynes et al., 1995;

Wang et al., 1995), whilst Fibroblast growth factor 8 is produced in a discrete region that corresponds to the midbrain hindbrain boundary (Crossley et al., 1996; Lee et al., 1997; Ye et al., 1998). The intersection and combination of signals provided by these two molecules gives rise to the induction of dopaminergic precursors rostrally to the isthmus organiser (Figure 1.3), (reviewed in Wallen & Perlmann, 2003; Arenas, 2002; Maxwell & Li, 2005).

The strength of the developmental influence of these two factors on the production of a midbrain dopaminergic cell fate is also recognised in the widespread usage of them in a variety of cell based methods and as such approaches founded on their usage offer a suitable starting point when considering dopaminergic differentiation *in vitro*.

Figure 1.3: Dopaminergic development in the mouse embryo (based on Simon et al, 2003

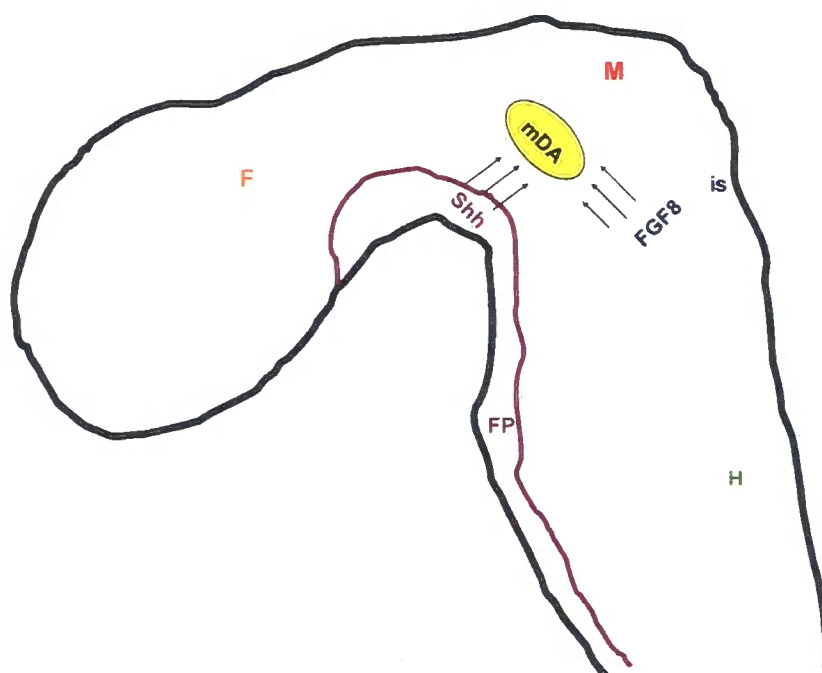


Figure 1.3: The intersection of Sonic hedgehog and Fibroblast growth factor 8 causes the induction of midbrain dopaminergic neurons in the mouse embryo at E8 to E9. FP = Floor Plate, is = isthmus, F = forebrain, H =hindbrain, M = midbrain, mDA = midbrain dopaminergic neurons (Simon et al., 2003).

The use of Sonic Hedgehog and Fibroblast Growth Factor 8 as tools to promote dopaminergic differentiation in vitro

These two key signalling molecules have been a part of many protocols aimed at generating dopaminergic neurons *in vitro*. Many of these methods have attempted to make use of these factors in synergy with other approaches or molecules known to have favourable effects on the induction of a dopaminergic cell fate and as such their use will also be referred to in later sections. However there are some key issues to their successful application which are to be focussed on here. Early work in mouse embryonic stem cells showed that neural differentiation could be achieved via formation of 3D aggregates in suspension culture, known as embryoid bodies, either in the presence of retinoic acid (Bain et al., 1995), or absence of it in serum free conditions (Okabe et al., 1996). Subsequently Lee et al., 2000 used a sequential protocol beginning with embryoid body formation from an expanded population of undifferentiated murine embryonic stem cells to act as a starting point to generate an enriched population of dopaminergic neurons. After the embryoid body stage nestin positive neural precursors were obtained via a medium based selection process, these cells were then expanded prior to a final differentiation step (Lee et al., 2000). The combined administration of Sonic hedgehog and Fibroblast growth factor 8 during the expansion phase (Stage 4 in Lee et al., 2000) gave rise to a more than twofold increase in the number of cells that were positive for tyrosine hydroxylase the rate limiting enzyme in dopamine biosynthesis. Interestingly treatment with either factor alone at this point showed a less significant effect than when both molecules were applied in a concerted manner. This is strongly indicative that as is the case *in vivo*, the presence of both signals is required to induce a dopaminergic phenotype. Another critical finding was that the collective application of these two molecules at other preceding or latter steps of the process had no effect on the yield of tyrosine hydroxylase positive cells obtained (Lee et al., 2000).

Further work in human embryonic stem and embryonal carcinoma cells further substantiated the importance of temporal factors when trying to elicit dopaminergic differentiation *in vitro*. Firstly using the human embryonal carcinoma stem cell line Ntera2, it was shown that Sonic hedgehog and Fibroblast growth factor 8 were not able to induce tyrosine hydroxylase expression. However this was in Ntera2

embryonal carcinoma cells pre treated with retinoic acid. Such culture of Ntera2 cells with retinoic acid results in the production of neurons with various neurotransmitter phenotypes and it is these hNT neurons that were the subject of investigation. Only small percentages (2%) of neurons were tyrosine hydroxylase immunopositive following application of Sonic hedgehog and fibroblast growth factor 8. Again highlighting the importance of developmental stage on the ability of cells in culture to respond to the cues provided (Stull & Iacovitti, 2001). Given that Sonic hedgehog and Fibroblast growth factor 8 are thought to act in the earlier stages of dopaminergic neuron development an approach involving their use to manipulate the embryonal carcinoma stem cell system towards a dopaminergic fate cannot be discounted if the exogenous application of them can be optimised to an appropriate temporal window.

Indeed in a similar protocol to that of Lee et al., 2000, the Ntera2.D1 cell line was used to first generate aggregates in suspension that appeared akin to embryoid bodies. Medium based selection for nestin positive precursors followed, prior to expansion and then sorting using neuronal cell adhesion molecule to enrich the neuroprogenitor pool. Finally differentiation was induced using a cocktail containing glial cell line derived neurotrophic factor and interleukin-1 β both agents being known to promote a dopaminergic phenotype (Ravindran & Rao, 2006). Interestingly unlike most other protocols involving experimentation using the embryonal carcinoma cell system, retinoic acid treatment is not involved. Although retinoic acid may possess a role in the specification of a dopaminergic phenotype it is also implicated in specifying other cellular fates and indeed can cause formation of both neurons and glia from certain embryonal carcinoma cell lines such as Tera2.cl.SP12 (Stewart et al., 2003). Therefore its use could be brought into question; however Sonic hedgehog itself is known to have a role in the formation of serotonergic hindbrain neurons. In conjunction with Fibroblast growth factor 8 in the protocol of Lee et al., 2000, it causes a fourteen fold increase in the number of serotonin positive cells when applied during the expansion phase. Even when Sonic hedgehog is applied alone a similar effect is observed (Lee et al., 2000). Therefore although it may be seen as desirable to avoid the use of certain molecules like retinoic acid, it is probably more important to control when and how they are used. Sonic hedgehog is regularly a constituent of procedures trying to induce dopaminergic neurons but it still has the ability to help generate alternative neuronal fates.

It is, though, noteworthy that embryonal carcinoma stem cells can be used in the absence of retinoic acid to generate dopaminergic neurons. It also demonstrates that Fibroblast growth factor 8 and Sonic hedgehog can be used effectively in the Ntera2 embryonal carcinoma cell system, and that using this embryoid body based method they do act in a similar fashion to the embryonic stem cells, for which they act as a model (reviewed in Przyborski et al., 2004).

In addition to their use in mouse embryonic stem and human embryonal carcinoma cells, Sonic hedgehog and Fibroblast growth factor 8 have also been utilised in human embryonic stem cells. This work highlighted the importance of the correct timing of application of exogenous compounds (Figure 1.4). When Fibroblast growth factor 8 was applied alongside Sonic hedgehog to Sox1 positive neuroepithelial cells, the output was a set of tyrosine hydroxylase positive, γ -aminobutyric acid co-expressing, bipolar cells that lacked expression of the midbrain marker Engrailed 1. However if Fibroblast growth factor 8 was administered to Sox1 negative precursors, followed by combined Sonic Hedgehog/Fibroblast growth factor 8 treatment then a distinctly different population of tyrosine hydroxylase positive, Engrailed 1 co-expressing midbrain type dopaminergic neurons, with complex processes and large cell bodies were produced (Yan et al., 2005).

Figure 1.4: The temporal effects of Shh and FGF8 on the acquisition of a dopaminergic phenotype (based on Yan et al, 2005)

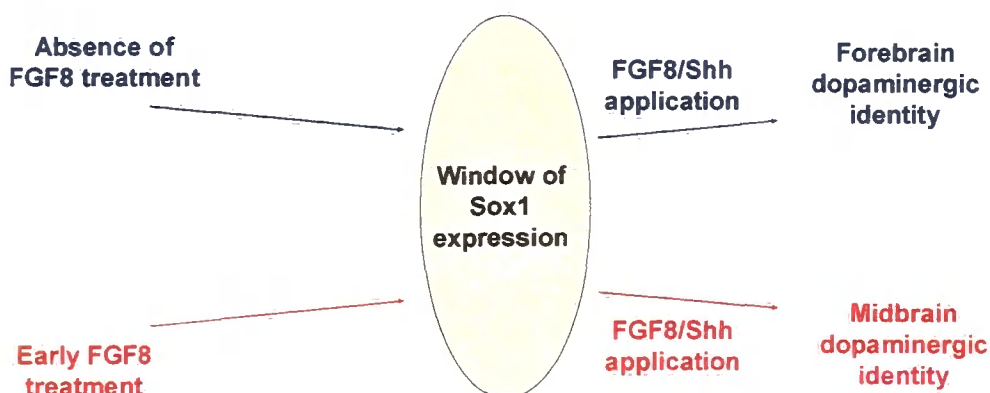


Figure 1.4: The effect of timing of administration of certain molecules is key in influencing the specification of particular cellular phenotypes, as exemplified here for Fibroblast growth factor 8 and Sonic hedgehog. When human embryonic stem cell derived neural precursors are given an early dose of Fibroblast growth factor 8 prior to Sox1 expression they are capable of generating midbrain type dopaminergic neurons, when this molecule is applied in conjunction with Sonic hedgehog after Sox1 expression is detectable, the resultant output is dopaminergic neurons characteristic of a forebrain identity (based on Yan et al., 2005).

In another protocol based on that of Lee et al., 2000, Sonic hedgehog and Fibroblast growth factor 8 were used to try and influence murine adult neural stem cells of the sub ventricular zone to produce midbrain phenotype dopaminergic neurons. The two factors were able to induce expression of midbrain markers but the adult neural stem cells still maintained a forebrain identity, giving rise to mixed phenotype neurons. However in their absence no tyrosine hydroxylase positive neurons were found, indicating that Sonic hedgehog/Fibroblast growth factor 8 were responsible for the induction of TH positive neurons and that adult neural stem cells do not default to, or show no predisposition to produce dopaminergic neurons. High performance liquid chromatography also showed that although adult neural stem cells could release dopamine, depolarisation with potassium ions (K^+) did not significantly increase this effect. In contrast neurons positive for tyrosine hydroxylase derived from embryonic stem cells displayed an evoked release of dopamine upon K^+ depolarisation (Papanikolaou et al., 2008).

Functional testing such as that carried out by high performance liquid chromatography is key if the dopaminergic neurons generated are to be validated for use in applications such as toxicological and pharmacological screens or if they are to be used in any kind of potential cell transplantation based strategy in the future. To this end dopaminergic neurons derived using Sonic hedgehog/Fibroblast growth factor 8 based approaches from a variety of sources do tend to display biological function. For example dopamine production, an ability to respond to neurotransmitters and spontaneous synaptic activity are all seen in mouse embryonic stem cell derived tyrosine hydroxylase positive neurons (Lee et al., 2000). Likewise human embryonic stem cell derived neurons exhibit activity dependent release of dopamine and electrophysiological properties, although the *in vitro* dopaminergic neurons derived in this human embryonic stem cell system convey the notion of a slow process of maturation as evidenced by atypical electrophysiology and poor uptake activity of dopamine. This may be due to a lack of signals and targets for the human cells in the culture conditions used (Yan et al., 2005).

Beyond the level of functional testing transplantation of enriched human Ntera2 derived neural cell adhesion molecule positive cells into the substantia nigra of a 6-hydroxydopamine treated rat model of Parkinson's disease resulted in behavioural improvement. Although the sample size was small, neural cell adhesion molecule positive sorted cells did not give rise to any tumours. Transplanted cells were both able to survive in the substantia nigra and differentiate as shown by expression of tyrosine hydroxylase and anti human nuclei, the presence of tyrosine hydroxylase being indicative of the dopaminergic differentiation of the transplanted cells. Though tyrosine hydroxylase could also be a marker of other neuronal phenotypes, the locality of the graft and procedure used to derive the transplanted cells would suggest a dopaminergic identity. Ideally as in any study a panel of dopaminergic markers would be used as supporting evidence. *In vitro* however neural cell adhesion molecule positive neuroprogenitors when differentiated did not express dopamine β hydroxylase as would be expected of non dopaminergic tyrosine hydroxylase positive neurons and did express Engrailed 1, Nurr1, and a range of other dopaminergic neuron associated elements. Therefore there is promise in this technique which underlines the importance of cell sorting prior to transplantation (Ravindran & Rao,

2006), to avoid the problem of teratoma formation as was found with mouse embryonic stem cell derived transplanted dopaminergic neurons. In this study one animal was found to have a teratoma in its brain post transplantation (Nishimura et al., 2003). Overcoming this safety issue is one of the many challenges facing the successful use of embryonic stem cells. Given the malignant nature of embryonal carcinoma stem cells their role is more realistically confined to more basic fundamental research to refine the procedures that could then be applied elsewhere.

Co-culture Methods to Achieve Dopaminergic Differentiation

For all the variety of strategies used to generate dopaminergic neurons *in vitro*, most of them at their core if not based on Sonic hedgehog and Fibroblast growth factor 8 are founded on co-culture approaches. In particular involving the use of bone marrow derived murine PA6 cells. Although alternative stromal cells, for example MS5 (Perrier et al., 2004), in addition to other cell types such as glia of the ventral midbrain (Castelo-Branco et al., 2006) and sertoli (Yue et al., 2006) as well as meningeal (Hayashi et al., 2008) cells have been used. The general advantages of such protocols are the relative ease of the technique and usually short time course needed to achieve induction of dopaminergic differentiation. The main drawback is in the use of animal cells that are coming into contact with or are in close proximity (if a membrane is used) to the cells of interest. This would need to be addressed before application in a clinical setting, and is prohibitive in the context of any kind of screening assay as the cellular population in direct co-culture approaches is heterogenous. Another key question that remains is what is the identity of the inductive molecules in such approaches in particular with PA6 cells what constitutes the Stromal Derived Inducing Activity known as SDIA?

In early work using mouse embryonic stem cells the nature of the stromal derived inducing activity was investigated. It was found that even after fixation with paraformaldehyde, PA6 cells could still cause neural differentiation. Similarly other stromal cell types also exhibited a comparable activity following fixation but prior to it only exerted a weak or intangible neural inducing effect, where as PA6 cells pre or post fixation displayed positive effects on neural cell specification. The speculation in this work was that other stromal cells produce a mixture of inductive and inhibitory

factors. PA6 cells lack the latter component that is lost upon fixation of alternative stromal cell types. Hence why PA6 cells can exert an effect in both conditions not just post fixation as is the case for their counterparts. The other important aspect of this work was it highlighted that at least in part the inductive activity arose from or was mediated by something on the cell surface, as fixed cells lack the capacity to secrete soluble factors.

However when a 0.4 μ M filter membrane was used to alleviate any physical contact between the embryonic stem and PA6 cells significant neural induction was observed. This is indicative that there is also a soluble constituent to the PA6 stromal derived inducing activity. Interestingly though media conditioned by PA6 cells was unable to exert a similar effect. Overall the initial suggestions were that the stromal derived inducing activity possessed two components, a membrane bound and soluble factor, or that there was an initial secretion of a product that then subsequently had an interaction at the cell surface. Although the identity of the stromal derived inducing activity is unknown, one intriguing aspect of this protocol is that detection of tyrosine hydroxylase occurs between day six to day eight of induction. Embryonic stem cells are equivalent to E4 cells of the inner cell mass and in foetal mouse midbrain tyrosine hydroxylase is first expressed at E11.5 (Foster et al., 1988). Therefore there is a seemingly comparable temporal schedule *in vitro* to that seen *in vivo* (Kawasaki et al., 2000).

Similar PA6 based protocols have also been investigated in embryonic stem cells from other species and in alternative cell types. From this there have arisen some consistent but also some contradictory findings. This may not only be accounted for by species specific differences but by the stromal derived inducing activity consisting of a number of factors, possibly more than the two identified using murine embryonic stem cells, however it may be that there are multiple activities that can either be classified as soluble or membrane bound. Different aspects of the stromal derived inducing activity may be critical depending on the system used.

Firstly in primate embryonic stem cells a similar ability was shown by PA6 cells to induce a broadly neuronal and more specifically a dopaminergic fate. There was a slightly longer period of induction required before the appearance of expression of

dopaminergic markers like tyrosine hydroxylase, and the efficiency of neural differentiation was approximately 50% of that found using mouse cells. However the process did work and was more rapid in its time course *in vitro* than might have been predicted from the *in vivo* setting, this rapidity is one advantage of the method (Kawasaki et al., 2002).

The PA6 based approach has also been tested in human embryonic stem cells, in addition to human embryonal carcinoma stem cells. Zeng et al., 2004 used the BG01 human embryonic stem cell line in conjunction with PA6 cells to generate tyrosine hydroxylase positive cells in just under 90% of embryonic stem cell colonies over a time course of three weeks. The differentiated cells expressed a range of dopaminergic markers such as dopamine transporter, Nurr1, and aromatic amino acid decarboxylase. They were dopamine β hydroxylase negative indicating that they were not noradrenergic and could release dopamine upon depolarisation by potassium ions (Zeng et al., 2004). Park et al., 2005 tested three other human embryonic stem cell lines in the PA6 co-culture system, only one of the three displayed neural precursor differentiation. After one week nestin and β III – tubulin expression were present in more than 90% of the HSF-6 derived cells, but given the same conditions the other two lines tested (SNU-hES3 and Miz-hES1) showed less than 10% of cells to be comparable in the expression of these markers (Park et al., 2005, reviewed in Taylor & Minger, 2005).

Given the variability in outcomes using human embryonic stem cells, at around the same period the observation was made that PA6 cells could influence the dopaminergic differentiation of the Ntera2 human embryonal carcinoma stem cell line (Schwartz et al., 2005). The efficiency and time course of this generation mirrored closely that in human embryonic stem cells, which is not dissimilar to the scenario found when using Sonic hedgehog/Fibroblast growth factor 8 based protocols. This offers promise in that the robust nature and relatively basic inexpensive methods for human embryonal carcinoma stem cell culture, combined with a clear likeness to the human embryonic stem cell system in relation to dopaminergic differentiation, could make embryonal carcinoma cells a useful tool to study dopaminergic neurogenesis whilst greater control and characterisation of human embryonic stem cell systems is developed. The findings could then be translated from one system into the other,

although subtle differences may be found, there are still advantages to be gained from the tandem use of both human cell systems; especially given the species specific differences that are observed between human and animal neuronal progenitor cells (Jin et al., 2006) that really underline the need for balanced experimentation across a range of models.

The work by Schwartz et al., 2005 using the Ntera2 cell line epitomised this, as PA6 driven differentiation occurred over a similar period to a comparable level and showed a sequence of marker expression resembling that in human embryonic stem cells (Zeng et al., 2004). However subtle variations were seen, for example the embryonal carcinoma cells developed as packed colonies upon seeding and remained as such during differentiation on a monolayer of confluent PA6 cells (Schwartz et al., 2005). Human embryonic stem cells in comparison normally migrate out of the colonies as differentiation proceeds (Zeng et al., 2004). In addition fewer tyrosine hydroxylase positive processes were seen after three weeks of Ntera2-PA6 co-culture (Schwartz et al., 2005) further highlighting the minor but noticeable disparity between the two systems.

The investigations in the Ntera2 embryonal carcinoma cell system though provided some interesting findings. First of all to reduce the stress on the cells in culture when differentiating, mitomycin C treated mitotically inactivated PA6 cells were used. These treated cells could produce dopaminergic differentiation in a manner analogous to their untreated counterparts. It was also possible to achieve dopaminergic neuron generation using media conditioned by PA6 cells exposed to mitomycin C, however more tyrosine hydroxylase positive cells were formed using standard co-culture than with conditioned medium (Schwartz et al., 2005). The observed effects with conditioned medium are though in contrast to the findings using murine embryonic stem cells where conditioned medium did not markedly induce tyrosine hydroxylase expression (Kawasaki et al., 2000).

This could be due to poor protein solubility (Kawasaki et al., 2002) or Ntera2 cells possessing a greater sensitivity to insoluble glyco or lipoglycoproteins for example Wnts, it may also reflect a species specific divergence in terms of responsiveness to stromal derived inducing activity as Schwartz et al in an unpublished observation,

2005 found a comparable result using human embryonic stem cells. It does however add support to the notion that secreted factors are a part of the dopaminergic differentiation enhancing ability of the stromal derived inducing activity of PA6 cells (Schwartz et al., 2005).

In a recent paper utilising human embryonic stem cells there was the suggestion that it is the secreted factors provided by the PA6 cells that are required to define a specific dopaminergic cell fate. In this work they found that fixed PA6 cells although in possession of a cell surface neural differentiation capacity lacked the ability to induce a significant number of tyrosine hydroxylase positive cells. This was recognised by a three fold decrease in tyrosine hydroxylase expression relative to co-culture with untreated PA6 cells. Irradiation and mitomycin C treatment also had strong effects in a congruous manner. However mitomycin C exposure had only a minor and fixation an extremely minimal effect on the neural inducing activity, assessed by β -III-tubulin expression. Irradiation though decreased it by 50%, thus indicating that the neural inducing ability was linked to the cell surface. Conditioned medium alone was ineffective as for mouse embryonic stem cells (Kawasaki et al., 2000) but in contrast to human embryonal carcinoma stem cells (Schwartz et al., 2005) in generating positive tyrosine hydroxylase expression. However when combined with heparin and/or fixed PA6 cells differentiation of tyrosine hydroxylase positive cells could be achieved, the extent of this though was still not as great as for untreated PA6 co-culture. In addition conditioned medium was also able to elicit two other effects, firstly it promoted the survival of human embryonic stem cells and secondly it was able to preserve Oct3/4 expression (Vazin et al., 2008). Other evidence has previously suggested that sustained Oct3/4 expression could be implicated in early neuronal differentiation (Shimozaki et al., 2003; Burgess et al., 2002). Together these findings reinforce the concept that the stromal derived inducing activity may be a compilation of multiple factors (Figure 1.5) that can exert an assortment of effects that may elicit varied responses in different experimental systems.

Figure 1.5: A summary of the elements of the stromal derived inducing activity

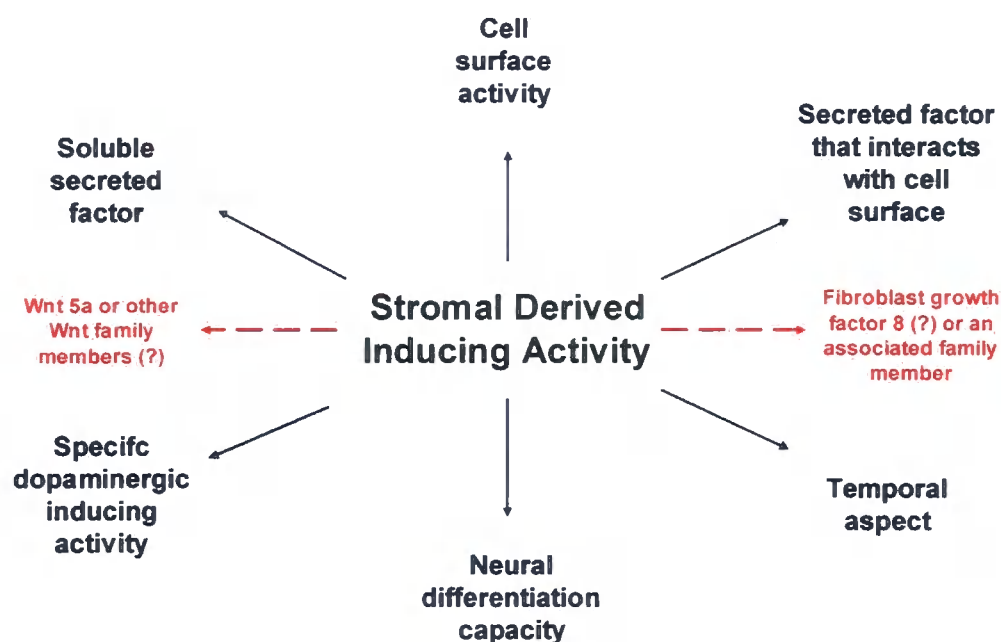


Figure 1.5: The different aspects of the stromal derived inducing activity that may have an effect on its neural or dopaminergic differentiation ability are shown in blue. Possible candidates that may comprise part of the SDIA are shown in red. It may be that factors such as Wnts or Fibroblast growth factor family members are involved in more than one of the aspects outlined in blue, for example Wnt 5a could potentially be a soluble factor, interact with the cell surface and have a specific effect on promoting the acquisition of a dopaminergic phenotype.

In fact the stromal derived inducing activity may have a temporal component to its action. It appears that the stromal derived inducing activity can instruct a dopaminergic fate in embryonic stem cell derived neural progenitors before or at the point of expression of Sox1. However it does not seem to exert an effect during the latter stages of neuronal differentiation (Parmar & Li, 2007). This is comparable to the situation where early Fibroblast growth factor 8 application prior to Sox1 expression is critical to the specification of a midbrain dopaminergic neuronal fate (Yan et al., 2005). Members of the Fibroblast growth factor family may be secreted then subsequently interact with the cell surface (Bernfield et al., 1999; Iozzo et al., 1998). This being one of the possible mechanisms of the stromal derived inducing activity identified by Kawasaki et al., 2000, who like Parmar & Li, 2007 carried out their work on murine embryonic stem cells. Therefore there is a strong suggestion

that Fibroblast growth factor 8 or an associated molecule could be a part of the instructive activity of the stromal derived inducing activity when using this embryonic stem cell system. However one would speculate that if Fibroblast growth factor 8 or a relation of it is involved, then it will most probably be acting in concert with at least one other factor, as when applied alone its effect was significantly less than when added in conjunction with Sonic hedgehog at stage four in the protocol of Lee et al., 2000.

Regardless of the molecular nature of the PA6 stromal derived inducing activity, any dopaminergic type cells derived using this approach must be able to show functional properties if they are ever to be able to be utilised in any relevant application. To this end the neuronal derivatives of mouse embryonic stem cells co-cultured with PA6 cells showed secretion of dopamine, assessed by high performance liquid chromatography and could upon transplantation maintain a tyrosine hydroxylase positive phenotype as well as integrate into the mouse striatum. There was around a 22% survival rate of such tyrosine hydroxylase positive cells in this 6-hydroxydopamine model after two weeks. In a similar 6-hydroxydopamine model the transplantation of neural precursors derived from a range of mouse and somatic cell nuclear transfer derived embryonic stem cells cultured on primary stromal cells or stromal cell lines, was able to show reasonable numbers of tyrosine hydroxylase positive cells surviving eight weeks after transplantation. In this model administration of 6-hydroxydopamine into the striatum causes a selective loss of dopaminergic neurons in the midbrain and a resulting deinnervation of the striatum. Apomorphine or amphetamine is then given to cause motor disturbances that lead to full body rotations depending on the drug used. Any measure that involves replacement of dopamine will give a recognisable decrease in the rotational behaviour. Within eight weeks of transplantation more than 70% of animals showed significantly diminished apomorphine or amphetamine induced rotational asymmetry (Barberi et al., 2003, reviewed in Taylor & Minger, 2005).

Like their murine counterparts primate embryonic stem cells showed dopamine release following potassium ion induced depolarisation. Again in a 6-hydroxydopamine mouse model two weeks post implantation there were some (approximately 8%) surviving tyrosine hydroxylase positive neurons (Kawasaki et al.,

2002). The PA6 method has also been utilised to create progenitors with the capacity to differentiate into dopaminergic neurons. 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) lesioned primates acting as a Parkinsonian model, upon receipt of transplants of these populations of cells, showed significantly reduced lesion related neurological defects and augmented fluorodopa uptake assessed by positron emission tomography relative to controls (Takagi et al., 2005). The percentage of dopaminergic cells surviving fourteen weeks post transplantation was also significantly increased over the levels observed using grafts of tissue from the post mitotic foetal midbrain (Taylor & Minger, 2005).

Finally when using human embryonic stem cell derived neurons for transplantation in a 6-hydroxydopamine model only small numbers (approximately 8 - 9%) of tyrosine hydroxylase positive cells survived five weeks post implantation (Zeng et al., 2004). Similarly HSF-6 derived cells although able to show functional signs and molecular markers of a dopaminergic phenotype, survived poorly upon transplantation; with few tyrosine hydroxylase positive cells remaining six weeks post implantation (Park et al., 2005; reviewed in Taylor & Minger). Therefore there are still clearly a large number of obstacles to overcome before cell transplantation can ever be effectively and safely used. However there have been attempts to address some of the issues. Dopaminergic neurons are particularly sensitive to enzymatic treatment and physical dissociation procedures. This means that loss of some of the cells of interest is likely in any protocol based on their derivation from a stem or progenitor cell population grown in standard adherent conditions, as the cells prior to transplantation have to be removed by some method from the culture surface used. By growing mouse embryonic stem cells in hollow fibres using PA6 conditioned medium for sixteen days Yamazoe & Iwata, 2006, were able to generate β -III-tubulin and tyrosine hydroxylase positive cells that could release dopamine upon depolarisation with potassium ions. This method allowed the formation of dopaminergic neurons without loss of cells as the fibres act to protect the neurons from mechanical pressures. Such fibres may also serve an additional role in protecting cells from the host immune system if they were to be transplanted (Yamazoe & Iwata, 2006). Such strategies offer a starting point for improvements in cell transplantation procedures.

Ultimately though for cell transplantation therapy and ideally for screening based assays it is both essential and desirable to remove the need for co-culture by elucidating the molecular nature of the stromal derived inducing activity of PA6 cells. This would eliminate possible transfer of murine pathogens which at present makes PA6 based approaches unviable in a clinical setting. To this end a comparison of PA6 and meningeal cells was made. Meningeal cells are able to induce dopaminergic differentiation in mouse and human embryonic stem cells, the derivatives express markers of a dopaminergic phenotype and potassium ion evoked dopamine release. Interestingly the developmental stage of the meningeal cells affects their dopaminergic neuron inducing activity with E18 cells displaying the greatest effect on the acquisition of a dopaminergic phenotype in comparison to their E14 and P4 counterparts. When expression of six soluble factors implicated in the differentiation of dopaminergic neurons (Fibroblast growth factor 8, Sonic hedgehog, Wnt1, Wnt3a, Wnt5a and transforming growth factor- β 3) was assessed only Wnt5a and transforming growth factor- β 3 mRNA were found to be expressed. Real time RT-PCR showed that transforming growth factor- β 3 expression increased with developmental stage and was greater in meningeal than PA6 cells; whilst E18 meningeal cells, which possessed the greatest inducing activity shared a similar level of Wnt5a expression to their PA6 counterparts, where as the less potent E14 and P4 stage meningeal cells expressed lower levels of this particular component.

The effects of Wnt5a as a soluble aspect of the stromal derived inducing activity were investigated further. PA6 conditioned medium could induce dopaminergic differentiation of mouse embryonic stem cells grown on matrigel relative to a control differentiation medium. If a Wnt5a neutralising antibody was added to the conditioned medium the levels of dopaminergic induction were decreased to control levels. However addition of recombinant Wnt5a to the differentiation medium could elicit an increase in the percentage of cells acquiring a dopaminergic phenotype. Therefore it appears that Wnt5a is an important part of the mechanism of the stromal derived inducing activity (Hayashi et al., 2008).

These findings are also consistent with other work that demonstrates that ventral midbrain glia express region specific transcription factors such as Engrailed 1 and Otx2 as well as secreting Wnt5a. In turn they are able to amplify the differentiation

of both mesencephalic and cortical Nurr1 positive precursors into tyrosine hydroxylase positive cells. Again using a Wnt5a neutralising antibody it was shown that at least part of the effect of ventral midbrain glia on Nurr1 positive precursors was attributable to the effects of Wnt5a (Castelo-Branco et al., 2006). Likewise earlier work had indicated that Wnt5a had a specific instructive role in driving Nurr1 positive precursors towards the acquisition of a dopaminergic phenotype (Castelo-Branco et al., 2003). When using human embryonic stem cells as described earlier it was observed that the specific dopaminergic inducing activity appeared to lie in the soluble factors produced by PA6 cells. When heparin was used to try and increase the activity of any secreted factors using conditioned medium there was a noticeable effect on dopaminergic differentiation (Vazin et al., 2008). Similarly in the study of Hayashi et al., 2008, conditioned medium was collected following incubation of PA6 cells for twenty four hours in a differentiation medium containing heparin. In this work Wnt5a was highlighted as one possible heparin binding soluble factor capable of causing dopaminergic differentiation (Hayashi et al., 2008). By analogy one might suspect that Wnt5a exerts a similar effect in human as well as mouse embryonic stem cells. Perhaps when considering possible constituents of the stromal derived inducing activity it may be worth examining dopaminergic inducing elements that share a high degree of conservation between species, as the stromal derived inducing activity can have an effect in murine, primate and human cell systems, despite there being evidence that there are species specific differences. Therefore it is logical that at least some of the aspects of the stromal derived inducing activity might be highly conserved. In addition the PA6 mediated stromal derived inducing activity if multifactorial as it appears to be opens up the question of whether a cell can be engineered to express in a spatially and temporally controlled manner, neurotrophic factors following implantation that could stimulate endogenous repair in a disease model.

Beyond the methods considered thus far, dopaminergic differentiation has also been achieved from primate embryonic stem cells in co-culture with sertoli cells. This protocol showed temporal efficiency and produced cells expressing a range of dopaminergic markers that could release dopamine upon potassium ion depolarisation. After transplantation into a 6-hydroxydopamine model tyrosine hydroxylase positive neurons were observed to survive for at least two months. When a filter membrane was used significant neural differentiation facilitated by the sertoli

cells was seen. This is indicative of soluble factors being responsible for the inductive effect. Interestingly though sertoli cell conditioned medium did not cause a significant level of differentiation. This could be indicative of two different components, one at the cell surface and one a labile soluble factor (Yue et al., 2006), this is analogous to the situation found with PA6 stromal derived inducing activity. However the neural inducing activity of sertoli cells could be highly attributable to glial cell line derived neurotrophic factor. Not only was it found in sertoli cell conditioned medium but following a weak dose of anti-glial cell line derived neurotrophic factor antibody, to act as a blocker of its activity, neural differentiation was reduced. Glial cell line derived neurotrophic factor may therefore act to assist or augment the efficiency of other factors in promoting differentiation (Yue et al., 2006). This is reflected in its usage in a number of protocols aimed at optimising the synergistic interactions between different known inducers of a dopaminergic phenotype. It is also worthy of note that a rapid differentiation of the human embryonal carcinoma cell line Ntera2.D1 can be achieved by growing tissue constructs of sertoli cells in conjunction with Ntera2 in a rotating wall bioreactor (Saporta et al., 2004). This is again analogous to the PA6 method in that the inductive factors may be highly conserved so as to be able to exert an effect on cell lines from different species. It also shows that retinoic acid is not necessarily an essential requirement when working with embryonal carcinoma stem cells, as was also the case in the work of Ravindran & Rao, 2006.

The role of Wnts in dopaminergic neurogenesis

This family of lipoglycoproteins consists of nineteen members, some of which have been shown to have effects on the development of dopaminergic neurons (for review see Arenas, 2005) and as such they are an area of great interest in the context of this thesis. Wnt5a as has been discussed may form part of the stromal derived inducing activity of PA6 cells and is secreted by ventral midbrain glia. However other Wnt family members may also play critical roles in the specification of a dopaminergic cell fate. In mice Wnt1 causes an increase in the expression of Otx2 which acts as a repressor of Nkx2-2, down regulating its activity. This then limits the acquisition of alternative neuronal fates and as such creates a permissive environment for other dopaminergic inducing agents such as Fibroblast growth factor 8 and Sonic hedgehog

to act (Prakash et al., 2006, reviewed in Burbach & Smidt, 2006). Wnt1 has also been shown to increase the number of Tuj1 positive cells in rat ventral midbrain precursor cultures (Castelo-Branco et al., 2003). Such an effect would be highly desirable to reproduce in other systems where mixed cell populations could be increasingly driven towards a neuronal fate to enrich them prior to their guidance towards a specific neurotransmitter phenotype. The same molecule is also capable of increasing the number of Nurr1 positive precursors in the rat E14.5 ventral midbrain cellular model. In addition it can have an effect on the differentiation of precursors into more mature dopaminergic neurons. This implies that it possesses a dual role controlling both the proliferation and differentiation of dopaminergic precursor cells (Castelo-Branco et al., 2003).

Another family member Wnt3a actually caused the number of dopaminergic neurons to decrease but increased the proliferation and helped to maintain the Nurr1 positive precursor pool. This was in contrast to Wnt5a which had little effect on the proliferation of the Nurr1 positive precursors but drove the differentiation of them towards a dopaminergic phenotype (Figure 1.6). Interestingly Wnt5a promoted the levels of the glial cell line derived neurotrophic factor receptor cRET mRNA in these cultures and maintained the expression of the mRNA for two glial cell line derived neurotrophic factor co receptors neural cell adhesion molecule and glial cell line derived neurotrophic factor family receptor $\alpha 1$ (Castelo-Branco et al., 2003). Glial cell line derived neurotrophic factor is involved in the post natal stages of development of dopaminergic neurons (Granholtm et al., 2000). This is indicative therefore that as is the case *in vivo* it may be possible to carefully control the acquisition and maintenance of a dopaminergic phenotype *in vitro* if the correct combination of molecules can be utilised in concert or at least more optimally than has been achieved at present.

The assessment of patterns of receptor expression may help provide a better understanding of how and when molecules known to influence dopaminergic differentiation act. In turn this may aid the design of new or adapted experimental protocols to achieve enriched populations of dopaminergic neurons *in vitro*. This is in principle neatly demonstrated in work using human embryonic stem cells differentiated towards a dopaminergic fate using co-culture with astrocytes or PA6

cells. First of all it was shown that co-culture of astrocytes from the embryonic striatum resulted in the production of more tyrosine hydroxylase positive cells in comparison to co-culture with astrocytes from the embryonic mesencephalon, indicating that there are region specific differences between cell types, at least in relation to their dopaminergic inducing activity. Secondly though co-culture with PA6 cells that may possess Wnt5a as a part of their stromal derived inducing activity (Hayashi et al., 2008) if carried out in the presence of glial cell line derived neurotrophic factor causes the number of tyrosine hydroxylase positive cells obtained to double. It also increased the size and survival of embryonic stem cell colonies containing the enhanced levels of tyrosine hydroxylase positive cells (Buytaert-Hoefen et al., 2004). Thus if Wnt5a is part of the stromal derived inducing activity it is possible in theory that it may be up regulating glial cell line derived neurotrophic factor receptor expression, and as such if the cells are primed to receive glial cell line derived neurotrophic factor then this would provide a plausible reason for the increased dopaminergic differentiation.

Figure 1.6: The roles of Wnt family members in specifying a dopaminergic fate (redrawn from Castelo-Branco et al, 2003)

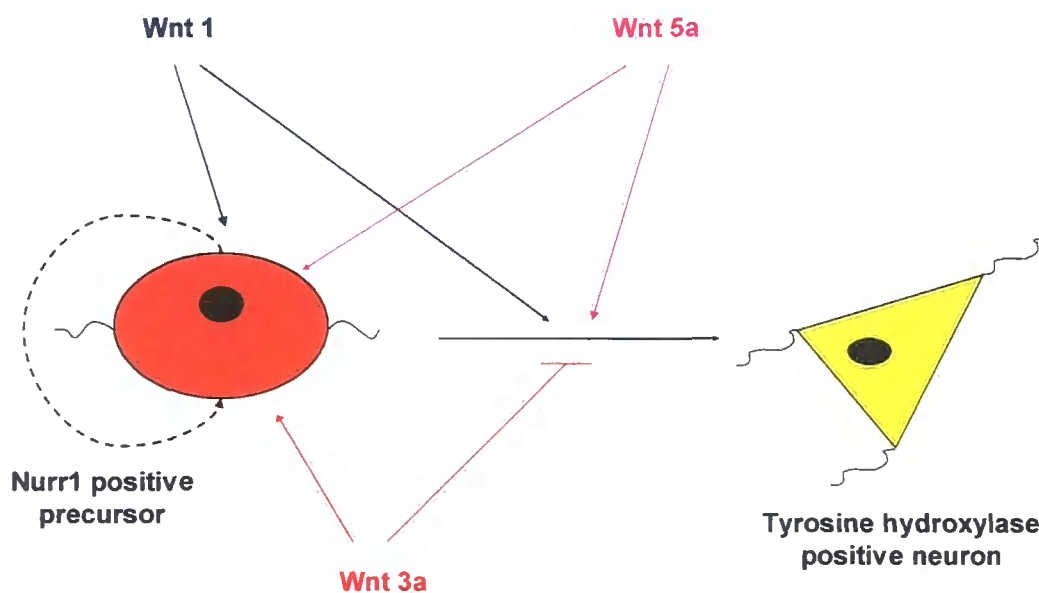


Figure 1.6: Specific Wnt proteins display different effects on the proliferation of Nurr1 positive precursors and their differentiation towards a more mature tyrosine hydroxylase positive neuronal phenotype. The greater the size of the arrow head the stronger the observed effect (based on Castelo-Branco et al., 2003).

Recently it has also been shown that Wnts may help in cell replacement strategies. Over expression of Wnt1 and Wnt5a in murine ventral midbrain cultures enhanced Nurr1 expression, and in the case of Wnt5a also increased the levels of tyrosine hydroxylase. This is indicative of Wnt1 up regulating the number of dopaminergic precursors but would suggest that Wnt5a plays a greater role in the specific differentiation of dopaminergic neurons, which is similar to the findings of Castelo-Branco et al., 2003. The effect of Wnt5a over expression is matched by exogenous application of the molecule and can be blocked by an antibody to it. Inhibitor experiments showed the effects of Wnt5a to be mediated by a non canonical signalling pathway, which is supported by other work in a dopaminergic cell line (Schulte et al., 2005; Parish et al., 2008). In addition to displaying markers of and an electrophysiological profile akin to midbrain dopaminergic neurons, Wnt5a treated cells upon transplantation into a 6-hydroxydopamine mouse model resulted in a marked recovery. They also showed enhanced integration and differentiation and could therefore be a highly efficient source of dopaminergic neurons for use in cell based replacement strategies (Parish et al., 2008).

Similarly positive effects were seen when Wnt5a in conjunction with Fibroblast growth factor 2 and 20 were used in the final stage of the *in vitro* dopaminergic differentiation of a parthenogenetic non human primate embryonic stem cell line. The stem cell derived neurons when transplanted into a 6-hydroxydopamine model displayed improved survival and enhanced the degree of motor recovery. In this and the work of Parish et al., 2008 there was no detectable tumour formation which is of great importance if this is to be developed further for clinical use (Sanchez-Pernaute et al., 2008).

Suspension and Lowered Oxygen Culture as mediators in the acquisition of a dopaminergic phenotype in vitro

Both the use of a physiological oxygen culture environment and growth of cells in suspension have provided a means of producing or even enhancing the generation of dopaminergic neurons *in vitro*. At the core of many protocols using embryonic stem cells is embryoid body formation. This has been extended to the generation of

homogenous structures referred to as spherical neural masses (Cho et al., 2008). These can subsequently be rapidly and efficiently differentiated into dopaminergic neurons using a protocol involving Fibroblast growth factor 8/Sonic hedgehog and ascorbic acid. In addition to this no feeder cells are required during the differentiation process and long term passaging is possible without the spherical neural masses losing their capacity for dopaminergic differentiation. When transplanted into a 6-hydroxydopamine model behavioural recovery is apparent and no tumour formation is observed. No Oct 4 positive cells were detected suggesting the efficient differentiation protocol eliminates any residual undifferentiated embryonic stem cells, thus providing a solid foundation for future development (Cho et al., 2008).

In an earlier study also using human embryonic stem cells dopaminergic differentiation was achieved in a serum free suspension culture system without addition of any dopaminergic phenotype inducing agents. This would suggest that there was sufficient signalling within the cellular aggregates to drive the process of differentiation. In addition bone morphogenetic protein 4 could be used to manipulate the system, demonstrating the potential for use of other exogenous factors to drive the system towards specific cellular fates (Schulz et al., 2004).

This highlights the flexibility of the suspension culture model. By mimicking the 3D environment *in vivo*, 3D cell growth *in vitro* not only allows for more cell-cell interactions but also creates a more complex microenvironment whereby diffusible factors may more readily access their targets; in turn creating a situation that may more closely resemble the scenario *in vivo*. The fact that the determination of cell fate in this system though can still be orchestrated by the application of exogenous compounds, adds to the potential benefits of it. A case may be made that suspension cultures are due to the more complex intrinsic interactions of which they may be capable subsequently more able to respond to external stimuli. However the opposite may also be true as gaining control of an increasingly intricate system might not so easily be achieved.

In the context of dopaminergic differentiation suspension culture may not only be advantageous in that it provides an *in vitro* representation of 3D cell growth but in doing so it creates a physical variable, oxygen pressure, known to have an effect on

the acquisition of a dopaminergic phenotype. When cells are grown as aggregates they are likely to be subjected to different ambient conditions depending on their local position. Cells at the edge of a sphere are likely to receive a greater oxygen pressure than those in the centre (Figure 1.7), (Gassmann et al., 1996). It is therefore possible that at least in part suspension culture favours production of neural cell types as oxygen pressures in the brain are typically lower (Silver & Erecinska, 1998) than those used in standard *in vitro* culture, and this situation may be to some degree recapitulated in the centre of a cellular aggregate.

Figure 1.7: The variation in oxygen pressure within a spherical cellular aggregate (based on Gassmann et al, 1996)

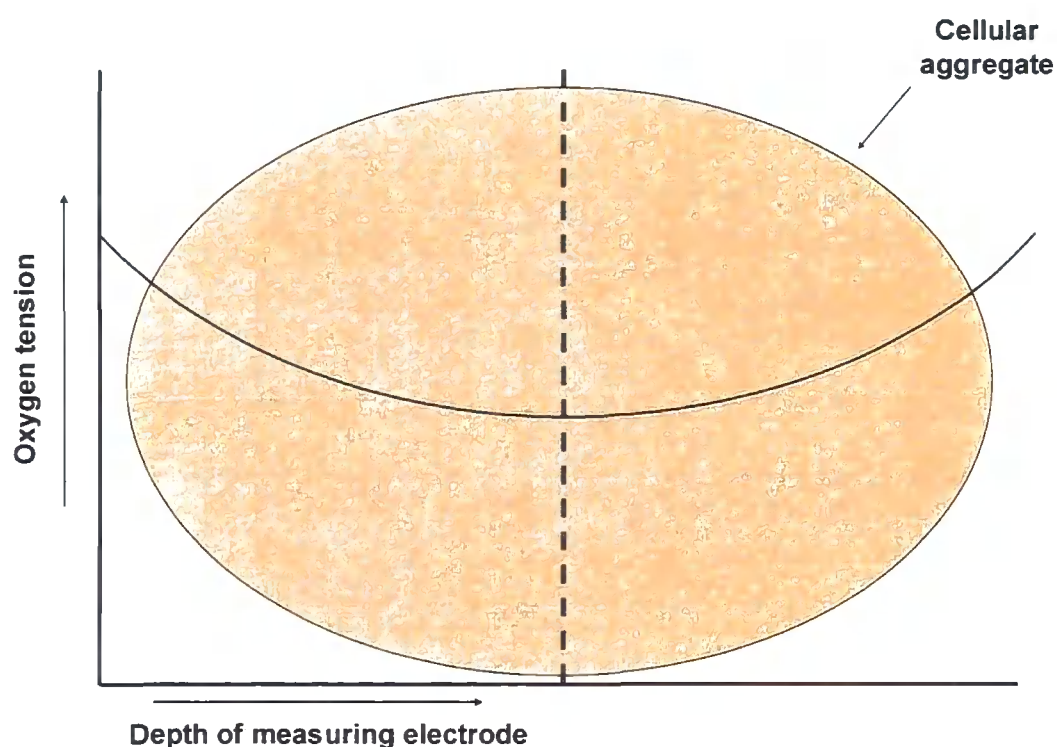


Figure 1.7: Oxygen tension is greatest at the periphery and decreases towards the centre of a spherical cellular aggregate (based on Gassmann et al., 1996)

There has indeed been evidence that may be indicative of this in E12 rat ventral midbrain cultures. In this work a mix of growth factors and a fusion protein TAT-Pax6 were used to promote dopaminergic differentiation of either intact neurospheres or dissociated neurosphere derived cells. Intact neurospheres treated with a combination of growth factors and TAT-Pax6 yielded a significantly higher percentage of tyrosine hydroxylase positive neurons than their dissociated neurosphere derived cell counterparts. Interestingly the tyrosine hydroxylase positive neurons were concentrated in the central region of the neurospheres with long axonal tyrosine hydroxylase positive projections protruding away from them. In contrast more limited axonal projections and a random distribution of tyrosine hydroxylase positive neurons were observed in the dissociated cell cultures (Spitere et al., 2008). This suggests there to be some advantageous effects on dopaminergic differentiation that are attributable to the growth of cells in 3D and the seemingly favourable environment that this provides. It is possible that these effects may be at least in part due to oxygen pressure as the centre of a sphere where dopaminergic differentiation was most pronounced in the work of Spitere et al., 2008 is also the region that would be predicted to receive the lowest partial pressure of oxygen. Other earlier work carried out in an essentially comparable rat E12 mesencephalic precursor model demonstrated a substantially increased capacity for dopaminergic differentiation in $3\pm 2\%$ oxygen in comparison to standard 20% oxygen culture. Thus although there may be an array of other factors involved the notion that 3D growth supports dopaminergic differentiation through a mechanism involving oxygen pressure is feasible, at least in the rat E12 midbrain precursor cell model.

In fact lowered oxygen has a number of favourable effects in the rat E12 mesencephalic cell system. These include increased proliferation of progenitors and a reduction in apoptosis, both of which lead to an increase in total cell number. There was also an increase in the percentage of cells specifically acquiring a dopaminergic phenotype (56% in $3\pm 2\%$ oxygen versus 18% in 20% oxygen). The combined effect of all these factors being a highly desirable nine fold increase in the yield of dopaminergic neurons produced. Progress towards a differentiated state was also hastened (Studer et al., 2000), which is in itself valuable in the context of temporal efficiency, which may be a key requisite in the design of any type of assay system.

It has also been shown that lowered (3%) oxygen can facilitate long term human mesencephalic precursor cell proliferation giving rise to a larger yield of cells that can be subsequently differentiated (Storch et al., 2001). Similarly murine mesencephalic neurospheres were viable and able to proliferate in 3% oxygen for a number of weeks. Contrastingly a 21% oxygen culture environment was prohibitive to long term expansion. It appears from microarray data that a range of genes implicated in cell maturation, the cell cycle and apoptosis are regulated in a differential manner in precursors exposed to 3 as against 21% oxygen over a one to two month period (Milosevic et al., 2005). Differential gene expression was also seen in lowered oxygen cultures in the work of Studer et al., 2000. In particular there was an up regulation of the erythropoietin transcript in 3±2% oxygen, as well as that of Fibroblast growth factor 8. When recombinant Fibroblast growth factor 8b and erythropoietin were applied to the system under 20% oxygen it was found that Fibroblast growth factor 8b increased proliferation in the precursors and that erythropoietin caused a notable dose dependent increase in the number of tyrosine hydroxylase positive cells. A blocking antibody to erythropoietin markedly reduced the dopaminergic neuron yield in both oxygen environments suggesting that application of erythropoietin can mimic the effects of lowered oxygen on the production of dopaminergic neurons (Studer et al., 2000)

The higher oxygen tensions in standard culture conditions are likely to cause the production of reactive oxygen species which may be damaging to sensitive midbrain cell types. Thus it is unsurprising that the survival of embryonic rat mesencephalic neurons is enhanced when they are protected from oxidative stress. Survival of tyrosine hydroxylase positive neurons was increased approximately twofold in 5% oxygen culture conditions. In addition in the presence of three different antioxidants there was also a favourable effect in terms of the number of tyrosine hydroxylase positive cells. The combination of 5% oxygen culture and one of the three antioxidants N-acetyl cysteine promoted tyrosine hydroxylase positive cell numbers further giving a four fold increase (Colton et al., 1995). Therefore it would appear that when dealing with mesencephalic cultures, the use of lowered oxygen is likely to assist in the production of dopaminergic neurons via a range of possible mechanisms.

The employment of lowered oxygen has also recently been applied to mouse embryonic stem cell derived neural precursors in a bid to elicit dopaminergic differentiation. In this work 3.5% oxygen culture resulted in a 1.34 fold increase in tyrosine hydroxylase positive cells; there was an increase in the number of colonies positive for tyrosine hydroxylase expression; however colony size was reduced in comparison to 20% oxygen. In addition expression of tyrosine hydroxylase mRNA was up regulated 1.68 fold in lowered oxygen conditions (Kim et al., 2008). Therefore it would appear that the use of more physiologically matched oxygen culture environments may be of use in other cell systems, which may in turn justify a more widespread usage of the technology.

Engineering a Dopaminergic Phenotype *in vitro*

There are many factors involved in the specification of a dopaminergic fate (Figure 1.8), some of which have been successfully utilised to engineer a dopaminergic phenotype *in vitro*. The transcription factor Nurr1 a member of the orphan nuclear receptor family exemplifies this and has been employed in conjunction with a number of other methods to elicit dopaminergic differentiation. Firstly using the protocol of Lee et al., 2000 involving Sonic hedgehog/Fibroblast growth factor 8 and ascorbic acid, it was possible to increase the production of tyrosine hydroxylase positive neurons via over expression of Nurr1. This was also reflected by a greater ability to produce and release dopamine in Nurr1 transduced cells (Chung et al., 2002). A similar study again in mouse embryonic stem cells demonstrated a consistent enhancement in dopamine release in stage five cultures of Nurr1 transfected embryonic stem cells relative to their wild type counterparts. In this study the percentage of tyrosine hydroxylase positive neurons was increased from 5 to 50% by over expression of Nurr1 alone and this effect was augmented in the presence of Sonic hedgehog and Fibroblast growth factor 8 (Kim et al., 2002). Such positive synergistic effects were built upon in subsequent work involving PA6 co-culture of murine embryonic stem cells in tandem with Nurr1 over expression. This procedure led to an approximately twofold rise in the number of neurons in relation to wild type controls, as well as increasing the proportion of these neurons that were tyrosine hydroxylase positive to more than 50%. When Sonic hedgehog/Fibroblast growth factor 8 and ascorbic acid were added to further facilitate this differentiation around

90% of Tuj1 positive neurons were found to be tyrosine hydroxylase positive, indicating not only that PA6 stromal derived inducing activity and Nurr1 can act synergistically but that the addition of other dopaminergic inducing agents can supplement this effect (Kim et al., 2006a).

Investigations in a mouse neural stem cell model using Nurr1 over expression were also able to show that Fibroblast growth factor 8 and Sonic hedgehog could act in concert with Nurr1 to yield a large number of cells displaying positive immunoreactivity for tyrosine hydroxylase. In the same study human foetal astrocytes could elicit a smaller but significant effect which could be slightly amplified by addition of forskolin and retinoic acid. These two molecules alone though could not exert any tangible effects in this system. The observations with Sonic hedgehog and Fibroblast growth factor 8 intimate that Nurr1 may not act downstream of these molecules, but rather it may operate independently of or in synergy with them to stipulate a dopaminergic phenotype (Kim et al., 2003).

The use of Nurr1 over expression has in addition been coupled to that of other known determinants of a mesencephalic dopaminergic cell fate. The use of retroviral gene delivery to express neurogenin 2 in foetal mouse ventral midbrain progenitors caused increased neuronal differentiation, but did not in itself boost formation of dopaminergic neurons. Generation of cells expressing tyrosine hydroxylase but with an immature morphology and a lack of dopaminergic marker co-marker expression was mediated by expression of Nurr1 alone; however when combined, neurogenin 2 and Nurr1 over expression produce mature tyrosine hydroxylase positive neurons that express a range of other markers (Andersson et al., 2007). These two factors have also been investigated in rat as well as mouse neural precursors. In rat cultures Nurr1 over expression could efficiently yield tyrosine hydroxylase positive cells. However in the murine system generation of tyrosine hydroxylase positive cells was diminished and showed a large disparity dependent upon the embryonic age and brain regions from which the precursors were derived. The variability between the two systems was sustained upon co-expression with neurogenin 2. In rat cultures the production of tyrosine hydroxylase positive cells induced by Nurr1 was curbed in the presence of neurogenin 2. The opposite was true in murine cultures where the dual action enhanced the output of dopaminergic cells (Park et al., 2008), which is similar to the

findings of Andersson et al., 2007; the critical aspect of the work of Park et al., 2008, being to identify clear species specific differences in the development of midbrain dopaminergic neurons. This again highlights the necessity of a range of experimental systems to provide a broad and balanced overview of the process of dopaminergic differentiation in different models.

On this theme Nurr1 over expression has also been utilised to induce a dopaminergic phenotype in rodent adult neural precursor cells from the subventricular zone and white matter. The use of adult cells carries not only ethical advantages but practical ones such as a lack of problems with tumour formation. The difficulty though lies in having the ability to instruct such cells towards specific neurotransmitter phenotypes. Therefore Nurr1 over expression in this instance is to be favoured, especially given that it can be combined with co-expression of Mash1 as well as brain derived neurotrophic factor and neurotrophin 3 treatment to increase the yield of dopaminergic neurons which display functional properties. Although to a limited extent Nurr1 and Nurr1 plus Mash1 engineered subventricular zone adult neural precursor cells were also able to help reverse the behavioural deficit in a Parkinsonian rat model having shown an ability to survive, integrate and differentiate into tyrosine hydroxylase positive neurons *in vivo*. This study gives support to the notion that autologous cell transplantation may in the future be possible (Shim et al., 2007).

Furthermore in both murine and human embryonic stem cell models Nurr1 has been shown to cooperate with Pitx3, a homeodomain transcription factor, in promoting the terminal maturation of such cultures to a midbrain dopaminergic phenotype (Martinat et al., 2006). Moreover these components were investigated in work using neural stem cells from rat, where neither Nurr1 nor Pitx3 expression alone was capable of initiating the production of dopaminergic neurons, indicating that they are both necessary but not sufficient to cause dopaminergic differentiation in this model. When cultured in the presence of E11 ventral midbrain explants, Pitx3 but not Nurr1 transduced neurospheres displayed a clear increase in the number of dopaminergic neurons present. Of these neurons in the explant cultures over half were attributable to the Pitx3 expressing neurospheres (O’Keeffe et al., 2008). This is one case where Nurr1 seems to exhibit little effect, in contrast to what has been found elsewhere, although it reinforces the usage of Pitx3 to achieve acquisition of a dopaminergic

phenotype. A Pitx3-eGFP knock in mouse embryonic stem cell line united with fluorescence activated cell sorting has also been successfully used to purify out an enriched population of dopaminergic neurons from a mixed population of the *in vitro* differentiated embryonic stem cells. This type of selection holds future promise in helping to produce refined populations of cells for use in transplantation strategies (Hedlund et al., 2008).

Figure 1.8: Factors and Signals involved in the specification of a dopaminergic fate (redrawn from Arenas, 2008)

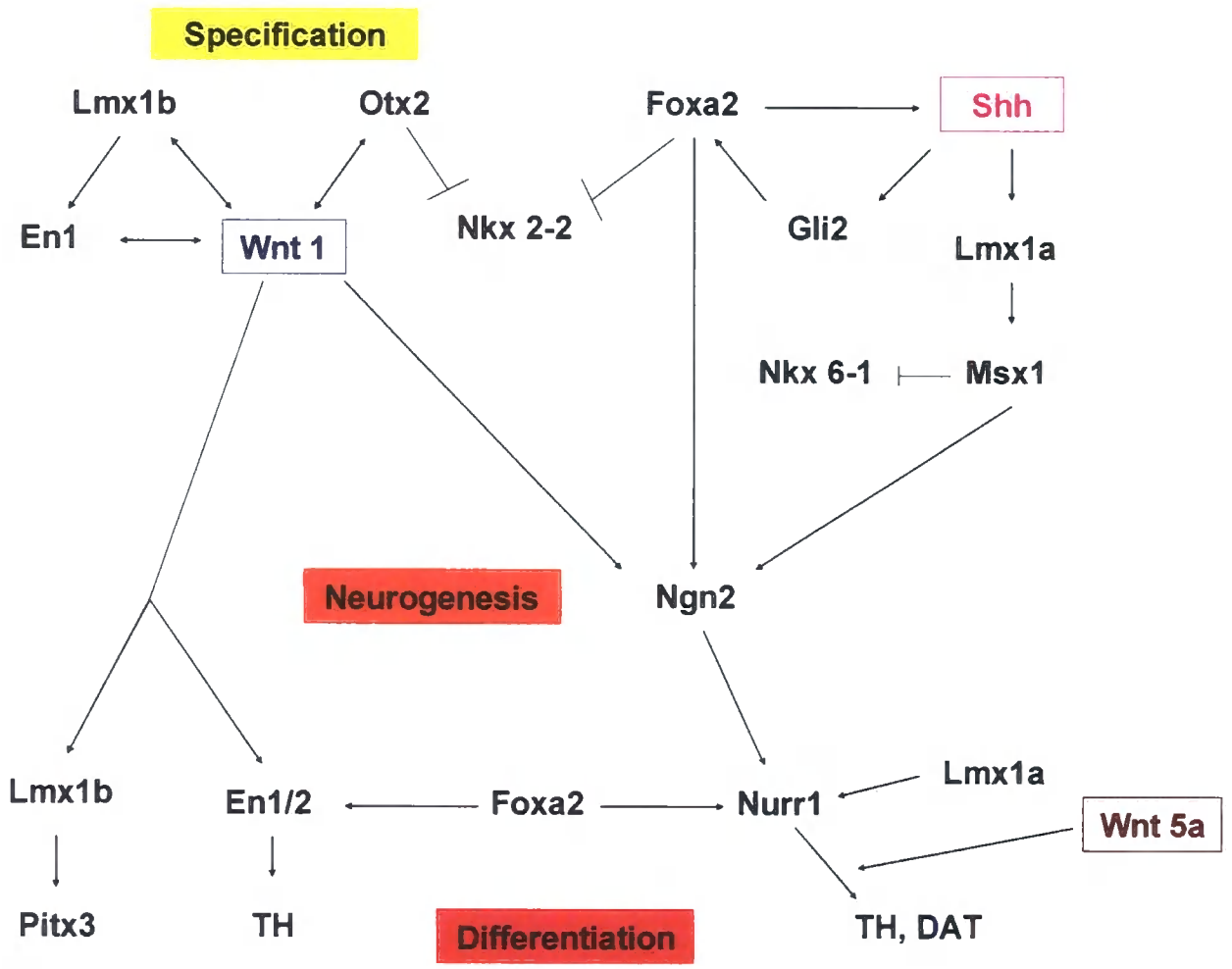


Figure 1.8: Some of the myriad of factors that may play a role in defining a dopaminergic phenotype. This scheme is a reflection of the current understanding of the process and will most probably change with time as new developments continue to arise (redrawn from Arenas, 2008).

Another key determinant of a dopaminergic phenotype *Lmx1a* was successfully over expressed in mouse embryonic stem cells in an expression vector driven by a nestin enhancer that is active in neuronal progenitor cells. The undifferentiated mouse embryonic stem cells were differentiated into nestin positive neuroprogenitors by culturing them on gelatin coated culture ware in the presence of Fibroblast growth factor 2, Fibroblast growth factor 8 and a 1.7mM dose of Sonic hedgehog. The concomitant use of this differentiation protocol and *Lmx1a* over expression lead to a strong induction of *Msx1*, an accompanying reduction in *Nkx 6-1* and the production of a substantial number of *Tuj1* positive neurons that also expressed tyrosine hydroxylase. More importantly these neurons co expressed a panel of other markers that together give a signature unique to dopaminergic neurons. It was also of interest that ventralisation by Sonic hedgehog was essential for *Lmx1a* to induce the formation of dopaminergic neurons, as mouse embryonic stem cells cultured in conditions where this signalling molecule was omitted expressed the dorsal marker *Pax7* the expression of which was absent in Sonic hedgehog treated samples, the result of this absence being an induction in *Msx1* expression but not in tyrosine hydroxylase positive neurons (Andersson et al., 2006). In the same study *Lmx1b* and *Msx1* were also over expressed. *Lmx1b* transfection gave rise to only a small number of tyrosine hydroxylase positive neurons, indicating that it is not as potent an inducer of them as *Lmx1a*. *Msx1* in a comparable fashion to that observed *in vivo* was able to induce *neurogenin2* but not dopaminergic neurons when used alone, but when co-transfected with *Lmx1a* was capable of causing a noticeable induction in tyrosine hydroxylase positive neurons after only 6 days of culture, suggesting it may possess a temporal effect on the dopaminergic differentiation process (Andersson et al., 2006).

When *Lmx1a* had its expression reduced by siRNA in human embryonic stem cell derived neural precursors, there was a congruous decline in the expression of a range of other dopaminergic markers, suggesting that this particular transcription factor is essential for the acquisition of a mature dopaminergic phenotype in human embryonic stem cell derived neural precursors (Cai et al., 2008). This evidence is not only consistent with that detailed previously in defining *Lmx1a* as an important determinant of a dopaminergic fate but also indicates that it plays a role in both murine and human systems, although its activity may be slightly different between species. In the work of Cai et al., 2008 the lack of efficient methods for the

transfection of *Lmx1a* expression vectors into human embryonic stem cell colonies leads to a situation whereby it is still at present unclear if *Lmx1a* is able to induce a dopaminergic phenotype in human embryonic stem cell derived neural precursors. Therefore in the same study monolayers of a human neuroblastoma cell line and primary human neural precursor cells were used to investigate the effects of *Lmx1a* over expression. In contrast to the findings in mouse embryonic stem cells (Andersson et al., 2006), transfected cells did not display an induction in dopaminergic marker expression. This may be due to the human neuroblastoma cell line used being derived from post natal tissue and the primary cultures from midgestational foetal brain. Given the importance of using/applying dopaminergic inducing agents at the optimal temporal window, usually early in development it is possible the cell types used were too far progressed in their development to be responsive to the effects of *Lmx1a* over expression. Alternatively it may be there is a species specific difference with other inductive partners being required for *Lmx1a* to exert an effect in the human system (Cai et al., 2008).

In other work using retroviral mediated transgene delivery, the over expression of *Lmx1a* and *Msx1* was investigated in the human embryonic derived progenitor cell line NGC-407. *Lmx1a*, but not *Msx1*, was able to cause a threefold increase in the yield of tyrosine hydroxylase immunopositive cells, although this rise represented 3% as opposed to 1% of the population, in essence meaning the quantity of such cells was too minimal to be of value in the context of any potential applied usage. These two factors along with *Pitx3* and *neurogenin2* were also over expressed in neurospheres obtained from E14.5 rat ventral mesencephalic progenitor cells. *Neurogenin2* in accordance with it possessing a proneural function exhibited a strong effect on neuronal induction. *Pitx3* was able to reduce expression of *Sox2* and *nestin*, markers of more immature neural cells, but displayed no clear effect on neuronal or glial differentiation. Of further interest were the observations that *Lmx1a* caused very few cells to form neurons and over expression of *Msx1* resulted in increased oligodendrocytic rather than neuronal differentiation. In addition over expression of any of these four factors either alone or in concert with each other had no effect on the ability of the rat neural stem cells to acquire a dopaminergic phenotype (Roybon et al., 2008). This work along with that described previously again reinforces the concept of the need for awareness of species specific differences, the methods of over

expression used and the developmental stage of the cell type chosen for experimentation. It also highlights that certain factors are more optimal in some model systems than others, even so such work is important in helping to highlight idiosyncrasies, such as *Lmx1a* over expression having effects on cell proliferation that are not easy to predict (Roybon et al., 2008). In turn this may temper the usage of comparable approaches in other cell types where this factor exerts a greater effect, if for example the aim was to produce neurons for transplantation, as such cells may have a propensity for tumour formation based on the findings of Roybon et al., 2008. That is not to say though in other models this would be the case but given the potentially large number of unknown variables, it is perhaps still advantageous to try and gain as broad an understanding as possible across the entire range of available experimental systems.

One other possible component of the pathway(s) specifying a dopaminergic fate that has successfully been over expressed to help engineer such a phenotype *in vitro* is *Foxa2*. An expression plasmid containing it was transferred into cultured murine E10.5 mesencephalic explants, resulting in a 4 fold increase in the ratio of tyrosine hydroxylase positive cells. In a similar manner a mouse embryonic stem cell line that had been designed to inducibly express a *Foxa2* transgene was able to show a 7 fold enhancement in the production of tyrosine hydroxylase positive cells (Kittappa et al., 2007).

In an alternative spin on the over expression strategy method, human neural progenitor cells were produced that could release either glial cell line derived neurotrophic factor or insulin like growth factor 1 which may both have neuroprotective effects. These cells were then implanted into a rat 6-hydroxydopamine Parkinsonian model. The cells secreting these two factors upon transplantation were shown relative to human neural precursor or sham controls to help diminish the loss of dopaminergic neurons (Ebert et al., 2008). This approach gives support to the concept that when transplanting cells that possess properties of dopaminergic neurons to try and directly combat the loss of them in models of Parkinson's disease, you could also transfer some supporting cells that express neuro protective or neurotrophic factors. It may even be possible to engineer dopaminergic type cells to fulfil this role, eradicating the need for a secondary cell population. In

turn such approaches may offer synergy with the capacity for endogenous repair within the brain.

Overall therefore efforts to engineer a dopaminergic phenotype *in vitro* or even to support or protect one *in vivo* hold some potential. It may be seen as undesirable to transplant cells that are genetically engineered, but this is debateable and does not restrict the value of such approaches in broadening our understanding of the mechanisms of dopaminergic differentiation in a variety of cellular models.

Alternative means to achieve or augment dopaminergic differentiation

Although for the purposes of this work the procedures used to attempt to achieve a dopaminergic phenotype *in vitro* have been classified into distinct sections, the complexity of the process of dopaminergic differentiation and the myriad of factors involved, mean the vast majority of such methods fall into more than one category. There are also some studies that do not discretely fit into any such banding and these are to be focussed upon here.

First of all ascorbic acid can restore the dopaminergic differentiation efficiency of mesencephalic precursors that is lost when such cells are proliferated or passaged over an extended duration *in vitro*. It also gives rise to a more than 10 fold increase in tyrosine hydroxylase positive cells relative to untreated controls (Yan et al., 2001). As such the use of this molecule is often associated with other protocols for example that of Cho et al., 2008 who used it alongside Sonic hedgehog and Fibroblast growth factor 8 as part of a highly efficient procedure to generate dopaminergic neurons. Like wise it was used in conjunction with these two molecules, along with a cocktail of other dopaminergic inducing agents, including glial cell line derived neurotrophic factor, brain derived neurotrophic factor, transforming growth factor type β 3 and dibutyryl cAMP as well as an initial stromal cell co-culture step in the work of Perrier et al., 2004. The transgenic MS5-Wnt1 stromal cell line used as part of the Perrier et al., 2004 study was also utilised in concert with the bone morphogenetic protein antagonist noggin to enhance the differentiation of two human embryonic stem cell lines into neuroepithelial progenitors which were subsequently driven towards a dopaminergic phenotype in conditions similar to those described in Perrier et al.,

2004, that favour such differentiation (Sonntag et al., 2007). In a comparable fashion formation of dopaminergic neurons from mouse and human embryonic stem cells via co-culture with PA6 cells was enhanced in the presence of noggin (Chiba et al., 2008). The production of tyrosine hydroxylase positive neurons from human embryonic stem cells grown in co-culture with PA6 cells can also be enhanced 5 fold, from 3 to 15% when the culture medium is supplemented with Fibroblast growth factor 20 (Correia et al., 2008). One possible advantage of the use of molecules like Fibroblast growth factor 20 and noggin is that they can be exogenously applied or withdrawn at key points in a differentiation protocol to try and optimise their effect, where as co-culture based methods are far less controlled in that there may be any number of unknown factors being secreted but there is no means to try and control their action to achieve the maximal desired output. There have though quite recently been efforts to reduce or remove the need for the use of co-culture, conditioned media and the often complex culture media containing undefined components that are often associated with dopaminergic differentiation procedures. Iacovitti et al., 2007 used a range of human embryonic stem cell lines to produce neurons expressing markers of a dopaminergic phenotype in just three weeks *in vitro* using a sequential method that involves only the use of chemically defined media additives and substrata that are derived from human sources. Molecules such as noggin and dibutyryl cAMP used in the work outlined previously feature in this strategy, as does a suspension culture step. Although it was not possible to successfully harvest the more mature cells exhibiting a dopaminergic phenotype at the terminus of the differentiation protocol, earlier stage neuronal progenitor cells were able to be collected for transplantation into a 6-hydroxydopamine rat model and after two to three weeks *in vivo* exhibited attributes associated with a dopaminergic phenotype (Iacovitti et al., 2007).

This work by Iacovitti et al., 2007, also highlights the need for an adequate number of neurons or neuroprogenitors for use in transplantation strategies. However cell number may be important in terms of the seeding density used when setting up cultures for differentiation *in vitro* as well. This was exemplified in the work of Ko et al., 2005 using rat mesencephalic precursors, which gave rise to an enhanced yield of neurons displaying dopaminergic characteristics when the cells were plated out at higher densities.

Cell seeding density is just one factor that may influence the dopaminergic differentiation of cells *in vitro*, the substratum on which they grow may also have an effect and this is reflected in a wide number of protocols. Of interest though is the work of Yu et al., 2007 who showed using rat neural stem cells that in the presence of basic Fibroblast growth factor, Heparin and Laminin there was greater dopaminergic differentiation than when the adherent substrate Laminin was omitted. In addition in the work of Iacovitti et al., 2007 the human embryonic stem cell derived embryoid bodies were plated out onto collagen IV coated flasks as opposed to gelatin coated dishes as is the case in many published protocols. This alteration strongly enhanced the number of embryoid bodies that became adherent meaning there was less of a loss of neural progenitors at this stage, essentially enhancing overall cell number (Iacovitti et al., 2007).

In other recent work a range of factors have been identified that may be of value in promoting dopaminergic differentiation *in vitro*. The α -chemokines CXCL1, 6 and 8 have been shown via a range of mechanisms to promote the number of dopaminergic neurons formed in rodent ventral midbrain precursor and neurosphere cultures (Edman et al., 2008a). In a similar fashion the β -chemokines CCL2 and CCL7 appear able to promote the dopaminergic differentiation of rat ventral midbrain precursors. These molecules also demonstrated the capacity to enhance neuritogenesis in both the rat ventral midbrain precursor model and in neurosphere cultures of murine origin (Edman et al., 2008b).

Another possible determinant of a dopaminergic phenotype is the Delta/Notch protein family member Delta like 1. Treatment with this agent during the expansion of murine ventral midbrain progenitors was able to increase the proliferation of such cells and the amount of Nurr1 positive neurons that expressed tyrosine hydroxylase following subsequent differentiation. However when Delta like 1 was applied during this differentiation phase it had no effect on the number of tyrosine hydroxylase positive neurons. When endogenous levels of this molecule were down regulated there was some concomitant loss of dopaminergic marker expression, supporting the concept that it may play a role in or have a permissive effect on the process of dopaminergic differentiation (Bauer et al., 2008).

An additional group of factors that may also possess a role and be of use in driving future attempts to achieve dopaminergic differentiation *in vitro* are the glial cell line derived neurotrophic factor family ligands. In a murine model neurturin and persephin in the presence of endogenous Transforming Growth Factor – β are able to induce dopaminergic neurons, notably when Transforming Growth Factor – β was utilised alongside persephin tyrosine hydroxylase positive cells were produced that appeared less susceptible to the effects of MPP⁺ (1-methyl-4-phenyl pyridinium ion) toxicity (Roussa et al., 2008).

One final distinctly different approach has been based on endeavours to produce cells displaying a dopaminergic phenotype from mesenchymal stem cells. Bone marrow derived adult human mesenchymal stem cells were induced over 12 days *in vitro* using a differentiation procedure that involved the use of Sonic hedgehog and Fibroblast growth factor 8 to form cells reflective of a dopaminergic phenotype based on their marker expression profile. In addition such cells demonstrated an ability to produce and release dopamine in a depolarisation independent fashion, suggesting they are relatively developmentally immature (Trzaska et al., 2007). When RE-1 silencing factor a suppressor of mature neuronal genes in neuronal progenitors was silenced in mesenchymal stem cells which were then differentiated using the Sonic hedgehog/Fibroblast growth factor 8 based approach, more functionally mature cells exhibiting properties of dopaminergic neurons were produced (Trzaska et al., 2008). Like wise using a differentiation cocktail including an array of dopaminergic phenotype inducing agents, Barzilay et al., 2008 were able to produce derivatives from mesenchymal stem cells that expressed markers indicative of a neuronal and more specifically a dopaminergic fate. In this work the cells produced were also able to secrete dopamine upon depolarisation (Barzilay et al., 2008). Such approaches however rely on the ability of mesenchymal stem cells to trans differentiate, this competence of such cells is an area of some controversy and therefore at present these findings though promising need to be viewed in the light of other evidence on this contentious issue (for a broader review see for example Hardy et al., 2008). It may even be that mesenchymal stem cells are capable of acting in a similar manner to stromal cell lines such as PA6 and MS5 whereby they can exert an effect through the secretion of neurotrophic factors. When combined with Sonic hedgehog and Fibroblast growth factor 8 application bone marrow stromal cells from adult mice

were able to help influence embryonic stem cells to form tyrosine hydroxylase positive neurons that were most probably dopaminergic given that they were negative in their expression for the noradrenergic and adrenergic marker dopamine β hydroxylase (Shintani et al., 2008). Overall therefore it can be seen that although some procedures to produce dopaminergic neurons *in vitro* can be classified into generalised categories, many of them rely on the interplay of a number of factors, reflecting the possible diversity between species and universal complexity of the process of dopaminergic differentiation. This is neatly exemplified in two recent papers, one of which combines the use of PA6 co-culture, growth of cells as aggregates and Fibroblast growth factor 20 application (Shimada et al., 2009). Whilst the other is based upon *Lmx1a* over expression in conjunction with Sonic hedgehog and Fibroblast growth factor 8 administration as a means to influence the differentiation of murine and human embryonic stem cells towards a dopaminergic fate (Friling et al., 2009).

Concluding Remarks

There has been a wide array of strategies employed to achieve the production of dopaminergic neurons from a variety of cell types *in vitro*, many of which have been reviewed in this Chapter. There may still be a range of obstacles to overcome in recognising the potential of such *in vitro* derived dopaminergic neurons to act as possible therapeutic agents in transplantation based treatments for Parkinson's disease. However the diversity of cellular based models gives a broad foundation for furthering our understanding of the process of dopaminergic differentiation. They also often neatly complement much of the *in vivo* work that has been carried out elsewhere. If the various means available to promote dopaminergic differentiation and the currently available technologies such as cell sorting are optimised in their usage, it is not too hard to envisage that *in vitro* stem cell derived dopaminergic neurons could be produced, that if shown to be functional may be suitable for use in pharmacological screening of test compounds, that may be of benefit in developing novel drug based therapies for Parkinson's disease in the not inconceivable future. In turn this may help reduce the number of animals used in research and as such this is a highly desirable goal. In addition the process of refining the current protocols and where possible maximising any potential synergistic interactions may help further our

understanding of the underlying developmental process, which may again result in even more productive methods being designed to enhance the yields of dopaminergic neurons it is possible to obtain *in vitro*. There are also as described in the previous section, different factors being identified which themselves may offer fresh insights into the specification of a dopaminergic fate that may subsequently be able to be translated into new protocols aimed at producing more enriched populations of dopaminergic neurons *in vitro*. This area due to its direct relevance to Parkinson's disease is one of active research and may well remain so for the coming years, especially given the many avenues for potential investigation on offer. Some of these routes are pursued experimentally in the remainder of this thesis which is primarily focussed on furthering our current understanding of the process of dopaminergic differentiation *in vitro*. The Tera2.cl.SP12 cell line is the primary vehicle for these studies as it may both help elucidate further details about the pathways involved in the specification of this particular neurotransmitter phenotype and in addition give further insight into embryonal carcinoma cells as a means to study dopaminergic differentiation for those who have ethical objections to the use of human embryonic stem cells.

Chapter 2

Materials and Methods

2.1 Cell Culture

Maintenance of Tera2.cl.SP12 and Ntera2 Embryonal Carcinoma cell cultures

Embryonal carcinoma cells (clone Tera2.cl.SP12 and Ntera2.cl.D1) were maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma or PAA) supplemented with 10% (v/v) fetal calf serum (Gibco or Sigma), 2mM L-glutamine (Cambrex) and 100U/ml penicillin/streptomycin (Gibco) (referred to here in as DMEMFG) in T75 culture flasks (Nunc) at 37°C in a 5% CO₂ incubator (Sanyo) (Przyborski et al., 2000; Przyborski, 2001). Beads were used to passage cells 1:3, every 2-3 days, once they had grown to a confluent state.

Passage 46, 47, 49, 50, 51 and 54 Ntera2 cells were used as these passages were most available and all the passages showed characteristic embryonal carcinoma cell morphology as in Figure 2.1. Figure 2.2 shows the range of passages of Tera2.cl.SP12 cells used during this project. The bulk of the cells used came from passages 15 to 24 and were deemed suitable for use based on their morphology and the time taken for them to reach a confluent state. A flask of cells reaching a confluent state in a T75 in 2 to 3 days after passaging or growing from frozen was seen as viable for use (if the morphology was consistent with that in Figure 2.1).

Maintenance of MG63 cell cultures

The MG63 Osteoblast human cell line (Hattar et al., 2002; Liu et al., 2004) was used as a negative control. Cells were cultured in DMEMFG in T75 culture flasks (Nunc) at 37°C in a 5% CO₂ incubator (Sanyo). Cells were passaged either 1:2 or 1:3, by incubating them at 37°C in a 5% CO₂ incubator (Sanyo), for 5 minutes, in 1ml of 0.25% (w/v) Trypsin/0.1% (w/v) EDTA (in HBSS) (Cambrex). Passage 9, 13, 16, 24, 25, 26 and 27 cells were used experimentally as these passages were readily available in the laboratory at the time of carrying out the work using this cell type.

Please note culture methods for PA6 and mesenchymal stem cells are covered in Chapter 6.

Figure 2.1: Characteristic EC cell morphology



Figure 2.1: A morphological image of a confluent culture of Tera2.cl.SP12 embryonal carcinoma stem cells, this kind of morphology if observed would have been deemed suitable for use based on the advice given of those in the laboratory at Durham who had significant experience of working with this cell line.

Figure 2.2: The different passages of Tera2.cl.SP12 cells used in this project

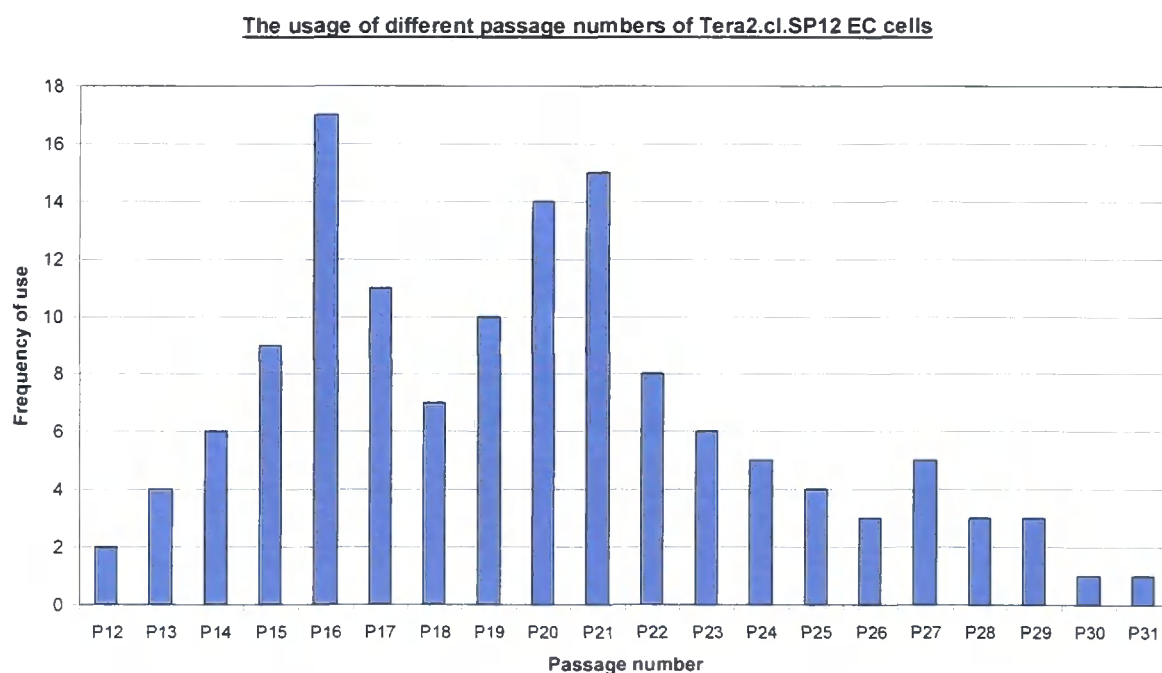


Figure 2.2: The frequency of use of different passage numbers of Tera2.cl.SP12 embryonal carcinoma stem cells in this project. Some of these cells may have been disposed of if not suitable for use judged against the guidelines outlined in the main text and some may have been generated in triplicate due to the process of passaging 1 in 3, this figure though gives an idea of the spread of passage numbers used. In general low passage numbers are preferred as this was seen as good practice in the laboratory at Durham.

2.2 Intracellular staining for Flow Cytometry

Note: All solutions in this and the subsequent methods are made up in sterile distilled water unless stated otherwise in the particular method.

Cultures of Tera2.cl.SP12 EC cells and their differentiated derivatives as well as MG63 controls were examined by flow cytometry. The media they were differentiating or proliferating in was removed and a 1x PBS wash was given, before incubation in 1ml of 0.25% (w/v) Trypsin/0.1% (w/v) EDTA (in HBSS) for 5 minutes in a 37°C, 5% CO₂ incubator (Sanyo), to release the cells from the surface of the culture flask. The Trypsin was then neutralized by addition of 4ml of DMEMFG and any cells remaining loosely bound washed off the surface of the flask by pipetting the DMEMFG/Trypsin-EDTA mix against the side of the flask where cell attachment occurs. A further wash with Phosphate Buffered Saline (PBS) was carried out to optimize the retrieval of the cells of interest. The cell containing mix was then transferred to a 15ml Falcon tube and spun in a centrifuge (Eppendorf 5810R (swing bucket rotor A-4-62)) at 800rpm, 4°C, for 2 minutes and excess fluid was removed to leave a cell pellet. This was re-suspended in PBS and cell number was determined by using a haemocytometer. Once cell number was known the appropriate volume of PBS containing the cells of interest was aliquoted into the required number of Falcon tubes to give 1 million cells per tube. Samples were then centrifuged using the settings above, the supernatant aspirated and the cells re-suspended in 0.875ml of cold PBS. 0.125ml of cold 2% (w/v) paraformaldehyde (Sigma) was added prior to a brief vortex of the mixture. The suspension was incubated for at least 4 hours at 4°C before further centrifugation at 250g for 5 minutes after which the supernatant was removed and the samples were permeabilised as follows.

Pellets were gently re-suspended in permeabilising solution (0.2% (v/v) Triton X-100 (Fisher Scientific) in PBS for cytoskeletal or cytoplasmic antigens, 0.5-1% (v/v) Triton X-100 in PBS for nuclear antigens) at room temperature. Prior to incubation for 30 minutes at 37°C in a water bath, followed by addition of 1ml of blocking buffer (1% (v/v) goat serum (Sigma), 0.2% (v/v) Tween 20 (Sigma), 0.1% (w/v) sodium azide (Sigma) in PBS) before another round of centrifugation at 250g for 5 minutes.

The supernatant was removed and pellets were dispersed in 100µl volumes of diluted primary antibody (see Table 2.1 for dilutions) in blocking buffer or blocking buffer alone for controls, this solution was then transferred to a 96 well plate, where samples were left to incubate in primary antibody for 30 minutes on ice. Post incubation cells were washed 3 times by adding blocking buffer before centrifuging at 250g for 5 minutes (4°C) and removing the supernatant. After the final wash samples were dispersed in 100µl of appropriate secondary antibody diluted in blocking buffer (see Table 2.2), and left to incubate in the dark for 30 minutes on ice. Samples were then washed 3 times using the above method. After washing cell samples were re-suspended in 200µl of blocking buffer and transferred to FACS tubes (BD Biosciences). The volume in each sample tube was then made up to 400µl by adding a further 200µl of blocking buffer to give an ample volume for analysis. This was carried out on a BD Biosciences FACS Calibur machine used in accordance with the manufacturer's guidelines.

Table 2.1: Primary Antibody Dilutions for Flow Cytometry

Antibody Name	Dilution
Nestin (Chemicon) (mouse)	1:600
Nurr1 (Cambridge Bioscience) (rabbit)	1:200
Tuj1 (Covance) (rabbit)	1:1000
Tyrosine Hydroxylase (Sigma) (mouse)	1:1000

Table 2.2: Secondary Antibody Dilutions for Flow Cytometry

Antibody	Dilution
Allophycocyanin conjugated anti-mouse IgG (Jackson Laboratories)	1:600
Allophycocyanin conjugated anti-rabbit IgG (Jackson Laboratories)	1:600

2.3 Cell Surface Antigen Detection Flow Cytometry

Cultures of Tera2.cl.SP12 EC cells and their differentiated derivatives were examined by flow cytometry. The media they were differentiating or proliferating in was removed and a 1x PBS wash was given, before incubation in 1ml of 0.25% (w/v) Trypsin/0.1% (w/v) EDTA (in HBSS) for 5 minutes in a 37°C, 5% CO₂ incubator (Sanyo), to release the cells from the surface of the culture flask. The Trypsin was then neutralized by addition of 4ml of DMEMFG and any cells remaining loosely bound washed off the surface of the flask by pipetting the DMEMFG/Trypsin-EDTA mix against the side of the flask where cell attachment occurs. A further wash with Phosphate Buffered Saline (PBS) was carried out to optimize the retrieval of the cells of interest. The cell containing mix was then transferred to a 15ml Falcon tube and spun in a centrifuge (Eppendorf 5810R (swing bucket rotor A-4-62)) at 800rpm, 4°C, for 2 minutes and excess fluid was removed to leave a cell pellet. This was re-suspended in PBS and cell number was determined by using a haemocytometer. Once cell number was known the appropriate volume of PBS containing the cells of interest was aliquoted into the required number of Falcon tubes to give 1 million cells per tube. Samples were then centrifuged using the settings above, the supernatant aspirated and the cells re-suspended in 1ml of blocking buffer (1% (v/v) goat serum, 0.2% (v/v) Tween 20, 0.1% (w/v) sodium azide in PBS) before centrifugation at 250g for 5 minutes to wash the cells. The supernatant was removed and cells were re-suspended in 100µl of primary antibody diluted in blocking buffer (see Table 2.3 for dilutions) or blocking buffer alone for controls, this solution was then transferred to a 96 well round bottomed plate, where samples were left to incubate in primary antibody for 30 minutes on ice. Post incubation cells were washed 3 times by adding blocking buffer before centrifuging at 250g for 5 minutes (4°C) and removing the supernatant. After the final wash samples were dispersed in 100µl of appropriate secondary antibody diluted in blocking buffer (see Table 2.4), and left to incubate in the dark for 30 minutes on ice. Samples were then washed 3 times using the above method. After washing cell samples were re-suspended in 200µl of blocking buffer and transferred to FACS tubes (BD Biosciences) on ice. The volume in each sample tube was then made up to 400µl by adding a further 200µl of blocking buffer to give an ample volume for analysis. 10µl propidium iodide solution (1.0mg/ml, Sigma)

was also added to each sample to label dead cells. The remaining live cell population was analysed using a BD Biosciences FACS Calibur machine used in accordance with the manufacturer's guidelines.

Table 2.3: Primary Antibody Dilutions for Live Cell Flow Cytometry

Antibody	Dilution
SSEA-3 (IgM, Developmental Studies Hybridoma Bank)	1:5
TRA-1-60 (IgM, a gift from Prof. P. Andrews, University of Sheffield)	1:6
VIN-IS-53 (IgG, Developmental Studies Hybridoma Bank)	1:5
A2B5 (IgM, R & D Systems)	1:100

Table 2.4: Secondary Antibody Dilutions for Live Cell Flow Cytometry

Antibody	Dilution
α -mouse IgG (Cappel)	1:100
α -mouse IgM (Cappel)	1:100

2.4 Immunocytochemistry

Immunocytochemical detection of proteins was performed according to previous methods (Stewart et al., 2003). Cells were cultured on 22mm poly-D-lysine (10 μ g/ μ l (Sigma)) coated glass coverslips in the wells of a six well plate, under a variety of induction conditions. Alternatively they were grown in 12 or 24 well plates (Nunc). Once cells had received the relevant treatment and were ready for analysis they were rinsed in PBS (Cambrex). They were subsequently fixed in ice cold methanol (BDH Biosciences) for 5 minutes, prior to 3 further washes in PBS. The cells were then permeabilised by incubation in 0.1% (w/v) saponin (Sigma), and 0.3% (w/v) bovine serum albumin (Sigma) in PBS (referred to here in as SBP) for 60 minutes at room temperature. Following this the cells were incubated with either primary mouse or primary rabbit monoclonal antibodies diluted in SBP, overnight at 4°C or for 2 hours at room temperature. The following dilutions were used, nestin (mouse, 1:400, Chemicon), TH (mouse, 1:600, Sigma), Tuj1 (rabbit, 1:600, Covance). Cells then received three further 5 minute washes in SBP before incubation with fluorescein-isothiocyanate-conjugated anti-mouse or anti-rabbit IgG secondary antibody (Sigma, diluted 1:400 in SBP) as appropriate for the primary antibody used, for 1 hour at room temperature. To remove unbound secondary antibodies cells were given five 3 minute washes in PBS. Stained coverslips were inverted and mounted onto microscope slides using Vectashield (Vector Laboratories, Inc.) with added Hoescht nuclear stain. Samples were analysed by fluorescence microscopy using an inverted Nikon Diaphot 300 microscope. For cells grown in 12 or 24 well plates Hoescht nuclear stain was added to one of the final PBS washes, prior to analysis via fluorescence microscopy.

Cell Counts

Cell counts based on immunocytochemical observations were made using Image J software with the cell counter add in installed (<http://rsbweb.nih.gov/ij/plugins/cell-counter.html>).

2.5 Western Blotting Protocol

Cultures of Tera2.cl.SP12 EC cells and their differentiated derivatives as well as MG63 controls were examined by Western Blot Analysis. The media they were differentiating or proliferating in was removed and a 1x PBS wash was given, before incubation in 1ml of 0.25% (w/v) Trypsin/0.1% (w/v) EDTA (in HBSS) for 5 minutes in a 37°C, 5% CO₂ incubator (Sanyo), to release the cells from the surface of the culture flask or six well plates (Nunc). The Trypsin was then neutralized by addition of 4ml of DMEMFG and any cells remaining loosely bound washed off the surface of the flask by pipetting the DMEMFG/Trypsin-EDTA mix against the side of the flask where cell attachment occurs. A further wash with Phosphate Buffered Saline (PBS) was carried out to optimize the retrieval of the cells of interest. The cell containing mix was then transferred to a 15ml Falcon tube and spun in a centrifuge (Eppendorf 5810R (swing bucket rotor A-4-62)) at 800rpm, 4°C, for 2 minutes and excess fluid was removed to leave a cell pellet. This was resuspended in 1ml of PBS and transferred to a 1.5 ml eppendorf tube, prior to being spun down in a bench top centrifuge (Eppendorf Minispin Plus (rotor F45-12-11)) at 800rpm for 2 minutes. The supernatant was removed to leave a cell pellet that was subsequently snap frozen in liquid nitrogen, whilst awaiting analysis such samples were stored at -80°C.

Western blot analysis was carried out in accordance with previous methods (Stewart et al., 2003). Collected samples were treated with protein isolation buffer (1% IgePal (Sigma), 50mM Tris-HCl (pH 8.0), 150mM HCl, 1mM MgCl₂ and protease inhibitors (Roche) in distilled water). Protein concentrations were determined using a Bradford based assay, bovine serum albumin (Sigma) was used as a standard; sample volumes were adjusted as appropriate. Sodium dodecyl sulphate-PAGE gels were made according to the method of Laemmli, 1970, using the Bio-rad mini gel system. Loading gels (4% (w/v) polyacrylamide) and separating gels (10% (w/v) polyacrylamide) were poured to a thickness of 0.75mm. Before samples were loaded 20µg of protein were denatured for 3 minutes at 95°C in sample loading buffer (0.5mM Tris-HCl (pH 6.8), 10% (v/v) glycerol, 70mM sodium dodecyl sulphate, 5% (v/v) 2-mercaptoethanol, 15µM bromophenol blue in distilled water). Samples were electrophoresed for 1 hour at 100 volts, prior to the immediate transfer of resolved

proteins onto a nitrocellulose membrane (Whatman) at 100 volts for 2 - 2.5 hours (on ice) using the Bio-Rad mini gel transfer apparatus. Transfer of protein was confirmed by staining membranes with amido black (in distilled water), this was washed out by diluting in water.

For immunoblotting nitrocellulose membranes were blocked in a solution of 5% (w/v) bovine serum albumin or 5% (w/v) dried milk powder (Tesco) in 1x TBS (Appendix A) for 30-45 minutes at room temperature, before being incubated overnight at 4°C with the primary antibody of interest, diluted in either 5% (w/v) bovine serum albumin or 5% (w/v) dried milk powder in 1x TBS as outlined in Table 2.5. Nitrocellulose membranes were subsequently washed every 10 minutes for 1 hour in 1x TBS or 1x TBS-T when using the Nurr1 antibody (Appendix A). Prior to incubation for 45 minutes at room temperature with either mouse or rabbit IgG-horseradish peroxidase linked secondary antibody (Amersham) diluted 1:2000 in either 5% (w/v) bovine serum albumin or 5% (w/v) dried milk powder in 1x TBS. This was followed by three five minute washes in 1x TBS, before the membrane was gently dried using soft tissue and treated with a mixture of equal volumes of ECL detection reagents 1 and 2 (Amersham), that facilitated imaging of the membrane, which was carried out in a Fujifilm LAS 1000 Intelligent Dark Box. Images were also taken of the molecular weight marker ladder to allow confirmation of band size where detected.

Membranes were blocked once again in a solution of 5% (w/v) bovine serum albumin or 5% (w/v) dried milk powder in 1x TBS for 10-15 minutes at room temperature, before being incubated for 1 hour at room temperature in the presence of a primary antibody for Beta-actin diluted in either 5% (w/v) bovine serum albumin or 5% (w/v) dried milk powder in 1x TBS. Blots were then washed every 10 minutes for 40-50 minutes in 1x TBS, before incubation for 45 minutes at room temperature with mouse IgG-horseradish peroxidase linked secondary antibody (Amersham) diluted 1:2000 in either 5% (w/v) bovine serum albumin or 5% (w/v) dried milk powder in 1x TBS. This was followed by three five minute washes in 1x TBS, before the membrane was gently dried using soft tissue and treated with a mixture of equal volumes of ECL detection reagents 1 and 2 (Amersham), prior to imaging of the membrane, which was carried out in a Fujifilm LAS 1000 Intelligent Dark Box.

Table 2.5: Primary Antibody Dilutions for Western Blotting

Antibody Name	Dilution	Blocking/Antibody Incubation Solution	Molecular weight (kDa)
Beta-actin (Sigma) (mouse)	1:5000	5% (w/v) milk powder/5% (w/v) bovine serum albumin	42
Nurr1 (Cambridge Bioscience) (rabbit)	1:500	5% (w/v) bovine serum albumin	67
Tuj1 (Covance) (rabbit)	1:5000	5% (w/v) bovine serum albumin	55
Tyrosine Hydroxylase (Sigma) (mouse)	1:10000	5% (w/v) milk powder	62

2.6 MTS Assay

To assess cell proliferation, the commercially available colorimetric CellTiter 96® Aqueous One Solution Cell Proliferation Assay kit (Promega) was used. This assay contains a tetrazolium compound, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) and phenazine ethosulfate (PES), which combine to make a stable solution. Cells reduce the reagent and this gives a coloured formazan compound.

The amount of product formed was quantified using a Nanodrop spectrophotometer at an absorbance of 490nm, with the measured absorbance being directly proportional to the number of viable cells in culture. For every 1ml of media, 200µl of MTS reagent was added to the culture well of a 24 well plate containing the sample of interest and left to incubate for 4 hours at 37°C. Media was changed prior to adding the MTS reagent to remove variation in the colour of the media that was covering the cells. A control well that did not contain any cells was set up to take into account the background absorbance of the MTS reagent. After the 4 hour incubation, absorbance was measured at 490nm. The absorbance reading for the highest (on average) expressing sample was normalised to an arbitrary value of 100 when presenting the data.

2.7 RNA Isolation Using Tri-Reagent[®] (Sigma)

Homogenisation

Cultures of Tera2.cl.SP12 EC cells and their differentiated derivatives as well as MG63 controls grown as monolayers had the media in which they were differentiating or proliferating in removed, prior to being washed in 1x PBS. Suspension culture samples were collected by collecting the media containing the cellular aggregates into a Falcon tube, prior to centrifugation at 800rpm, 4°C, for 2 minutes (Eppendorf 5810R (swing bucket rotor A-4-62)), to give a sample pellet. This was resuspended once in 1x PBS, before further centrifugation at 800rpm, 4°C, for 2 minutes (Eppendorf 5810R (swing bucket rotor A-4-62)). Cells (or aggregates of them) were then lysed by incubating them for 10 minutes at room temperature in an appropriate volume of Tri-Reagent (1ml for a single well of a six well plate, T25, or Falcon tube, 3ml for a T75). The cell lysate was then passed through a pipette several times before 1ml of it was transferred to a 1.5ml eppendorf tube. Insoluble materials were removed from the homogenate by centrifugation at 12,000g for 10 minutes at 4°C (Eppendorf 5415R (cooled standard rotor F45-24-11) all subsequent centrifugation steps recorded with a g value were carried out using this centrifuge and rotor combination). The supernatant (containing RNA) was transferred to a fresh tube.

Phase Separation

0.2ml of Chloroform (Fisher Scientific) was added (per 1ml of Tri-reagent used in the homogenisation step) to the sample containing eppendorf tubes, which were securely capped and shaken vigorously by hand for 15 seconds. Samples were then incubated at room temperature to allow separation into layers, prior to being spun in a cooled bench top centrifuge at 12000g for 15 minutes at 4°C. After this centrifugation, 3 layers were present; the RNA resides exclusively in the uppermost aqueous phase.

RNA Precipitation

The RNA containing aqueous phase was carefully transferred to a fresh tube, with care being taken, not to carry over material from the other DNA and protein containing phases. 0.5ml of isopropanol (Fisher Scientific) (per 1ml of Tri-Reagent used in the homogenisation step) was added to the aqueous phase to precipitate the RNA and this sample was mixed by inverting it 2-3 times, before incubating for 10 minutes at room temperature, prior to centrifugation at 12000g for 10 minutes at 4°C. A pellet containing the RNA precipitate is formed on the outer side of the sample containing eppendorf tube.

RNA Wash

The supernatant was removed to leave the pellet which was then washed once with 75% (v/v) ethanol (made up from a mix of 3 parts 100% ethanol (Sigma) to 1 part RNase free water (Sigma)) (at least 1ml of 75% (v/v) ethanol was used per 1ml of Tri Reagent used in the homogenisation step). The pellet in 75% (v/v) ethanol washing solution was centrifuged at 10000g for 10 minutes at 4°C.

Redissolving the RNA

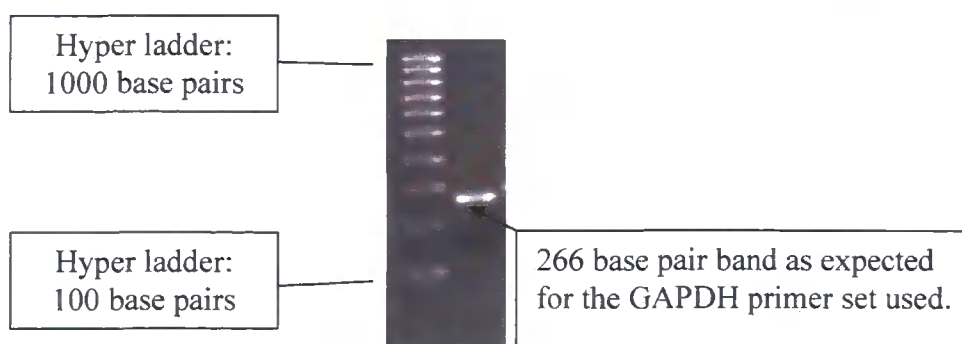
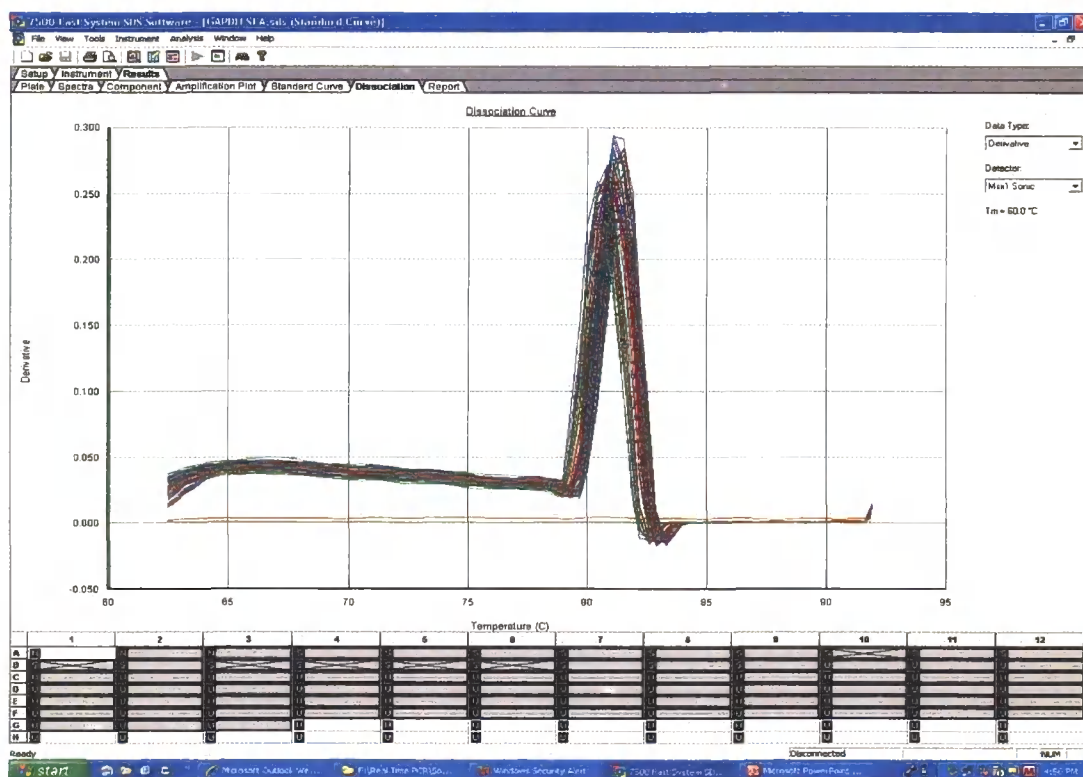
After centrifugation the supernatant was removed to leave just the RNA pellet which was briefly air dried for 5-10 minutes. The RNA was then dissolved in RNase free water (15-75µl) by passing the solution through an RNase free pipette tip a few times or by flick mixing. Samples were then incubated for 5 minutes at 55-60°C. The purity (A260/280nm) and concentration (A260nm) of the RNA sample were then measured using a Nanodrop 1000 spectrophotometer. RNA samples were stored at -80°C prior to subsequent usage.

2.8 Reverse transcription & Real Time Polymerase Chain Reaction

For mRNA expression analysis by real time PCR, total RNA was obtained from three different passages of cells. Total RNA was isolated using Tri-Reagent (Sigma) according to the method presented previously, which was based upon the manufacturers' instructions. The amount of RNA was quantified (A260nm) and the purity (A260/280 ratio) assessed using a Nanodrop analyser. Purity values were deemed suitable within the range 1.7 – 2.1. 4µg of total RNA was reverse transcribed to cDNA using the commercially available Taqman reverse transcription kit (Applied Biosystems) in accordance with the manufacturer's instructions. Real time PCR was performed on an Applied Biosystems 7500 Fast Real Time PCR instrument using SYBR green technology. Primers were synthesised by VHBio (sequences are provided in Table 2.6) for the following genes: β -actin, β -catenin, D1, D2, DAT, Dopa Decarboxylase, Engrailed 1, FGFR4, Frizzled 8, GAPDH, Lmx1a, Msx1, Nestin, Ngn2, Nkx 6-1, Nurr1, Oct4, Otx2, Patched, SLC23A1, Sox1, TBP, TH, Tuj1(Beta III tubulin). A standard curve was constructed for each primer set to facilitate absolute quantification of sample cDNA; samples were normalised by comparing the mean quantity of the gene of interest against the mean quantity of the housekeeping gene GAPDH. To further clarify that the expression of this gene (GAPDH) was relatively consistent and thus suitable for use samples were also normalised against another house keeping gene which possessed a broadly different cellular function usually β -actin but on occasion TBP. The results for the gene of interest were compared to see if the trends were comparable when normalised against the different housekeeping genes, to try to remove some of the uncertainty that the housekeeping gene normalisation process was not affecting the results due to differences in its expression that could have arisen from the varying sample growth conditions. Each standard curve was constructed from a range of dilutions (1:5, 1:10, 1:20, 1:50, 1:100, 1:1000 and NTC), with R^2 values exceeding 0.99. Standards and samples were amplified on the same reaction plate. Three technical repeats of three biologically independent samples were run; results show the average across the three independent biological samples. Each individual reaction mix consisted of the following: 5µl SYBR green mastermix (Applied Biosystems), 3.2µl RNase free water (Sigma), 0.4µl of forward and reverse primer (made up in RNase free water) and 1µl

of cDNA diluted 1:5 in RNase free water. The thermal cycling parameters were as follows: 50°C for 2 minutes, 95°C for 10 minutes, 95°C for 15 seconds followed by 60°C for 1 minute (x40 cycles or x50 cycles for Otx2 only), a dissociation step of 95°C for 15 seconds, 60°C for 15 seconds and 95°C for 15 seconds was also added. This additional stage was included to check primer specificity and for the absence of primer dimers. PCR product size and specificity were also checked on an agarose gel, as shown in Figure 2.3.

Figure 2.3: Dissociation Curve and Agarose gel image for GAPDH



The hyper ladder marker gives a size reference, and the presence of a single band at 266 base pairs indicates that there is only one product of the predicted correct size. This is in agreement with the single peak on the dissociation curve that indicates one specific product has been formed.

Note: This method was used to produce the real time PCR data in Figures 3.1, 3.2, 3.3, 3.4, 4.4, 4.6, 4.7, 4.10, 4.11, 4.15, 4.16, 4.17, 4.18, 4.19, 4.20, 4.30, 5.3, 5.4, 5.5, 5.6, 5.9, 5.10, 5.11, 5.12, 5.13, 5.14, 5.15, 5.16, 5.17, 5.18, 5.19, 5.20, 5.21, 6.2, 6.3, 6.4, 6.5, 6.16 and 6.17.

This method and method 2.9 that follows have never been used in tandem; they have only ever been used in isolation hence why both are included separately in this Thesis.

Table 2.6: Primer Sequences

Gene	Forward	Reverse	Source/Reference
B-actin	5' CGCCCCAGGCACCAGGGC 3'	5' GCTGGGGTGTGAAGGT 3'	RTPrimer DB ID:2238
B-catenin	5' TGCAGTTCGCCTTCACTATG 3'	5' ACTAGTCGTGGAATGGCACC 3'	Tulac et al., 2003
D1	5' ATCCTCACTGCCTGTTTC 3'	5' TGTCGGAACCTGATAACG 3'	Dr. V. Lakics
D2	5' TGAATCTGTCCTGGTATGATGATG 3'	5' GTGTGGCATAGTAGTTGTAGTGG 3'	Dr. V. Lakics
DAT	5' TACCTGAGCGTTGACTTC 3'	5' TGGTGAACCTGTTGTAGC 3'	Dr. V. Lakics
Dopa Decarboxylase	5' TGTCCAGCTGTCCCATGAGTT 3'	5' GAATGACTTCCACACAGATTTCAA 3'	
Engrailed 1	5'-CCCTGGTTTCTCTGGGACTT-3'	5'-GCAGTCTGTGGGGTCGTATT-3'	Yan et al., 2005
FGFR4	5' GGGTCCTGCTGAGTGTGC 3'	5' TGGGTAACGTGCCTATTCG 3'	Mawrin et al., 2006
Frizzled 8	5' GACACTTGATGGGCTGAGGT 3'	5' CAAATCTCGGGTCTGGAAA 3'	Rozen & Skaletsky, 2000
GAPDH	5'ATGGGGAAGGTGAAGGTCGGAG 3'	5'TCGCCCCACTTGATTTGGAGG 3'	RTPrimer DB ID:2072
Lmx1a	5' TGAGGAAGGCAAGGACCATAA 3'	5' ATGCTCGCCTCTGTTGAGTTG 3'	
Msx1	5' AAGAGACTACAAGAGGCAGAG 3'	5' CCGAGAGGGAAGGAGAGG 3'	Dr. V. Lakics
Nestin	5' CAGCGTTGGAACAGAGGTTGG 3'	5' TGGCACAGGTGTCTCAAGGGTAG 3'	Strojnik et al., 2007
Ngn2	5'TGTCCTCCAATTCCACCTC 3'	5' CTGCCAATAGTCCATGTCTG 3'	Dr. V. Lakics
Nkx6-1	5' AAGAAGCAGGACTCGGAGACAG 3'	5' CAGAGGCTTATTGTAGTCGTCGTC 3'	Dr. V. Lakics

Nurr1	5' CCTGGCTGTTGGGATGGTC 3'	5' TGTGGGCTCTTCGGTTTCG 3'	Dr. V. Lakics
Oct4	5' GAGAACCGAGTGAGAGGCAACC 3'	5' CATAGTCGCTGCTTGATCGCTTG 3'	Willems et al., 2006
Otx2	5' AGAGGAGGTGGCACTGAAAA 3'	5' ATTGGCCACTTGTTCCTC 3'	Boon et al., 2003
Patched	5' TCCTCGTGTGCGCTGTCTTCCTC 3'	5' CGTCAGAAAGGCCAAAGCAACGTGA 3'	Regl et al., 2002
SLC23A1	5' AAAGCCTTGCAGCATACCTACAT 3'	5' TGTCAGCATCTAGGAAGAACAGCT 3'	RTPrimer DB ID:2566
Sox1	5' CAATGCGGGGAGGAGAAGTC 3'	5' CTCTGGACCAAAGTGTGGCG 3'	Kim et al., 2006b
TBP	5' TGCACAGGAGCCAAGAGTGAA 3'	5' CACATCACAGCTCCCCACCA 3'	RTPrimer DB ID:2627
TH	5' CCTGCTTCTCAGCGCAACAG 3'	5' GAGCCAGGACAGGACCTCAC 3'	Dr. V. Lakics
Beta III tubulin	5' GGCCAAGTTCTGGGAAGTC 3'	5' CGTTGTAGTAGACGCTGATCC 3'	Kumar et al., 2007

Table 2.6: Primer sequences and sources of reference, RTPrimer DB refers to primers found at <http://medgen.ugent.be/rtpimerdb/>. Primers with no source/reference were designed using Primer Express 3.0 (<https://products.appliedbiosystems.com/ab/en/US/adirect/ab;jsessionid=JXpzKJFHZ2hLvyFdddbpnsMfPNm7XB23jTFLy0yw61GKSQsgz4!950160382?cmd=catNavigate2&catID=605537>).

2.9 Real Time Polymerase Chain Reaction (Lilly)

For mRNA expression analysis by real time PCR, total RNA was obtained from three different passages of cells. Total RNA was isolated using Tri-Reagent (Sigma) according to the method presented previously, which was based upon the manufacturers' instructions. The amount of RNA was quantified (A260nm) and the purity (A260/280 ratio) assessed using a Nanodrop analyser. Purity values were deemed suitable if they lay within the range 1.7 – 2.1. Samples were then DNase treated with the commercially available Turbo DNA-free™ Kit. After this the TaqMan Reverse Transcription Kit (Applied Biosystems) was used to synthesise first strand cDNA. The final concentration of RNA in the reaction was 1 µg / 100µl for all samples. Quantitative real-time RT-PCR was performed using SYBR Green chemistry with a final concentration of 1000 ng/ml template in the PCR on an ABI Prism 7900HT real-time PCR system (Applied Biosystems, 384-well plate, 5 ml final reaction volume, in quadruplicates). The following cycling conditions were used: 50°C for 2min (stage1); 95°C for 10min (stage 2); 95°C for 15sec; 60°C for 1min (stage 3) x 40. At the end of the run threshold cycles (Ct) were determined for each PCR reaction on the plate. To establish the relative expression of TH, Nurr1, Nkx6-1, Msx1, DAT, D1 and D2 genes in various samples, the normalisation procedure described by Vandesompele et al., 2002 has been used. Briefly, dCt values of a given target gene were calculated for each sample, relative to a selected calibrator (the highest expresser sample for that gene), then converted into fold differences by raising them to the power of 2. Similarly, the dCt values of selected reference genes (beta-actin, RNA-polymerase2, PSMB2), were created for each sample, relative to the same calibrator sample used for the respective target gene, and were also converted to fold differences. The expression of the target gene was then normalized with a factor created by using the geometric mean of the fold difference values of the 3 reference genes in each sample. Finally, the relative quantities of the various samples have been expressed as a percentage relative to the chosen calibrator sample, which was arbitrarily set to 100%.

Note: This method was used to generate the data presented in Figures 3.5, 3.6, 4.8, 4.12, 4.13, 4.14 and Appendix B. My thanks go to Dr. V. Lakics for providing this method and carrying out this analysis as mentioned in the acknowledgements.

2.10 Statistical Analysis

All bar charts were produced in Microsoft Excel; custom error bars representing either the standard deviation or standard error of the mean were added using this package. All statistical analyses were performed using SPSS 15.0 for Windows; the line graph shown in Chapter 3 was also produced using this package.

The statistical tests used can be categorised as a Student's T-test, a one way anova including Tukey post hoc analysis to compare differences between groups, or a two way anova. Significance is attributed in all these tests at a Sig. value ≤ 0.05 . T-test outputs where significant are shown on bar charts by lines linking the samples between which there is a statistically relevant difference, an exemplar T-test data output is shown in Figure 2.4. From here on in a one way anova output refers to the one way anova including Tukey post hoc analysis, for presentation purposes an example is given in Figure 2.5. Significant differences determined using this test are indicated by lines linking the samples between which there is a statistically relevant difference. Two way anova outputs are exemplified in Figure 2.6 which also highlights the relevance of the line graph (used in Chapter 3) to support such analysis.

Figure 2.4 Example T-test output

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
									Lower	Upper
Expression	Equal variances assumed	10.189	.033	-6.460	4	.003	-1093.660	169.30746	-1563.73	-623.587
	Equal variances not assumed			-6.460	2.067	.021	-1093.660	169.30746	-1799.88	-387.443

If the Sig. value for the Levene's test is greater than 0.10 then the variances can be assumed to be equal. Here it is not, so unequal variances are assumed.

In this example unequal variances are assumed based on the result of the Levene's test (blue box), the red box highlights the result is significant on this occasion. If equal variances had been assumed (i.e. if there was a value greater than 0.10 in the blue box) then the upper figure 0.03 would be used as an indicator of significance.

Figure 2.5: Example one way anova output

Multiple Comparisons

Dependent Variable: Expression
Tukey HSD

The condition column shows the conditions being compared.

(I) Condition	(J) Condition	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
EC	17 Day (R.A. + Wnt1)	-1.56700	2.71339	.837	-9.8924	6.7584
	3W R.A.	-11.28167*	2.71339	.014	-19.6071	-2.9562
17 Day (R.A. + Wnt1)	EC	1.56700	2.71339	.837	-6.7584	9.8924
	3W R.A.	-9.71467*	2.71339	.027	-18.0401	-1.3892
3W R.A.	EC	11.28167*	2.71339	.014	2.9562	19.6071
	17 Day (R.A. + Wnt1)	9.71467*	2.71339	.027	1.3892	18.0401

*. The mean difference is significant at the .05 level.

The mean difference column highlights any differences (between the samples outlined in the condition column (blue box)) that are significant at the 0.05 level, where an asterisk is present there is a correlation with the value in the Sig. column (red) being less than 0.05 as can be seen in this example.

The Sig. column shows whether there are significant differences between the sample groups outlined in the condition column (blue box).

Figure 2.6: Example two way anova output

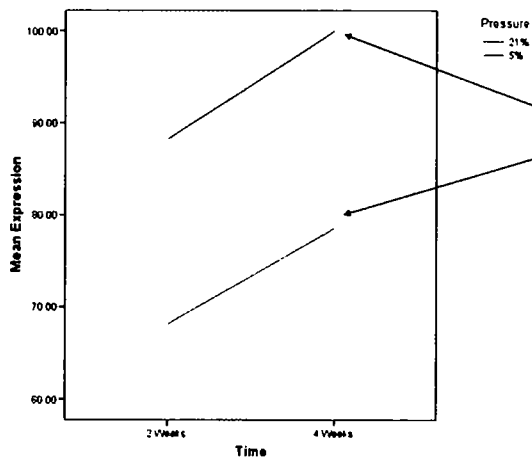
Tests of Between-Subjects Effects

Dependent Variable: Expression

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1667.109 ^a	3	555.703	.204	.891
Intercept	84097.139	1	84097.139	30.841	.001
Pressure	1293.179	1	1293.179	.474	.511
Time	372.604	1	372.604	.137	.721
Pressure * Time	1.326	1	1.326	.000	.983
Error	21814.080	8	2726.760		
Total	107578.329	12			
Corrected Total	23481.189	11			

a. R Squared = .071 (Adjusted R Squared = -.277)

The Sig. values in the red box if ≤ 0.05 would be deemed significant (in this case they are not). They display significance in relation to the variables defined in the Source column (blue), both individually for time and pressure and for the two combined, to see if there is an interaction between the variables being assessed.



Parallel lines on the line graph are indicative that there is no interaction between the variables (pressure and time). The almost perfectly parallel lines in this example correspond with the almost total lack of significance (Sig. value of 0.983) in the two way anova output above.

Chapter 3

Characterisation of the
Tera2.cl.SP12 Human EC
cell system as a model to
study dopaminergic
neurogenesis, in the
presence of retinoic acid
and in a lowered oxygen
culture environment

3.1 Introduction

In any cell culture system there are a number of variables, such as the media and physical environment. There are already well established protocols (for example Stewart et al., 2003) for the culture of Tera2.cl.SP12 cells and their retinoic acid treated derivatives, therefore a logical first step in this project was to characterise the embryonal carcinoma stem cell system pre and post retinoic acid differentiation to begin to gain an understanding of its potential as a model to study dopaminergic neurogenesis and this forms the basis of this Chapter. In addition given the availability and ease of use of lowered oxygen cell culture technology in the laboratory and the findings of Studer et al., 2000, who were able to show enhanced dopaminergic differentiation from rat mesencephalic precursor cells cultured in physiological oxygen conditions, it was also decided that the Tera2.cl.SP12 system would be investigated in a 5% oxygen environment as well. The rationale for doing these extra experiments being that if 5% oxygen could enhance dopaminergic differentiation of the embryonal carcinoma stem cells then it could be easily utilised to try and augment subsequent efforts to achieve the production of an enriched population of dopaminergic neurons. The evidence provided by Colton et al., 1995, that 5% oxygen culture could provide an approximately twofold increase in survival of tyrosine hydroxylase positive neurons in primary cultures of embryonic rat midbrain gave further support to the argument for using lowered oxygen, as in the initial stages of experimentation it is impossible to know when expression of dopaminergic markers by the cells under study will occur or peak, so any variable that may stabilise this expression to a detectable level at a measured time point is highly favourable.

This also highlights the need to define the time period of retinoic acid induced differentiation. Work using the four and five week old retinoic acid differentiated derivatives of the human embryonal carcinoma stem cell line Ntera2.D1 showed that there was significantly more tyrosine hydroxylase positive cells (60%) in four week retinoic acid treated cultures than in their five week ($\leq 15\%$) counterparts (Zigova et al., 2000). This finding, combined with the need for temporal efficiency if the Tera2.cl.SP12 system were to be viable for use in screening assays, lead to the decision that the longest time point to be used in this study would be four weeks of retinoic acid treatment. A two week retinoic acid treatment time point was included to bridge the gap between the

embryonal carcinoma stem cell and four week retinoic acid treated samples in the studies into the effects of different oxygen concentrations, and a series of short time points, 1, 3, 5 and 7 days were used to investigate the possible favourable effects of retinoic acid in normoxic conditions, as well as to gain greater insight into, and understanding of the Tera2.cl.SP12 cell line in the context of it as a model to study dopaminergic neurogenesis. The next two sections aim to outline the purpose of and background to the investigations that are subsequently reported in this Chapter.

The Induction of Nurr1 with Retinoic Acid

The transcription factor Nurr1 is expressed in a variety of regions of the brain such as the hippocampus, hypothalamus and cerebellum (Zetterstrom et al., 1996) and is a member of the orphan nuclear receptor family. In mouse the mRNA is expressed from embryonic day E10.5 in the ventral mesencephalic flexure and continues into adulthood (Zetterstrom et al., 1997). This study also showed by immunological methods that tyrosine hydroxylase (the rate limiting enzyme in dopamine biosynthesis) was not expressed in the ventral mesencephalon of Nurr1^{-/-} mice where it is usually found. Other dopaminergic cell groups for example those of the pontine locus coeruleus (A6), displayed no differences in tyrosine hydroxylase immunoreactivity between wild type and Nurr1 deficient mice. This coupled with an absence of positive tyrosine hydroxylase immunostaining at all pre-natal stages in the developing midbrain when it would normally be expected from E11.5 in mouse (Foster et al., 1988) strongly indicate Nurr1 as a critical determinant in the differentiation of ventral midbrain dopaminergic neurons (Groups A8-A10) (Zetterstrom et al., 1997).

High performance liquid chromatography analysis of the levels of dopamine and its metabolite DOPAC in portions of ventral mesencephalon and striatum showed an absence of dopamine in Nurr1 deficient mice, and a reduction in the amount of the metabolites in heterozygous new born animals, indicative of a gene dosage effect on nigrostriatal dopamine levels. This second group though appeared healthy and were not hypoactive as was the case for the Nurr1^{-/-} population which also died soon after birth (Zetterstrom et al., 1997). Overall these results suggest Nurr1 is not only a key factor in the production

of a ventral midbrain dopaminergic phenotype but is also a contributor to the maintenance of a differentiated phenotype in adult mice.

In a similar fashion Nurr1 is expressed in human Ntera2 embryonal carcinoma cells and their 3, 4 and 5 week retinoic acid differentiated derivatives. In addition all the observed tyrosine hydroxylase positive Ntera2 derived neurons in these cultures were Nurr1 positive (Misiuta et al., 2003). These findings therefore suggest that Nurr1 expression is maintained to some level throughout the retinoic acid driven differentiation process and that the human embryonal carcinoma cell system may possess similarities to the mouse model where tyrosine hydroxylase expression may be linked to that of Nurr1. Subsequent work also showed that after 1 day of retinoic acid treatment of Ntera2 cells it was possible to achieve a significant rise in expression of Nurr1 from approximately 12 to 20% (Misiuta et al., 2006). This is a rapid and therefore potentially desirable effect especially given the importance of time as a variable. Another study where Nurr1 was over expressed again carried out in the Ntera2 cell line showed that cultured Ntera2.Nurr1 transfected cells were nestin negative despite the fact 'wild type' Ntera2 cells are nestin immunopositive indicating that Nurr1 may trigger an early commitment towards the neuronal lineage. It was observed that after 4 weeks of retinoic acid treatment Ntera2 cells over expressing Nurr1 exhibited greater tyrosine hydroxylase expression than their non-transfected Ntera2 counterparts (Hara et al., 2007). These findings give additional support to the concept that Nurr1 may influence tyrosine hydroxylase expression in human embryonal carcinoma stem cells, this is in stark contrast to observations in human neural progenitor cells where tyrosine hydroxylase gene regulation appears to be independent of Nurr1 (Jin et al., 2006), showing there may not just be species specific differences in dopaminergic neurogenesis but cell type specific idiosyncrasies within a species. They also indicate that Nurr1 may help promote neuronal differentiation which is highly desirable given the mixture of cell types produced following retinoic acid treatment of Tera2.cl.SP12 cultures. Therefore the key aspects to test for were: can Nurr1 be induced rapidly with retinoic acid treatment in the Tera2.cl.SP12 system and if so does this have any effect on enhancing neuronal differentiation or can it be linked to the stimulation of neurogenesis in this cell line?

Cell Culture at Lower Oxygen Tensions

The use of lowered oxygen culture is founded on the principle that greater mimicry of the in vivo environment of the mammalian brain where oxygen pressures are much lower, around 1 to 5% (Silver & Erecinska, 1998) than those used in standard (20 to 21% oxygen) cell culture; may enhance the production of neuronal cell types. The main evidence that showed physiological oxygen conditions could help promote acquisition of a dopaminergic phenotype was provided by Studer et al., 2000, who used rat mesencephalic precursor cells to demonstrate four clear effects of 3±2% oxygen culture. First of all in lowered oxygen apoptosis was reduced, secondly progenitor cell proliferation was increased, with both of these factors helping to contribute to increased precursor cell numbers. A differentiated state was also achieved more rapidly in physiological oxygen conditions which would be a highly desirable effect to reproduce given the need for temporal efficiency. Finally lowered oxygen was able to alter the proportions of neurons displaying particular neurotransmitter phenotypes. The percentage of dopaminergic neurons increased from 18% in 20% oxygen to 56% in 3±2 oxygen. The combined effect of the increases in absolute cell number and the percentage of neurons of a dopaminergic phenotype gave rise to a nine fold increase in yield of dopaminergic neurons (Studer et al., 2000). Given that Tera2.cl.SP12 embryonal carcinoma stem cells are very highly proliferative and robust the first two effects are of lesser importance. However the ability to rapidly produce cells reflective of a differentiated state and the capacity to increase specifically the yield of dopaminergic neurons were both favourable concepts to attempt to duplicate in the context of the Tera2.cl.SP12 system. The main reasons being that the lowered oxygen conditions may act in synergy with retinoic acid to promote cells to adopt a differentiated, more mature phenotype and when set against the back drop of retinoic acid treated embryonal carcinoma stem cells producing a mixture of neural cell types, the capacity of physiological oxygen levels to influence a large proportion of cells to take up a dopaminergic phenotype was extremely appealing.

In the light of advice provided by other members of the Przyborski lab group and the technician for the lowered oxygen incubator, an oxygen pressure of 5% was chosen, as below this level the oxygen pressure may be susceptible to fluctuations. Although this is higher than in the work of Studer et al., 2000 it is in the range of oxygen pressures they

used in this work as $3\pm 2\% \equiv 1-5\%$ oxygen. In addition Colton et al., 1995 showed positive effects on tyrosine hydroxylase positive cell survival when using 5% oxygen. It therefore seemed reasonable especially given the effects in the work of Studer et al., 2000 were striking to think that using 5% oxygen would at least highlight any major differences between it and standard oxygen culture on the acquisition of a dopaminergic phenotype by retinoic acid treated Tera2.cl.SP12 embryonal carcinoma stem cells.

When set against the background of available knowledge a number of hypotheses could then be set up to test. The most basic of these are can retinoic acid induce expression of dopaminergic markers over time and do embryonal carcinoma stem cells themselves express any of these markers? To build on this is there any pattern to the way particular markers or sets of them are regulated? Also how are some of the key mechanistic determinants of a dopaminergic phenotype regulated in this system? In addition can lowered oxygen culture enhance the dopaminergic differentiation of retinoic acid treated Tera2.cl.SP12 embryonal carcinoma stem cells in vitro? By growing the Tera2.cl.SP12 cells under a range of conditions and by carrying out a range of molecular analyses these theories were investigated. Section 3.2 describes the specific culture methods and section 3.3 outlines the results obtained.

3.2 Cell Culture Methods

Retinoic Acid Induced Differentiation of EC cell cultures

Cultures of Tera2.cl.SP12 EC cells were grown to confluency, the media aspirated from them, before being washed with 1x PBS, prior to treatment for 5 minutes with 0.25% (w/v) Trypsin/0.1% (w/v) EDTA (in HBSS) (Cambrex), to release the cells from the surface of the culture flask. The Trypsin was then neutralized by addition of 4ml of DMEMFG and any cells remaining loosely bound washed off the surface of the flask by pipetting the DMEMFG/Trypsin-EDTA mix against the side of the flask where cell attachment occurs. A further wash with 1x PBS was carried out to optimize the retrieval of EC cells for use. The cell suspension was then transferred to a 15ml Falcon tube and spun in a centrifuge (Eppendorf 5810R (swing bucket rotor A-4-62)) at 800rpm, 4°C, for 2 minutes. The excess fluid was removed to leave an EC cell pellet. This was resuspended in DMEMFG and cell number was obtained by using a haemocytometer. Once the cell number was known the EC cells were seeded out at the following densities depending on the culture ware used:

- For a T75 – 1.5×10^6 cells per flask,
- For a T25 (Nunc) – 0.5×10^6 cells per flask,
- For a single well of a 6 well plate (Nunc) containing a 22mm poly-D-lysine coated glass coverslip – 0.2×10^5 cells per well.

Cultures were induced to differentiate by exposure to a final concentration of $10 \mu\text{M}$ all trans retinoic acid (made up in dimethyl sulfoxide (both Sigma)). They were then placed into either the same atmospheric (normal, 21%) oxygen, 5% CO_2 incubator that the EC cells were maintained in or depending on the investigations being undertaken a 5% O_2 , 5% CO_2 incubator (Sanyo) (for the studies into the effect of oxygen tension on the dopaminergic differentiation potential of retinoic acid induced EC cells). Media was changed every 2 – 4 days.

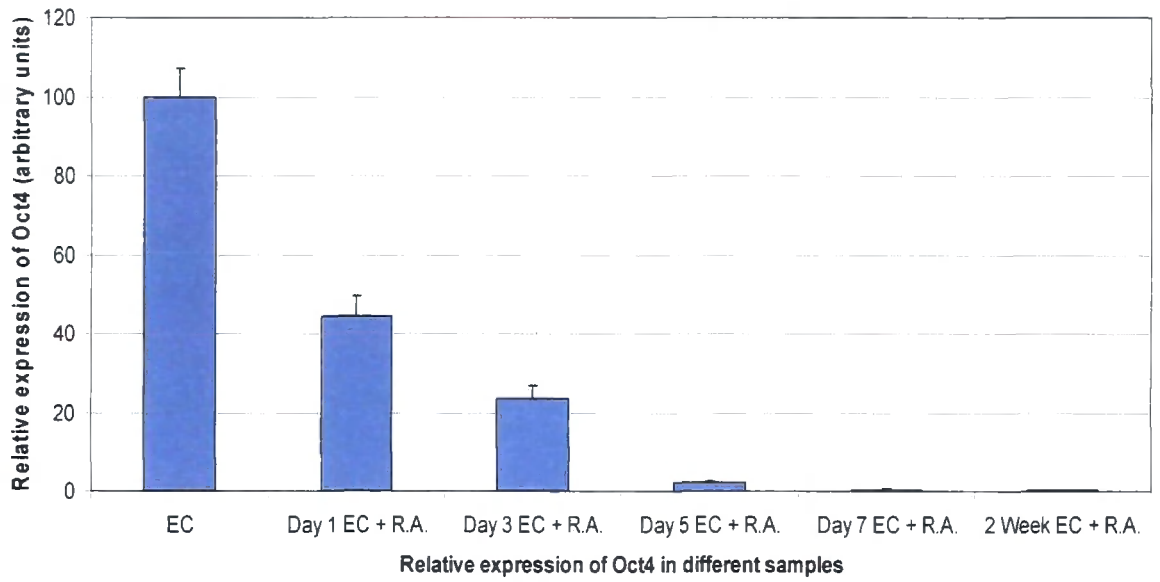
3.3 Results

Before starting to characterise the embryonal carcinoma stem cell system Tera2.cl.SP12 in both its undifferentiated state and in the presence of retinoic acid at different oxygen concentrations, some classical experiments were performed to act as controls to show that the cells were exhibiting predictable patterns of behaviour. The first experiment involved using real time PCR to show the drop in expression of Oct4 mRNA following retinoic acid treatment of the embryonal carcinoma cells (Figure 3.1A). This yielded similar results to those in Przyborski, 2001, where Northern blotting was used to demonstrate how this marker of human pluripotent stem cells was reduced following retinoic acid induction.

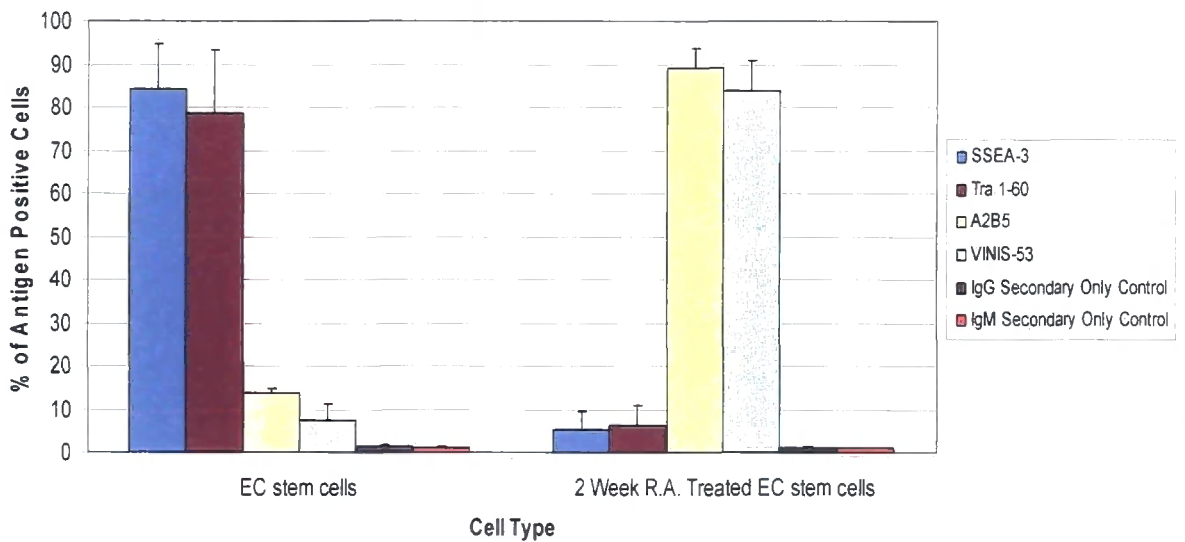
Figure 3.1B then shows the results of a flow cytometry based analysis of markers of human pluripotent stem cells and neural phenotypes. Undifferentiated Tera2.cl.SP12 embryonal carcinoma stem cells express high levels of SSEA-3 and Tra 1-60 (markers associated with pluripotent stem cells) (Andrews et al, 1982; Andrews et al, 1984) but express only minimal levels of A2B5 and VINIS-53 (markers of a neural phenotype) (Eisenbarth et al, 1979; Andrews et al, 1990). In contrast following treatment with retinoic acid for two weeks, the pattern of regulation is switched with high expression of the neural markers and low levels of the stem cell markers being observed in a consistent manner with earlier work (Przyborski, 2001). These results were therefore positive as they indicated that the embryonal carcinoma stem cells were behaving in a characteristic fashion and could respond to retinoic acid as expected, which in turn suggested any subsequent work with them would hopefully be representative of them acting in a similar way to that in previous studies. Different passages of cells were used and there did not appear to be any major passage number related effects.

Figure 3.1: Characterisation of stem cell differentiation

A.



B.



C.

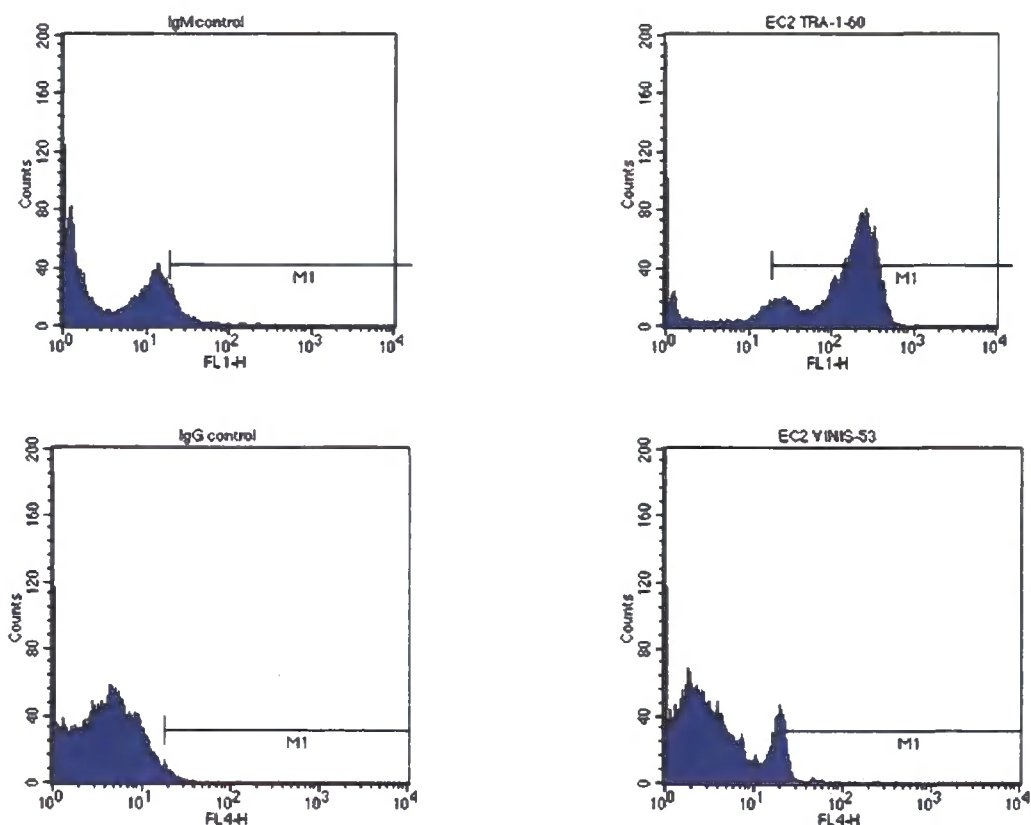


Figure 3.1A: The relative levels of Oct4 mRNA expression assessed by real time PCR in Tera2.cl.SP12 cells and their derivatives following 10 μ M retinoic acid treatment at different time points up to two weeks. **B:** A flow cytometry based analysis of changes in the pattern of human pluripotent stem cell and neural marker expression in Tera2.cl.SP12 cultures pre and post two weeks of 10 μ M retinoic acid induced differentiation. In all experiments n=3. Error bars represent the S.D. of the mean. **C:** Flow cytometry traces, first of all to gate out dead cells propidium iodide is used. This penetrates dead or dying cells and its signal can be detected using the 488nm argon-ion laser, live cells negative for propidium iodide (below a threshold) are gated out and further analysed by the flow cytometer. Prior to analysing the test samples a background fluorescence threshold is determined by analysing cells stained with only secondary antibody that are not stained for the antigen of interest. These control samples are used to define the threshold which identifies if a cell is antigen positive or not, any cell that shows a fluorescence value above this threshold is counted as positive. The traces above on the left hand side show how the secondary only control peak sits to the left of the threshold and essentially all the EC cells in these experiments are negative. In the upper right quadrant the single peak has moved to the right and shows positive expression of Tra 1-60 a marker that would be expected to be expressed by EC cells. The lower right quadrant shows that there is only a small movement of the peak to the right for VINIS-53, this results in only a few EC cells being positive for this marker which would be expected as it is more normally associated with cells showing characteristics of neural differentiation hence its greater expression after 2 weeks of exposure of the EC cells to retinoic acid.

After the initial experiments, the next step was to start to assess the dopaminergic differentiation potential of the Tera2.cl.SP12 cell line pre and post retinoic acid treatment. This was first undertaken at the mRNA level using real time PCR. In the light of the work of Misiuta et al., 2006 where retinoic acid caused a significant increase in Nurr1 protein expression in the Ntera2.D1 embryonal carcinoma stem cell line investigations were undertaken to assess if an analogous peak in mRNA expression was seen using the Tera2.cl.SP12 system. Figure 3.2 shows that there is a peak in Nurr1 mRNA expression after 1 day of retinoic acid exposure. It is not a statistically significant effect however which may be due to the variation in the expression of this transcription factor in the Tera2.cl.SP12 cells themselves. The trend though is consistent with the earlier observations of Misiuta et al., 2006 and it is also noticeable that expression although variable persists until the final two week time point. This is again a compatible trend with the concept that Nurr1 expression may persist throughout different stages of development with the transcription factor possibly possessing a range of time dependent roles.

Figure 3.2: Nurr1 mRNA Expression in Tera2.cl.SP12 cells and their derivatives

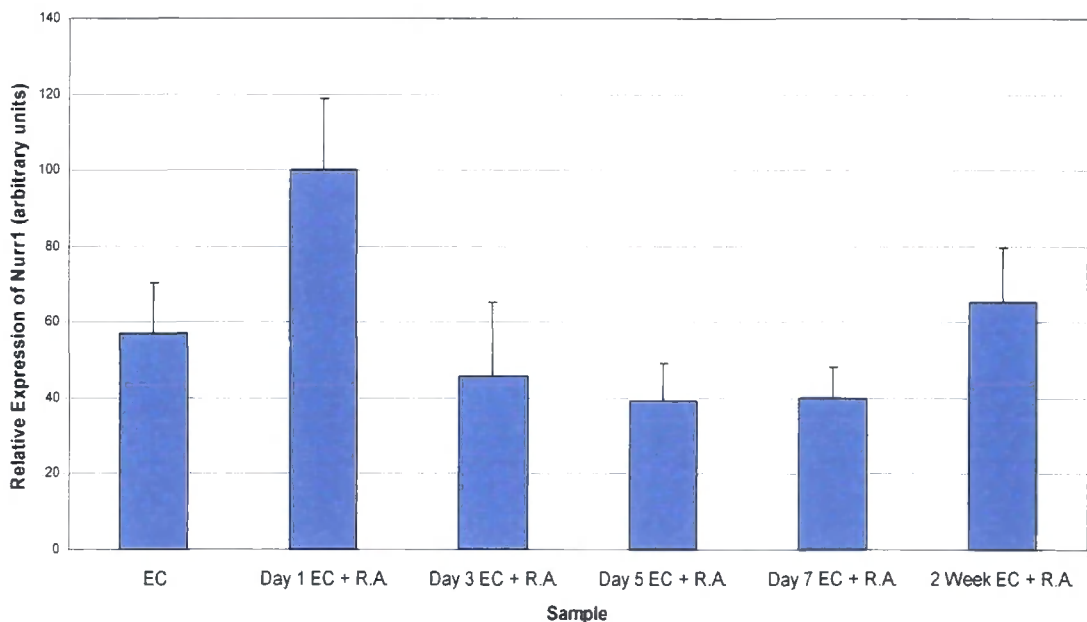


Figure 3.2: Real time PCR data showing the changes in the relative expression levels of Nurr1 mRNA in undifferentiated cultures of Tera2.cl.SP12 embryonal carcinoma stem cells and their 10 μ M retinoic treated derivatives over a two week period (n=3). Error bars represent the S.D. of the mean. Student's T-test revealed that there was not a statistically significant difference between EC and 1 day R.A. treated samples.

With a mind to future work the next factor to be assessed was Otx2, a homeodomain transcription factor expressed in dividing midbrain dopaminergic precursors that is essential for their formation (Puelles et al., 2004). In a mouse model Wnt1 was able to up regulate Otx2, in turn repressing expression of Nkx2-2 which is involved in the acquisition of alternative non dopaminergic neuronal fates (Prakash et al., 2006). Wnt1 could also help expand the number of Nurr1 positive precursors in rat mesencephalic cultures (Castelo Branco et al., 2003). Given the peak in Nurr1 expression at 1 day of retinoic acid treatment, the combined use of Wnt1 and retinoic acid was seen as a possible area for subsequent investigations. Therefore given the favourable effects Otx2 could potentially possess, its mRNA expression pattern following retinoic acid treatment was studied, see Figure 3.3. In the untreated embryonal carcinoma stem cells Otx2 mRNA is present, which would suggest that if it is a part of the machinery required to specify a dopaminergic fate, then it may be the case that embryonal carcinoma cells are a viable model for use in the study of dopaminergic neurogenesis. However upon retinoic acid treatment as is clearly seen in Figure 3.3, expression plummets and shows no signs of recovery within the two week time frame. Although this appears to be a negative finding it does pose the question of whether expression of Otx2 can be rescued following addition of retinoic acid by adding another compound such as Wnt1. It is also an interesting observation alongside the Nurr1 result in the sense that embryonal carcinoma stem cells are seemingly able to express markers that may indicate they are further towards a differentiated state than the embryonic stem cells for which they act as a model.

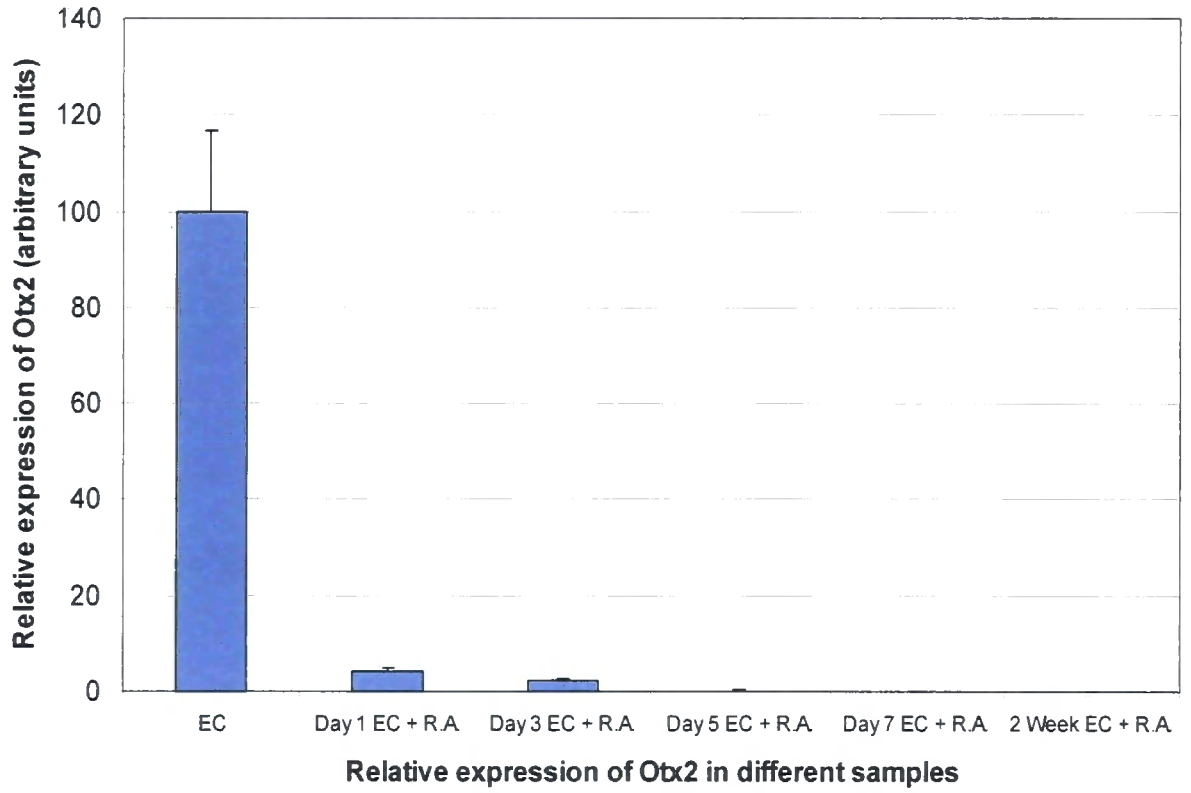
Figure 3.3: Otx2 mRNA Expression in Tera2.cl.SP12 cells and their derivatives

Figure 3.3: Real time PCR data showing the changes in the relative expression levels of Otx2 mRNA in undifferentiated cultures of Tera2.cl.SP12 embryonal carcinoma stem cells and their 10 μ M retinoic treated derivatives over a two week period (n=3). Error bars represent the S.D. of the mean.

Figure 3.4: Sox1 mRNA Expression in Tera2.cl.SP12 cells and their derivatives

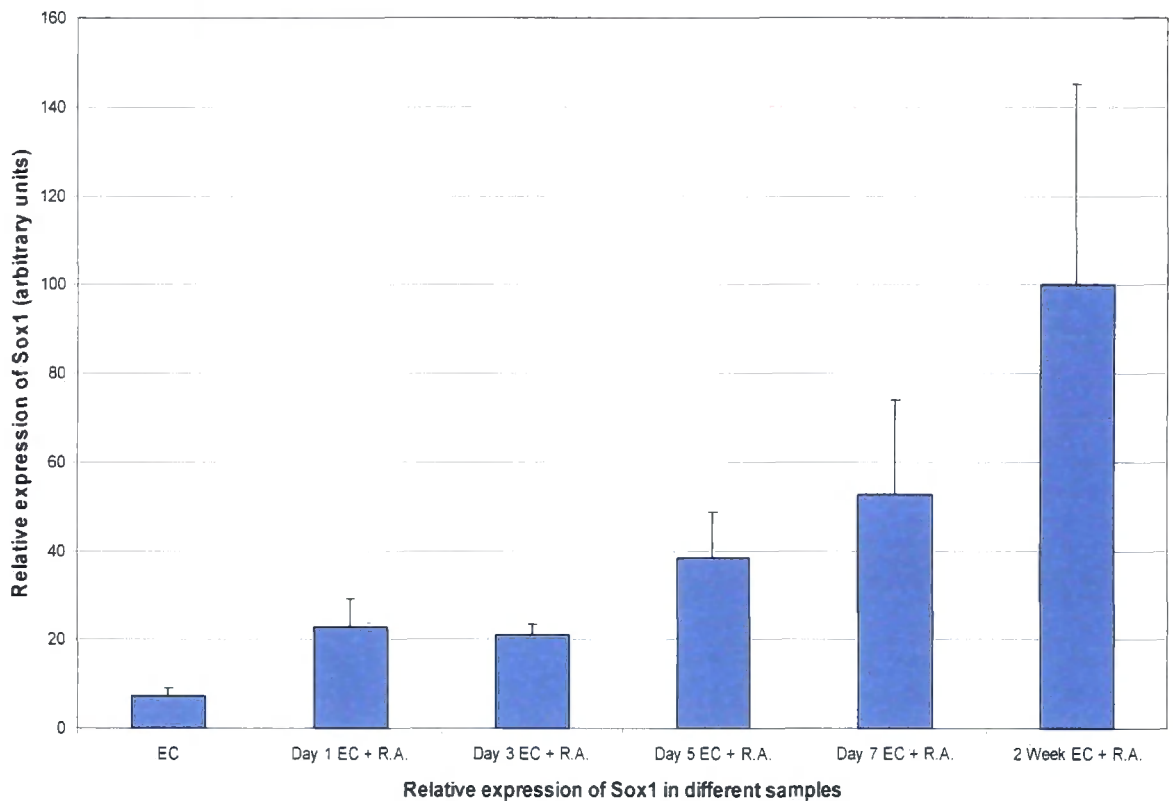


Figure 3.4: Real time PCR data showing the changes in the relative expression levels of Sox1 mRNA in undifferentiated cultures of Tera2.cl.SP12 embryonal carcinoma stem cells and their 10 μ M retinoic treated derivatives over a two week period (n=3). Error bars represent the S.D. of the mean.

Further analysis by the same technique of the embryonal carcinoma plus retinoic acid system focussed on Sox1, a marker of dividing neural precursors that is strongly associated with neuronal fate acquisition both in vivo and in vitro (Pevny et al., 1998). The expression of which was highlighted as being an important factor when studying the dopaminergic differentiation of human embryonic stem cells in the presence of Sonic hedgehog and Fibroblast growth factor 8 (Yan et al., 2005). The effect in this work was temporal and given the likelihood of using these molecules in later studies especially given that Wnt1 which was possibly another factor to be investigated may interact or create permissive conditions for them (Burbach & Smidt, 2006), it seemed prudent to gain an understanding of the regulation of Sox1 expression. Figure 3.4 clearly demonstrates

that expression is low in the embryonal carcinoma stem cells and rises in a predictable almost linear fashion with increasing exposure time to retinoic acid over the two week period of study. This is indicative that if Sonic hedgehog and Fibroblast growth factor 8 are to be utilised effectively then it is probably going to be necessary to apply them reasonably early in the differentiation process. It also illustrates that in general as was the case in Figure 3.1B, retinoic acid can cause increased neural marker expression over time. When real time PCR was put to further use to investigate the effects of retinoic acid over both two and four weeks in both normal and 5% oxygen, similar trends were observed for other markers associated with a dopaminergic cell fate. This work also started to give an insight into the effects of oxygen pressure as a dopaminergic phenotype inducing agent in Tera2.cl.SP12 embryonal carcinoma stem cells. Figure 3.5 shows that there is a predictable rise in the expression of tyrosine hydroxylase over time, up to four weeks in both oxygen environments. However there is no real difference in the expression of tyrosine hydroxylase between 5 and 21% oxygen cultures. The output of the two way anova performed to assess if there are any statistically significant effects indicates that between two and four weeks of retinoic acid application there is almost a significant effect due to time (p value of 0.051). Oxygen pressure though alone has no effect and there does not appear to be any favourable interaction between pressure and time.

Figure 3.6 illustrates the pattern of expression for dopamine receptor 2 under the same conditions; however although the trend here is the same as for tyrosine hydroxylase, the time of exposure to retinoic acid from two to four weeks has a less significant effect upon expression. Again oxygen pressure exhibits no statistically significant or tangible effect and the slight trend observed is that normal oxygen is the favourable condition at both two and four weeks which contradicts the hypothesis that lowered oxygen is likely to up regulate dopaminergic marker expression and the acquisition of that specific neurotransmitter phenotype. The parallel lines on the line graph (Figure 3.6) also neatly indicate that there is no obvious interaction between oxygen pressure and time period of retinoic acid treatment.

Figure 3.5: The effect of oxygen pressure and duration of R.A. exposure on the relative expression of tyrosine hydroxylase mRNA in Tera2.cl.SP12 cultures

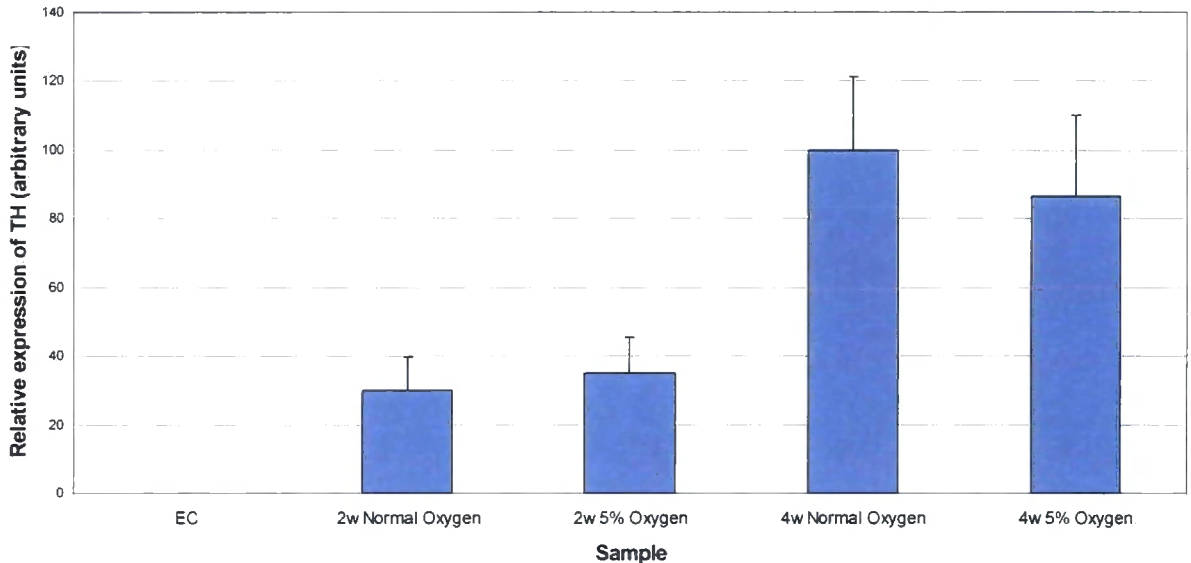


Figure 3.5: The relative expression of tyrosine hydroxylase mRNA in undifferentiated Tera2.cl.SP12 cells and their derivatives exposed to 10 μ M retinoic acid for 2 or 4 weeks in either a 5% or normal (21%) oxygen culture environment. The output of the 2 way anova indicated that between 2 and 4 weeks there is almost a statistically significant effect (p value of 0.51) due to time period of retinoic acid exposure but that there is no effect due to oxygen pressure. In addition it also shows that there is no significant synergistic interaction between oxygen pressure and the duration of retinoic acid treatment. Error bars represent the S.D. of the mean. In all experiments n=3.

Figure 3.6: The effect of oxygen pressure and duration of R.A. exposure on the relative expression of D2 mRNA in Tera2.cl.SP12 cells

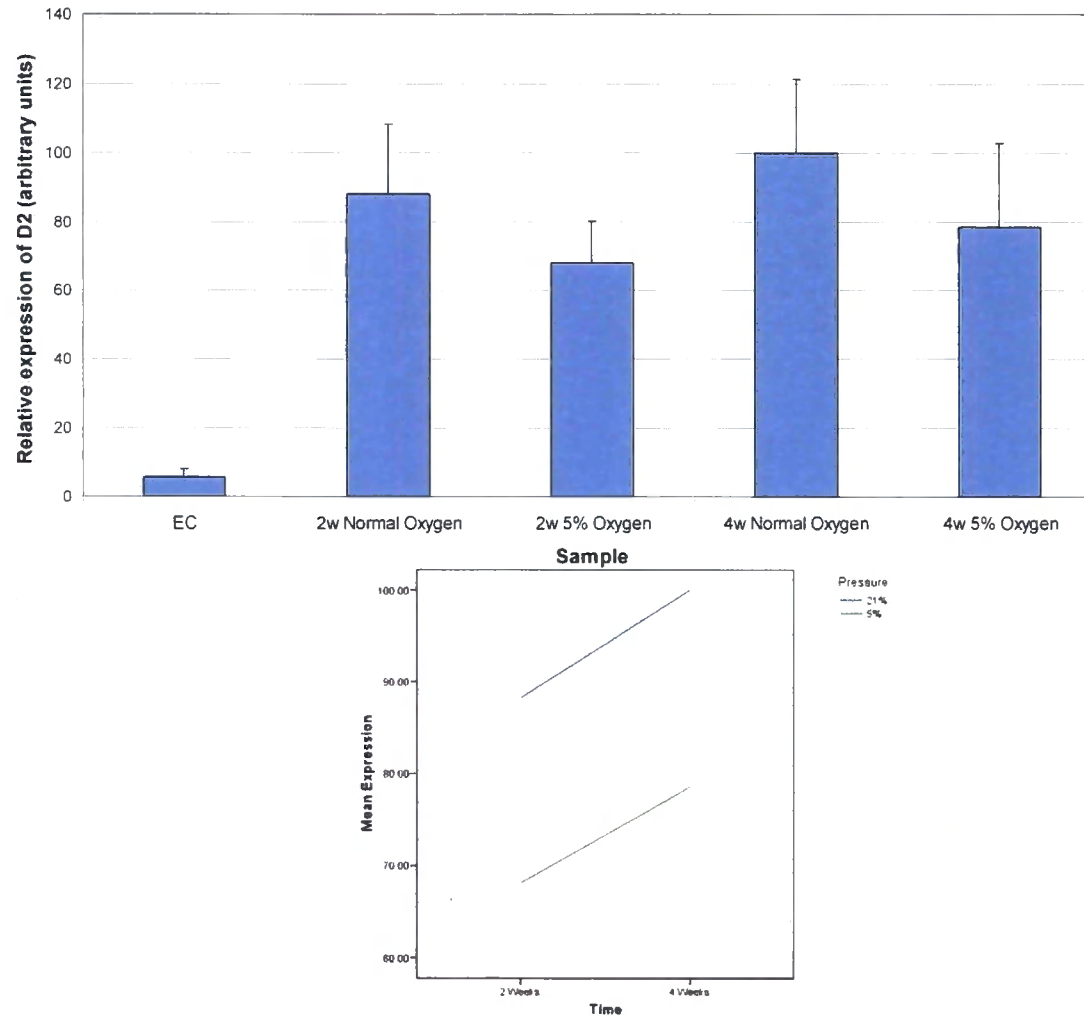


Figure 3.6: The relative expression of dopamine receptor 2 (D2) mRNA in undifferentiated Tera2.cl.SP12 cells and their derivatives exposed to 10 μ M retinoic acid for 2 or 4 weeks in either a 5% or normal (21%) oxygen culture environment. The output of the 2 way anova performed indicated that between 2 and 4 weeks there is no statistically significant effect due to time period of retinoic acid exposure and that there is also no effect due to oxygen pressure. In addition it indicates that there is no significant synergistic interaction between oxygen pressure and the duration of retinoic acid treatment. The almost perfectly parallel nature of the lines on the lower graph neatly supports this observation as this would be the expected result if there were no interaction between the two factors under investigation. Error bars represent the S.D. of the mean. In all experiments n=3.

Figure 3.7: Morphological Images

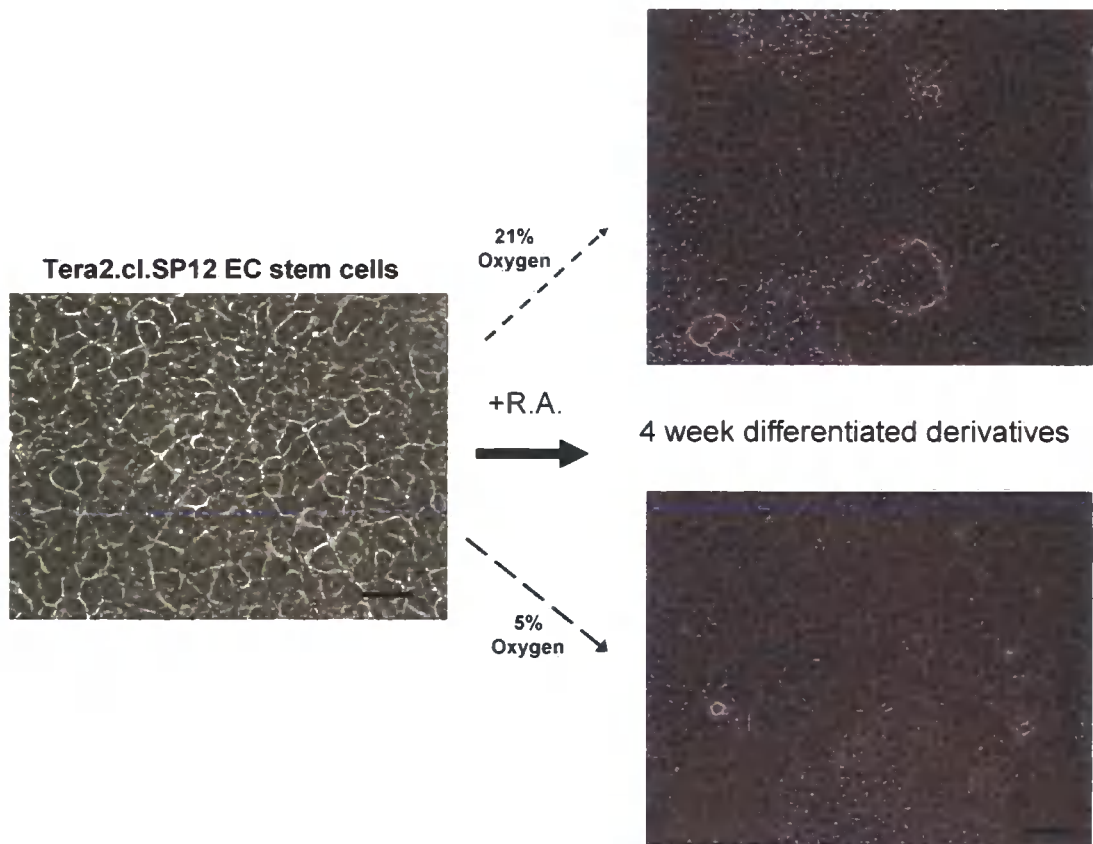


Figure 3.7: Morphological phase images of Tera2.cl.SP12 EC cells in their undifferentiated state and their 4 week 10 μ M retinoic acid induced derivatives in 5% and normal oxygen environments. The Tera2.cl.SP12 cells were viewed using a 40x objective lens (scale bar represents 25 μ m) and have a characteristic reasonably uniform appearance. Their 4 week retinoic acid treated derivatives were viewed using a 10x objective lens (scale bars represent 100 μ m). There are no clear morphological differences between the 4 week cultures in the different oxygen conditions but it is apparent that the cultures have lost their uniformity during the process of differentiation producing a mixture of cell types.

Some additional real time PCR was also performed on RNA samples sent to Dr. V. Lakics (Appendix B). These analyses indicated that in the embryonal carcinoma cells and their 1, 3, 5 and 7 day retinoic acid treated derivatives there was a generally low expression of the dopaminergic markers tyrosine hydroxylase, dopamine receptor 1, dopamine receptor 2, dopamine transporter and Nurr1. However dopamine receptor 1, dopamine transporter and Nurr1 could all be detected at four weeks in both 5 and 21% oxygen samples. These findings are congruous with the observations shown previously in that retinoic acid treatment of Tera2.cl.SP12 embryonal carcinoma stem cells is able to induce expression of a range of dopaminergic markers in such samples. Once again the

use of lowered (5%) oxygen as for tyrosine hydroxylase and dopamine receptor 2 has seemingly no effect in this cell system, at least at the oxygen pressure tested.

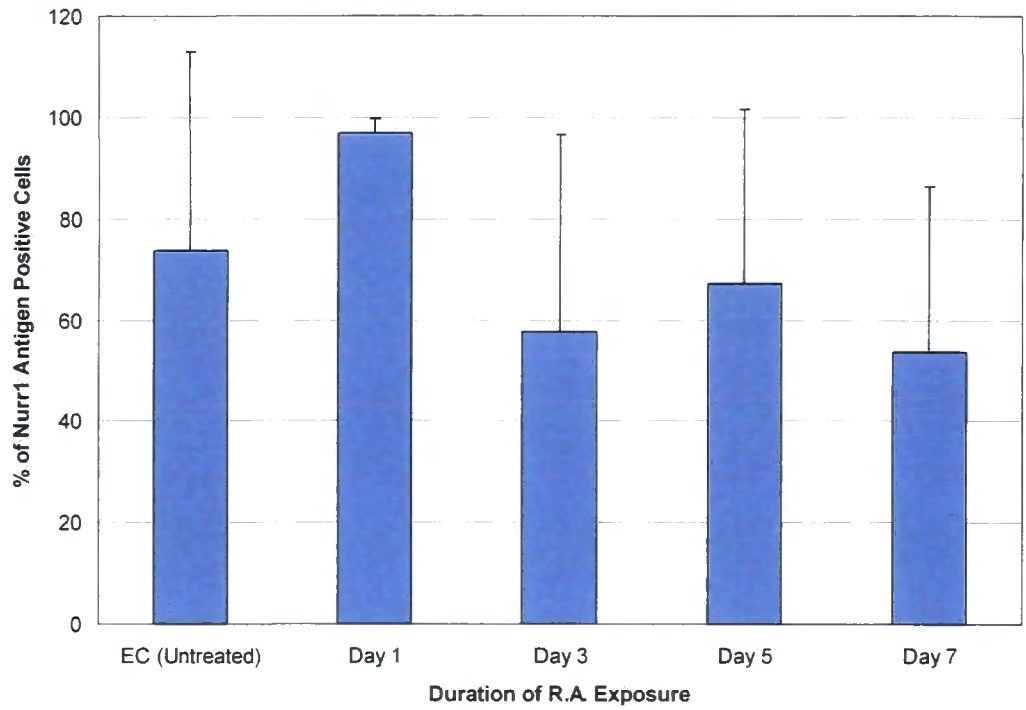
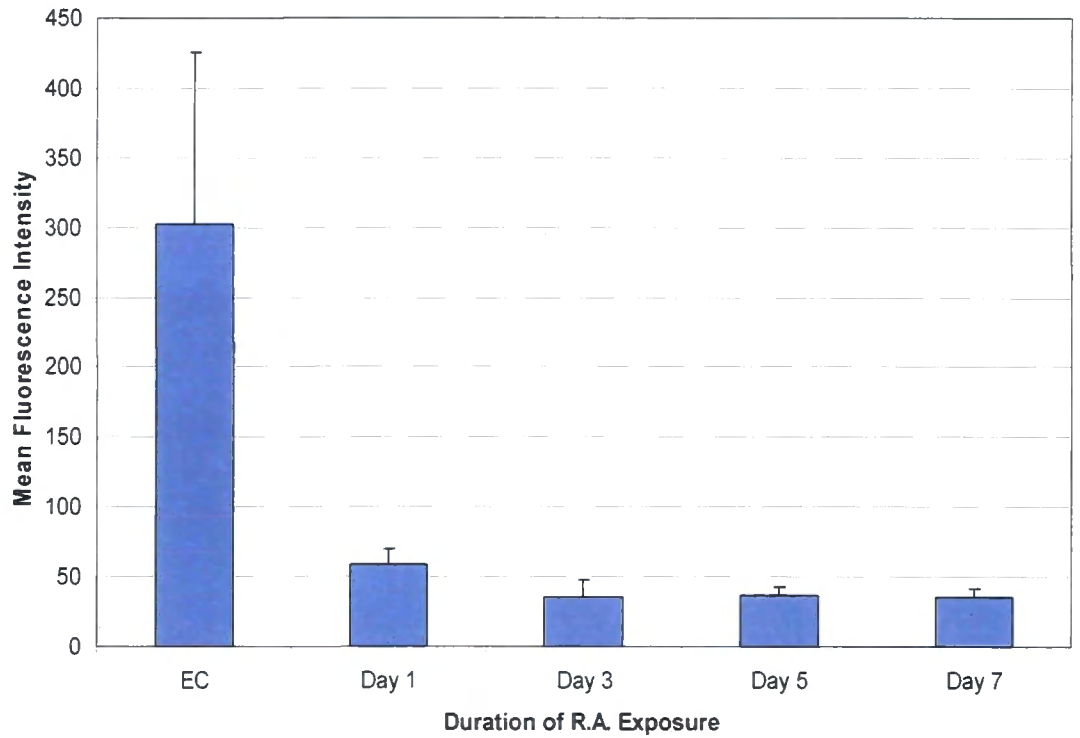
Figure 3.7 shows some morphological images of the embryonal carcinoma cells themselves and following four weeks of retinoic acid induced differentiation in both oxygen environments. Retinoic acid causes a mix of cell types to form from the original embryonal carcinoma stem cells but there are no obvious differences at the basic level of observable cell morphology. This too is in keeping with the results from the real time PCR analyses.

After making an assessment of the effects of retinoic acid and physiological oxygen at the morphological and mRNA level and in the light of the fact that proteins are the functional components of a cell the next step experimentally was to start to gain an understanding of the patterns of protein expression. Indeed although the mRNA was present for several dopaminergic markers this is not proof that the translated protein is expressed. Flow cytometry, Western blotting and immunocytochemistry were employed to confirm the expression of known neuronal and dopaminergic phenotypic indicators, as well as to gain an insight into the levels at which they are present (flow cytometry) and their cellular distribution (immunocytochemistry). After having seen a peak in the expression of Nurr1 mRNA this protein was the first to be investigated. In the light of this finding and the work of Misiuta et al., 2006, where Nurr1 protein expression rose significantly after one day of exposure of Ntera2.D1 embryonal carcinoma stem cells to retinoic acid, the expectation was that a peak may also be observed in the Tera2.cl.SP12 system. However using both flow cytometry and Western blotting as can be seen in Figure 3.8 it was impossible to gain any meaningful data. There are large fluctuations in the percentage of cells expressing Nurr1 and the intensity of expression is very low being comparable to that of negative controls. There is in addition little to no detection of the protein via Western blotting. There are a number of possible reasons for these results; one option is that in the Tera2.cl.SP12 cells the mRNA is expressed but the protein is not. Another is that the antibody used did not work particularly well. The antibody was however tested on some brain material where expression of Nurr1 is normally expected to be detectable. The data from this (Appendix C) display a quite diffuse band on a Western blot. This would suggest that it may well be the case that the immunological reagent used was part of the problem, as a positive control would usually be expected to give a strong clear band

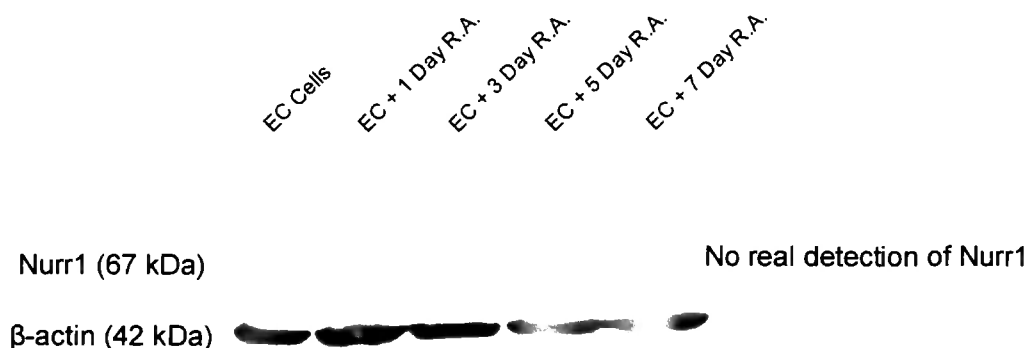
and this was not obtainable after a number of attempts. Beta-actin used as a loading control was clearly detectable and amido black staining of the nitrocellulose membrane confirmed there was protein present, giving support to the notion that the antibody was the limiting factor. There is also the possibility that the expression of the mRNA and protein do not correlate and thus different patterns are seen. The only small trends that can be seen from the flow cytometry results are that on average Nurr1 protein is expressed in more cells at a slightly greater intensity following one day of retinoic acid treatment than after three, five or seven days. The data though are too ambiguous to make any real conclusions from.

This is particularly highlighted by the Western blot data in Figure 3.8 which show that there is seemingly a faint non specific binding of the antibody resulting in detection of multiple bands. These could be break down products or complexes of proteins containing Nurr1 but there is a strong possibility that given the technical problems with the antibody that they result from non specific binding of it to non-target proteins. This reinforces the notion that the data provide insufficient evidence on which to make any firm judgements.

The expression of tyrosine hydroxylase, the rate limiting enzyme in dopamine biosynthesis was also assessed using the same techniques as for Nurr1. Figure 3.9 shows there are generally large numbers of cells expressing this protein. The intensity of this expression varies in the initial seven days of retinoic acid exposure but over time retinoic acid has an inductive effect. The Western blot (Figure 3.9C) is indicative of weak levels of expression where there is no major observable difference. The protein data for tyrosine hydroxylase show a basic correlation with the findings of the real time PCR analyses, with low expression in the starting material and in the first seven days leading to an eventual rise by four weeks. However the faint nature of the Western blot detection although limited to a discrete band may reflect a broad and possibly non specific binding of the antibody, this idea is supported by the widespread expression of this marker by a large number of cells in the flow cytometry analysis. The detection seen may also be more an artefact of the sensitivity of the technique than a reflection of a true robust expression of the protein. Overall therefore there is unlikely to be any significant level of tyrosine hydroxylase present at the early stages of differentiation.

Figure 3.8: Nurr1 protein expression in Tera2.cl.SP12 cells and their derivatives**A.****B.**

C.



D.

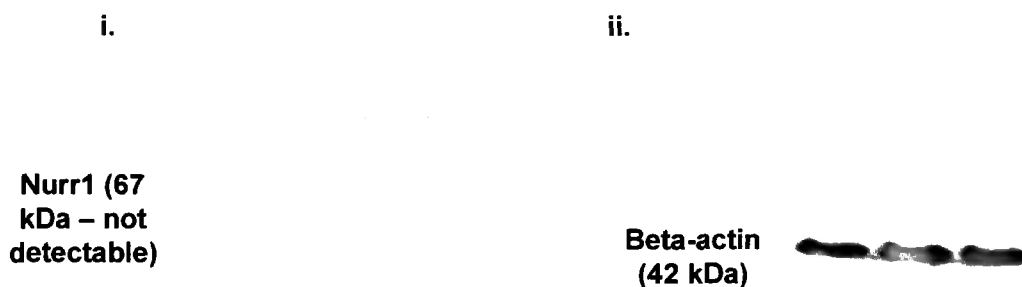
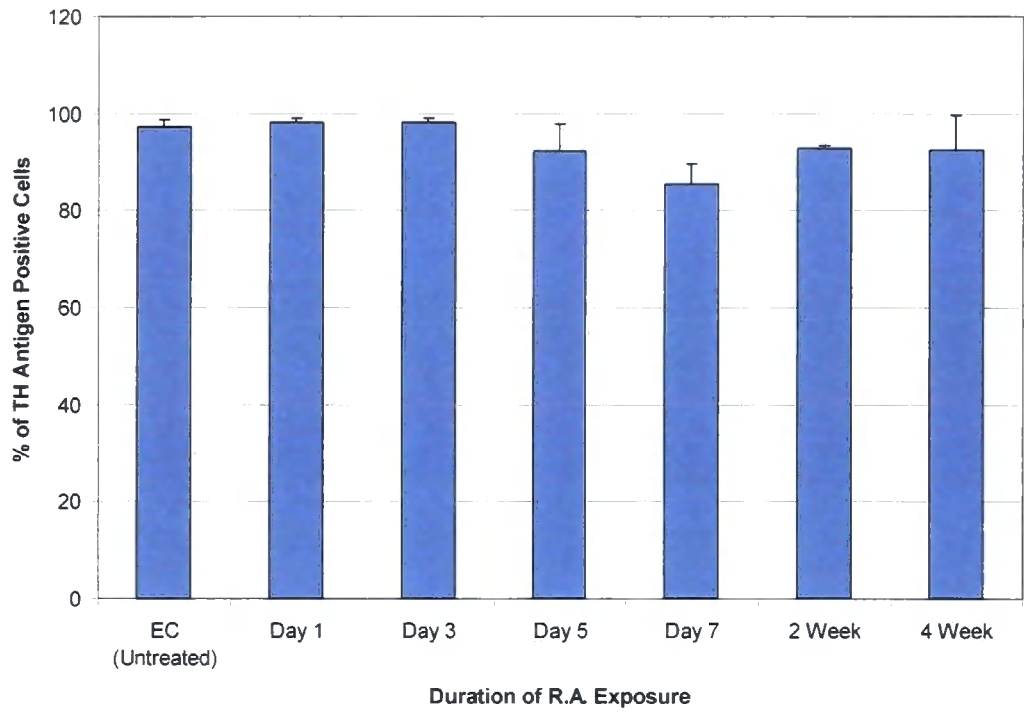
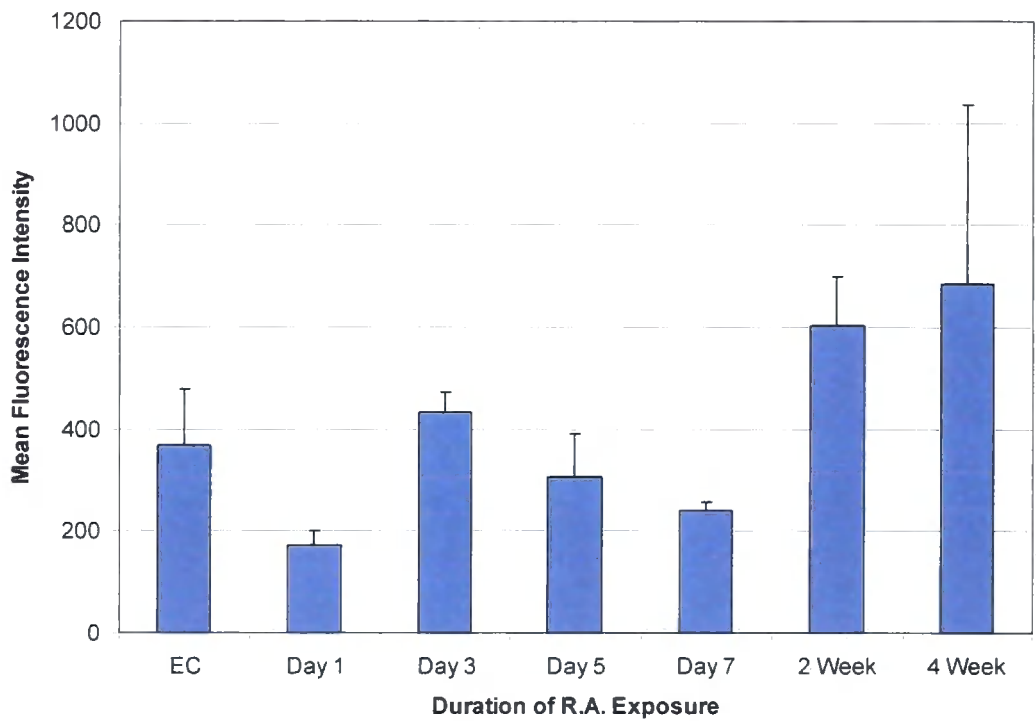


Figure 3.8A: Flow cytometric analysis of the number of cells expressing Nurr1 in Tera2.cl.SP12 cultures and their 10 μ M retinoic acid induced counterparts up to 7 days. Error bars represent the S.D. of the mean. **B:** The intensity of Nurr1 expression in such cultures. Error bars represent the S.E.M. **C:** A Western blot showing protein expression in identical samples. **D: (i)** A Western blot probed for Nurr1 carried out on 3 different passages of MG63 cell samples expected to be negative for Nurr1 expression. Although a band of Nurr1 expression is not seen there does appear to be some non specific binding of the antibody in other areas, this is also the case in C. above. **(ii)** Detection of beta-actin in the MG63 cell samples used as a loading control. Given the non specific binding of the antibody and technical problems with it as mentioned in the main text neither the Western blot nor flow cytometry data yielded any meaningful result. In all experiments n=3.

Figure 3.9: TH protein expression in Tera2.cl.SP12 cells and their derivatives**A.****B.**

C.

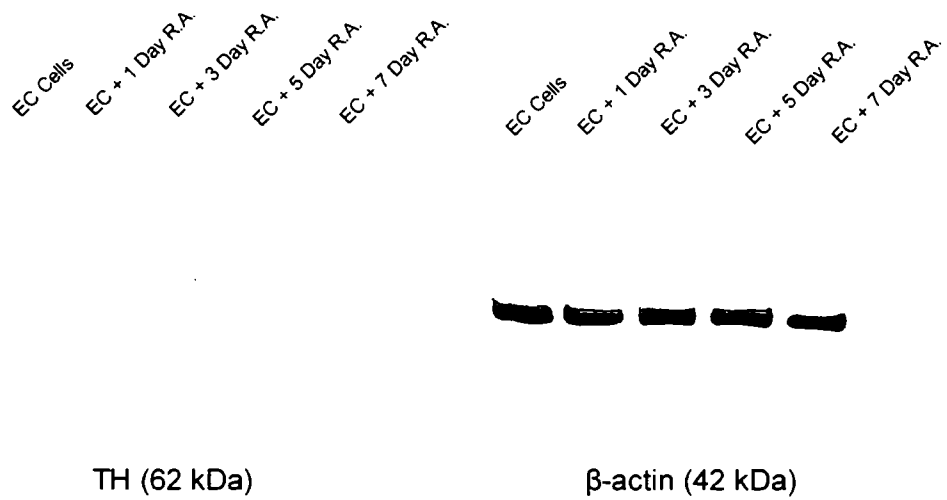


Figure 3.9A: Flow cytometric analysis of the number of cells expressing tyrosine hydroxylase in Tera2.cl.SP12 cultures and their 10 μ M retinoic acid induced counterparts up to 4 weeks. Error bars represent the S.D. of the mean. **B:** The intensity of tyrosine hydroxylase expression in such cultures. Error bars represent the S.E.M. **C:** A Western blot showing faint but detectable tyrosine hydroxylase protein expression in comparable samples up to 7 days of retinoic acid driven differentiation. Beta-actin was used as a loading control. In all experiments n=3.

Figure 3.10: Beta III tubulin protein expression in Tera2.cl.SP12 cells and their derivatives

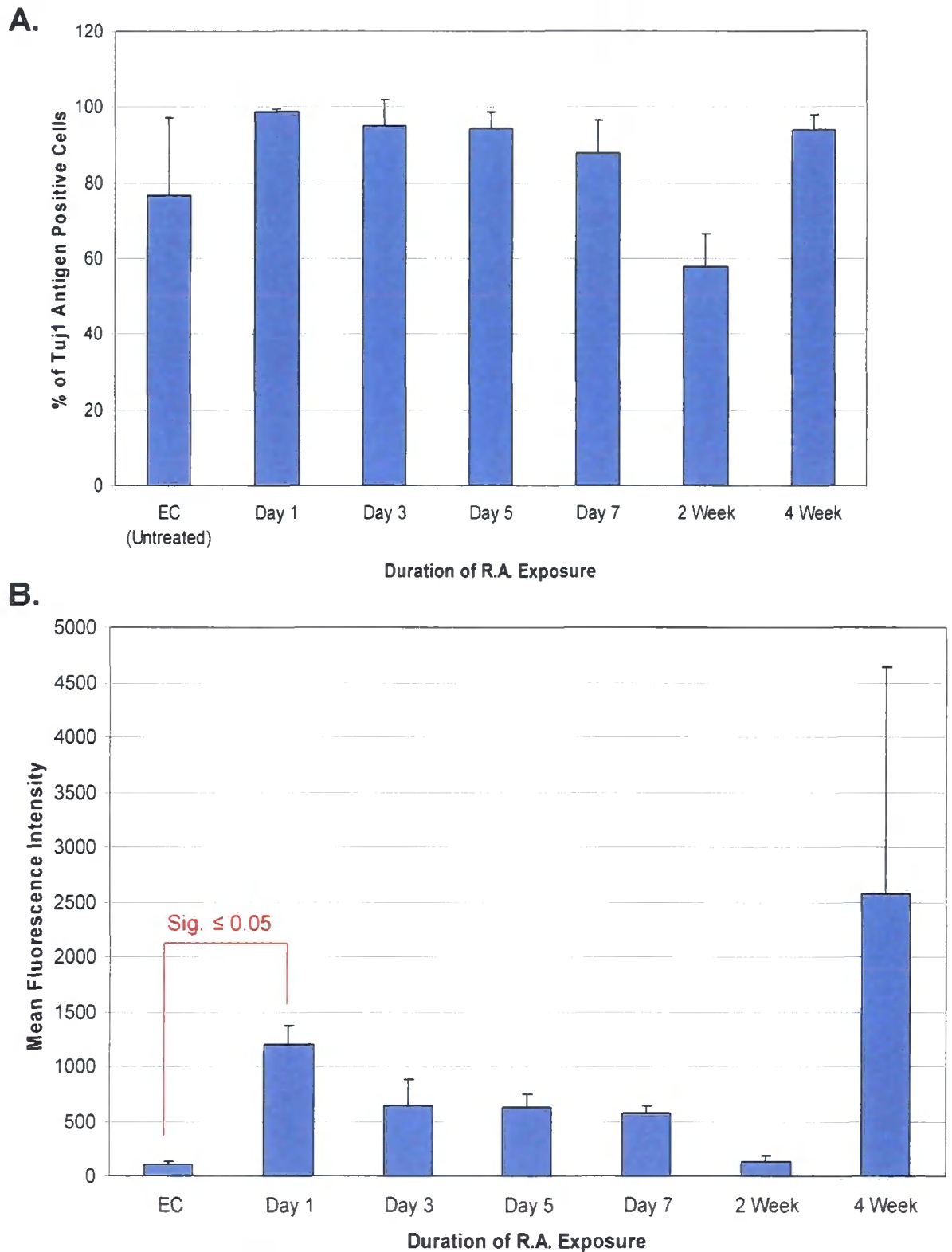


Figure 3.10A: Flow cytometric analysis of the number of cells expressing the neuronal marker Tuj1 in Tera2.cl.SP12 cultures and their 10 μ M retinoic acid induced counterparts up to 4 weeks. Error bars represent the S.D. of the mean. **B:** The intensity of Tuj1 expression in such cultures. Error bars represent the S.E.M. Student's T-test indicated that there was a statistically significant increase in mean fluorescence intensity from the EC cells to their derivatives treated with retinoic acid for 1 day. In all experiments n=3.

Figure 3.10 shows that the neuronal marker Beta III tubulin when assessed by flow cytometry displays a similar pattern of expression. However there are a couple of key differences. The first of these is that the starting material expresses tyrosine hydroxylase more strongly than the cells after one day of retinoic acid treatment. The opposite is true for Beta III tubulin which shows a statistically significant rise in expression following induction with retinoic acid for one day. This mirrors the rise in expression of Nurr1 and is in accordance with the concept that Nurr1 may help promote neuronal differentiation. The other main difference is that although Beta III tubulin, as for tyrosine hydroxylase, appears overall to be induced with lengthening exposure to retinoic acid, at two weeks there is a dip in both the amount and intensity of its expression. Therefore although predictable almost linear rises in certain markers can be observed upon retinoic acid treatment of Tera2.cl.SP12 cultures, this is not always the case and there may be subtle effects at different times when assessing the regulation of certain proteins of interest. Figure 3.11 summarises the mean fluorescence intensity data for all three markers displaying the overall trend of retinoic acid induction by four weeks and the peak in Beta III tubulin expression after one day.

Figure 3.11: Summary mean fluorescence intensity flow cytometry data

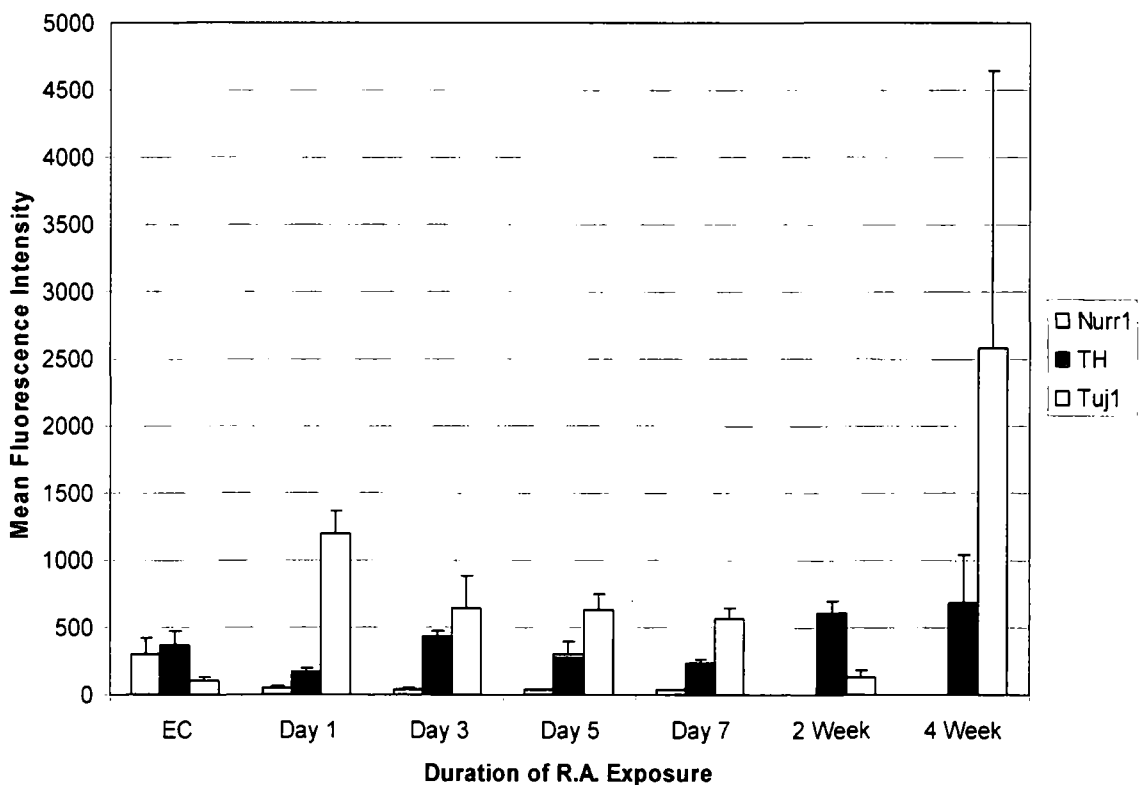
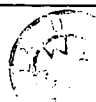


Figure 3.11: The intensity of Nurr1, TH and Beta III tubulin protein expression assessed by flow cytometry, in Tera2.cl.SP12 cultures and their 10 μ M retinoic acid induced derivatives over 4 weeks of differentiation. Error bars represent the S.E.M. (n=3).



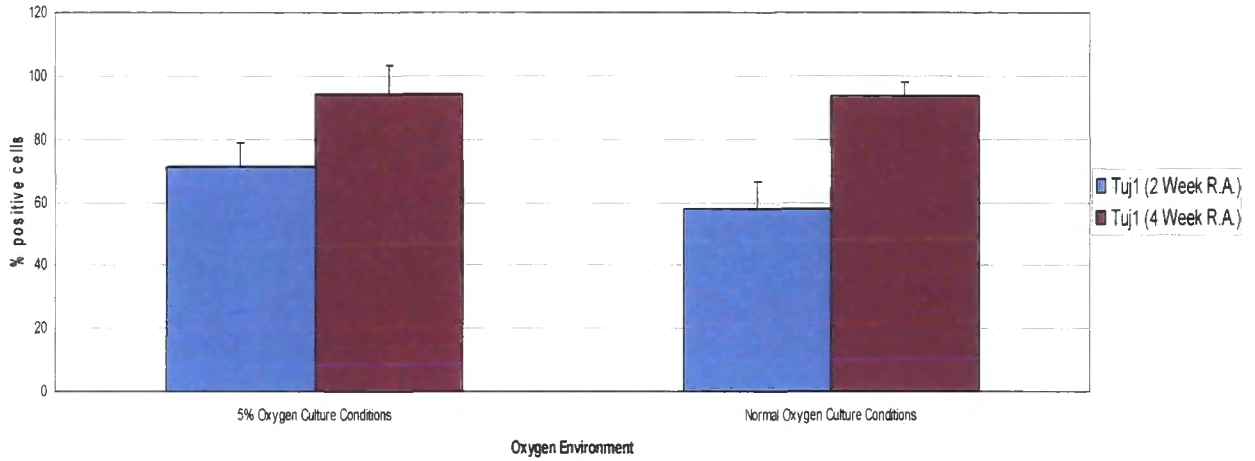
When the effect of lowered oxygen was also investigated utilising the same techniques a pattern of protein expression comparable to that for the mRNA was observed. Figure 3.12 shows that there is little difference in the number of cells expressing tyrosine hydroxylase from two to four weeks but there is a rise in the intensity of this expression. Oxygen pressure appears to exert no real or statistically significant effect and at the protein level time also has less of an impact on the induction of tyrosine hydroxylase. However overall as for the mRNA there is still the trend of oxygen pressure having little effect and increased duration of retinoic acid exposure leading to higher levels of expression of this dopaminergic marker.

Figure 3.13 shows that for the neuronal marker Beta III tubulin the situation is similar to that for tyrosine hydroxylase. There is more of an induction due to retinoic acid exposure between two and four weeks although this may in part be due to the observed dip at 2 weeks. There is also once again no tangible or statistical effect caused by using lowered oxygen conditions.

Western blotting was also used to try and give further support to these observations. Figure 3.14 neatly shows that for tyrosine hydroxylase there appears to be an induction of expression with retinoic acid treatment over time (although mainly at 4 weeks) and in a consistent manner with other techniques, the data indicate that oxygen pressure has no effect. Figure 3.15 shows that there is some very faint inconclusive expression of Nurr1 at two and four weeks but as for the earlier data no firm conclusions can be made for the reasons outlined previously, non specific binding of the antibody being one major problem as evidenced by the presence of multiple bands.

Figure 3.12: The effect of oxygen on TH protein expression in Tera2.cl.SP12 cells

A.



B.

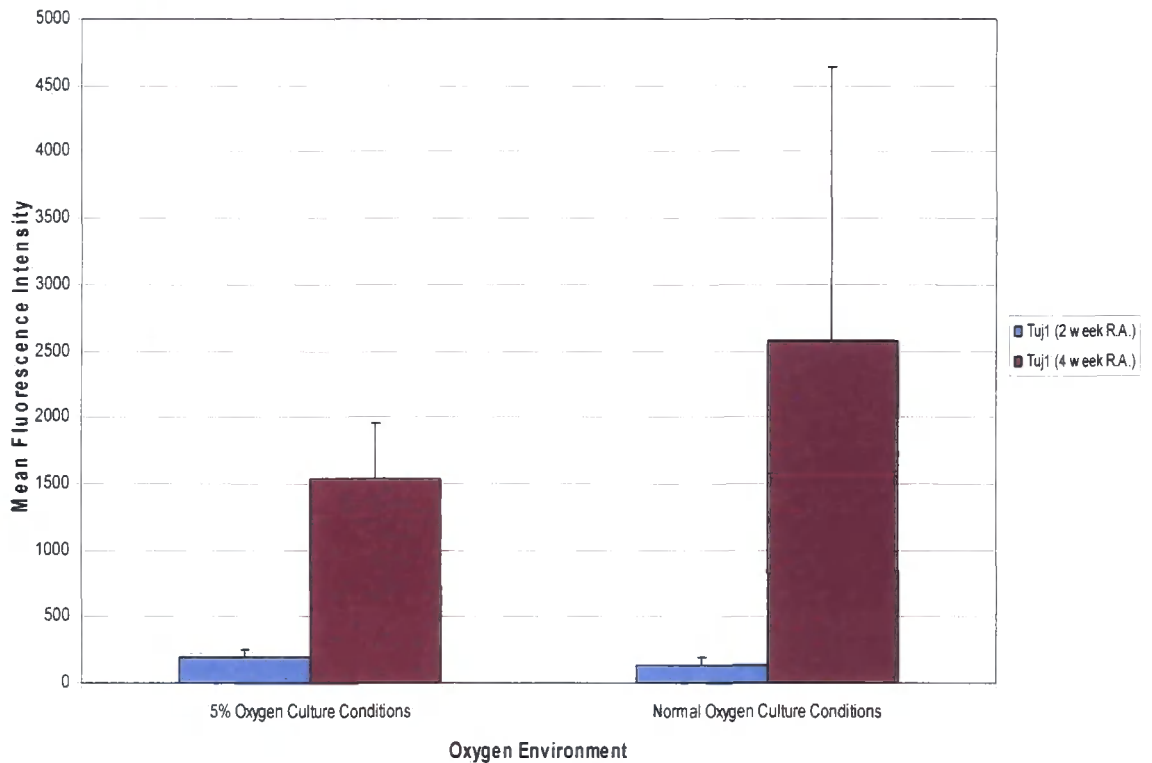


Figure 3.12A: Flow cytometry was used to assess the percentage of Tera2.cl.SP12 cells expressing TH protein after 2 or 4 weeks of 10 μ M retinoic acid induced differentiation in 5% or normal oxygen culture conditions. Error bars = S.D. of mean. **B:** The intensity of TH expression in these cell samples was also measured. Between 2 and 4 weeks neither the duration of retinoic acid exposure or oxygen pressure exerted any significant effect, either alone or in concert. Error bars represent the S.E.M. (n=3).

Figure 3.13: The effect of oxygen on Beta III tubulin protein expression in Tera2.cl.SP12 cells

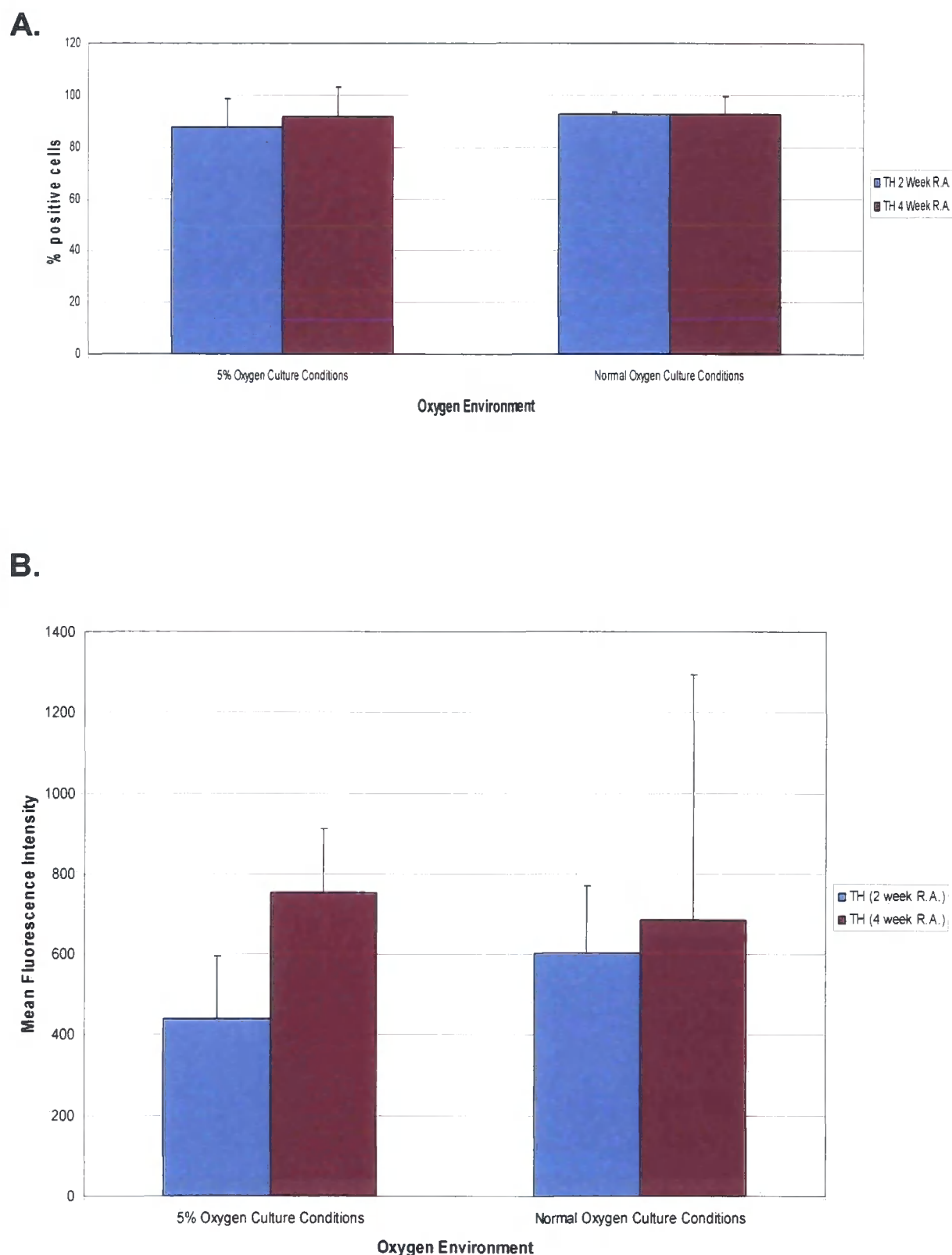


Figure 3.13A: Flow cytometry was used to assess the percentage of Tera2.cl.SP12 cells expressing TuJ1 protein after 2 or 4 weeks of 10 μ M retinoic acid induced differentiation in 5% or normal oxygen culture conditions. Error bars represent S.D. of mean. **B:** The intensity of TH expression in these cell samples was also measured. Between 2 and 4 weeks neither the duration of retinoic acid exposure or oxygen pressure exerted any significant effect, either alone or in concert. Error bars represent the S.E.M. (n=3).

Figure 3.14: Assessment of TH expression via Western Blotting

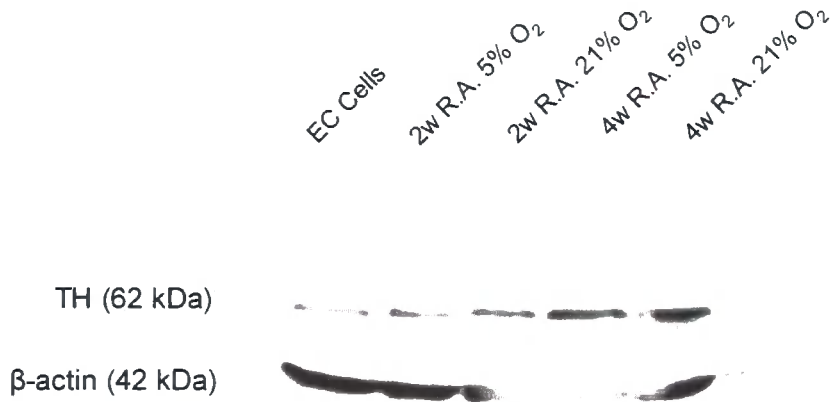


Figure 3.14: A Western blot showing tyrosine hydroxylase protein expression in Tera2.cl.SP12 stem cells and their 2 and 4 week 10 μ M retinoic acid treated derivatives in both 5% and normal oxygen culture environments. The best representation of n=3 blots each ran using biologically independent samples is shown. Beta-actin was used as a loading control. The presence of multiple bands may indicate non specific binding of the antibody or be the result of break down products given the bands are smaller in size than the main band. Non specific binding is more likely as the products are only slightly smaller than the main band of interest. This non specific binding would support the flow cytometry data that indicate many cells are antigen positive which could be accounted for by the binding of the antibody in a non specific manner. There does though appear to be some inductive effect particularly at 4 weeks due to retinoic acid exposure in a consistent manner with the flow cytometry (mean fluorescence intensity) and real time PCR data.

Figure 3.15: Assessment of Nurr1 expression via Western Blotting

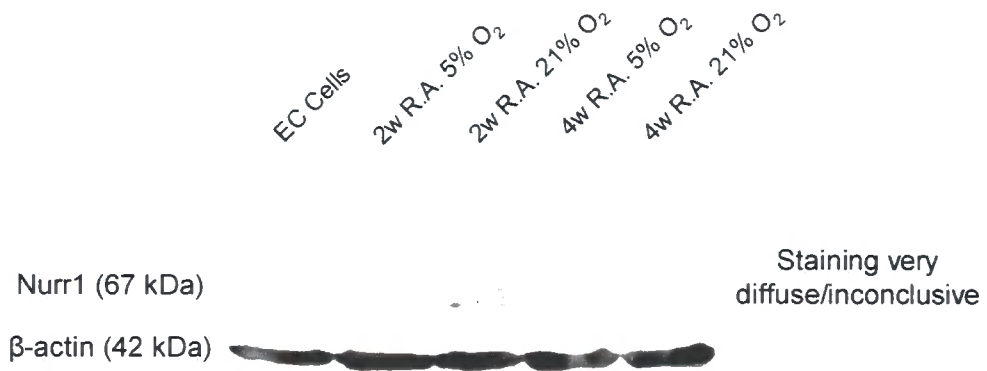


Figure 3.15: A Western blot showing Nurr1 protein expression in Tera2.cl.SP12 stem cells and their 2 and 4 week 10 μ M retinoic acid treated derivatives in both 5% and normal oxygen culture environments. The best representation of n=3 blots each ran using biologically independent samples is shown. Beta-actin was used as a loading control. The presence of multiple bands indicates that there may well be non specific binding of the antibody as mentioned previously. This in combination with the technical problems found when using the antibody essentially gives rise to a result that is far too ambiguous to base any meaningful conclusions on, as was the case in Figure 3.8 where the same reagent was used and where no real outcome was found.

The final technique used was immunocytochemistry. This gave an insight into the changing distribution and expression of Beta III tubulin and tyrosine hydroxylase. It was also used to assess nestin expression as the presence of this neuroprogenitor marker would confirm that retinoic acid treated cultures at four weeks were still populated by some cells that were less mature and relatively undifferentiated. In the longer term if nestin expression could be down regulated or turned off it may then favour acquisition of neuronal phenotypes as in Hara et al., 2007. Figures 3.16 and 3.17 show nestin expression in both sub confluent and confluent Tera2.cl.SP12 cultures, both conditions were tested as cells exist in both states at some point in their cycle of usage. In both scenarios nestin is clearly expressed, however tyrosine hydroxylase is expressed in a diffuse pattern and this too is the case for Beta III tubulin. The expression is so weak it is hard to detect in the merged images. This may be due to a degree of non specific binding of the antibodies used giving rise to a diffuse but detectable haze when recording the data. There is certainly no clear staining associated with neuronal cell body or process like structures. In the light of this positive staining is only viewed as such in the remainder of this Thesis if clear detection is observed alongside a characteristic morphological pattern indicative of potential neurites in culture (i.e. cell body and process like structures). Figure 3.18 shows the expression of these three markers following four weeks of retinoic acid treatment in both oxygen environments. Beta III tubulin staining is more intense and generally seen in neuronal process like structures leading from what could be neuronal cell bodies. It is hard by eye to discern any major differences due to oxygen tension. For tyrosine hydroxylase, which as a cytosolic protein is more diffuse in its appearance than the cytoskeletal Beta III tubulin, the scenario is somewhat different. There is expression associated with small process like structures emanating from possible cell bodies but the detection of such cells is scarce. Circles highlight possible candidates as in the merged image the Hoescht stained nuclei predominate heavily. This would suggest that there may be a low number of tyrosine hydroxylase positive cells that display neuronal type morphology present in such cultures. It does though highlight that the high numbers of cells detected as antigen positive in the earlier flow cytometry analysis are probably not a reflection of a high number of truly tyrosine hydroxylase positive cells of a potentially neuronal nature being present but are more likely the result of a broad non specific binding of the antibody. This is further supported by the detection of multiple bands in the Western blot in Figure 3.14, which are a further indication of non specific binding of the antibody. There once again at both time points appears to be little difference between

5 and 21% oxygen culture environments. The presence of nestin at four weeks shows that the cultures even after exposure to retinoic acid still possess some relatively immature cells. However the pattern of nestin expression as for tyrosine hydroxylase and Beta III tubulin is different from the undifferentiated to the differentiated cells which is compatible with the idea that as the cultures mature in the presence of retinoic acid there is a remodelling of the cellular architecture as more differentiated cell types form. Essentially these along with the results outlined previously indicate that retinoic acid can induce expression of neuronal as well as dopaminergic markers and give rise to cells reflective of a neuronal morphology. In addition they demonstrate that using 5% oxygen has no favourable effects over using standard (21%) oxygen culture conditions when utilising the Tera2.cl.SP12 cell line as a tool to study dopaminergic neurogenesis.

Figure 3.16: Immunostaining of Sub-Confluent Undifferentiated Tera2.cl.SP12 EC cell cultures

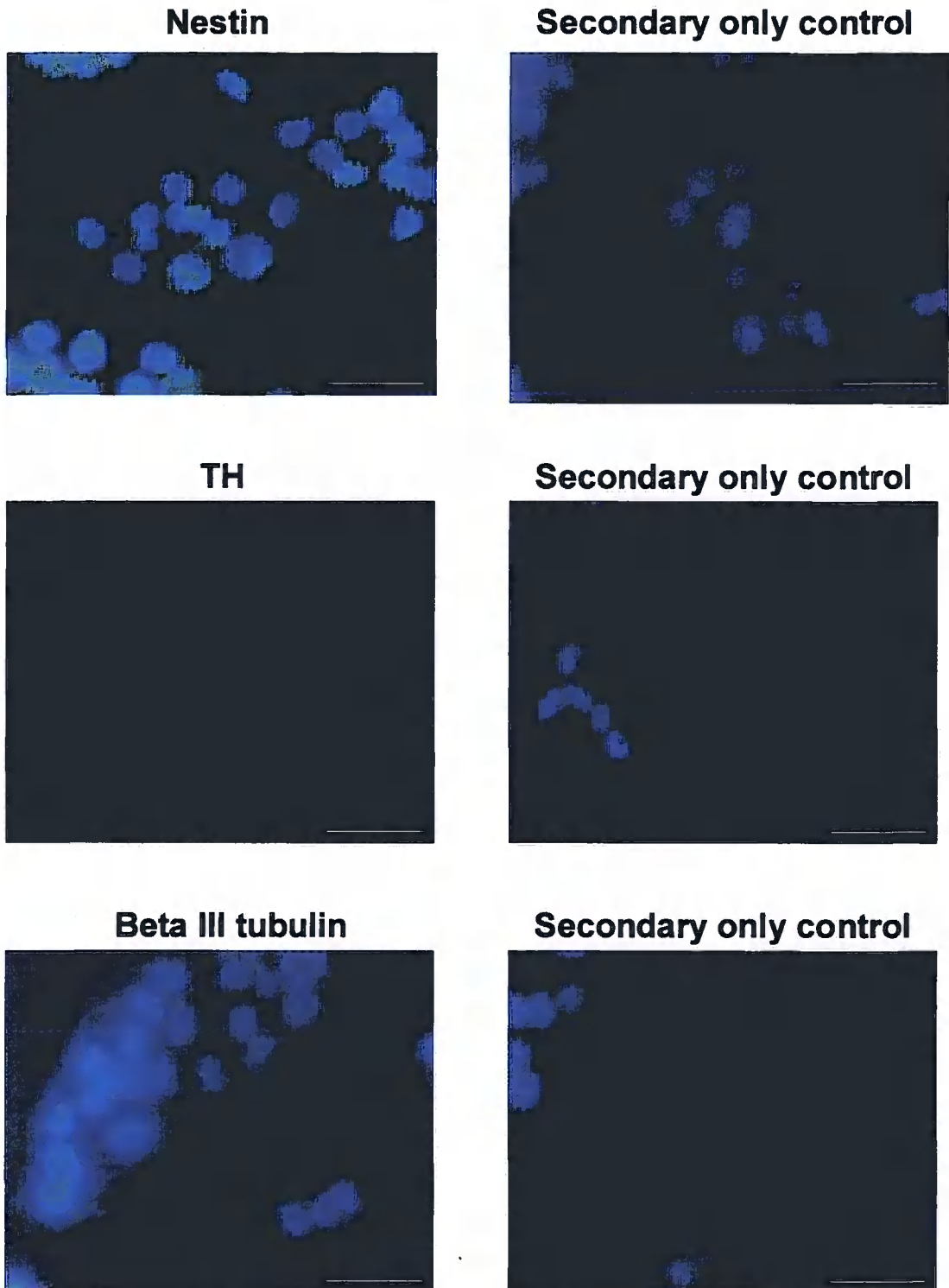


Figure 3.16: Immunostaining of sub-confluent Tera2.cl.SP12 cultures for Nestin, TH and Tuj1. Hoescht nuclear stain was used to confirm the presence of cells by staining their nuclei. Secondary only controls received no primary antibody treatment just the vehicle that primary antibodies were diluted in for the test conditions. All images were merged using Adobe Photoshop (scale bars represent 50 μ m). In all experiments n=3. Nestin is clearly detectable in EC cells. However TH and Beta III tubulin are only detectable as a weak haze on the image that is barely observable. This background level of detection is almost too faint to discern and may arise from a degree of non specific binding of the primary antibodies used.

Figure 3.17: Immunostaining of Confluent Undifferentiated Tera2.cl.SP12 EC cell cultures

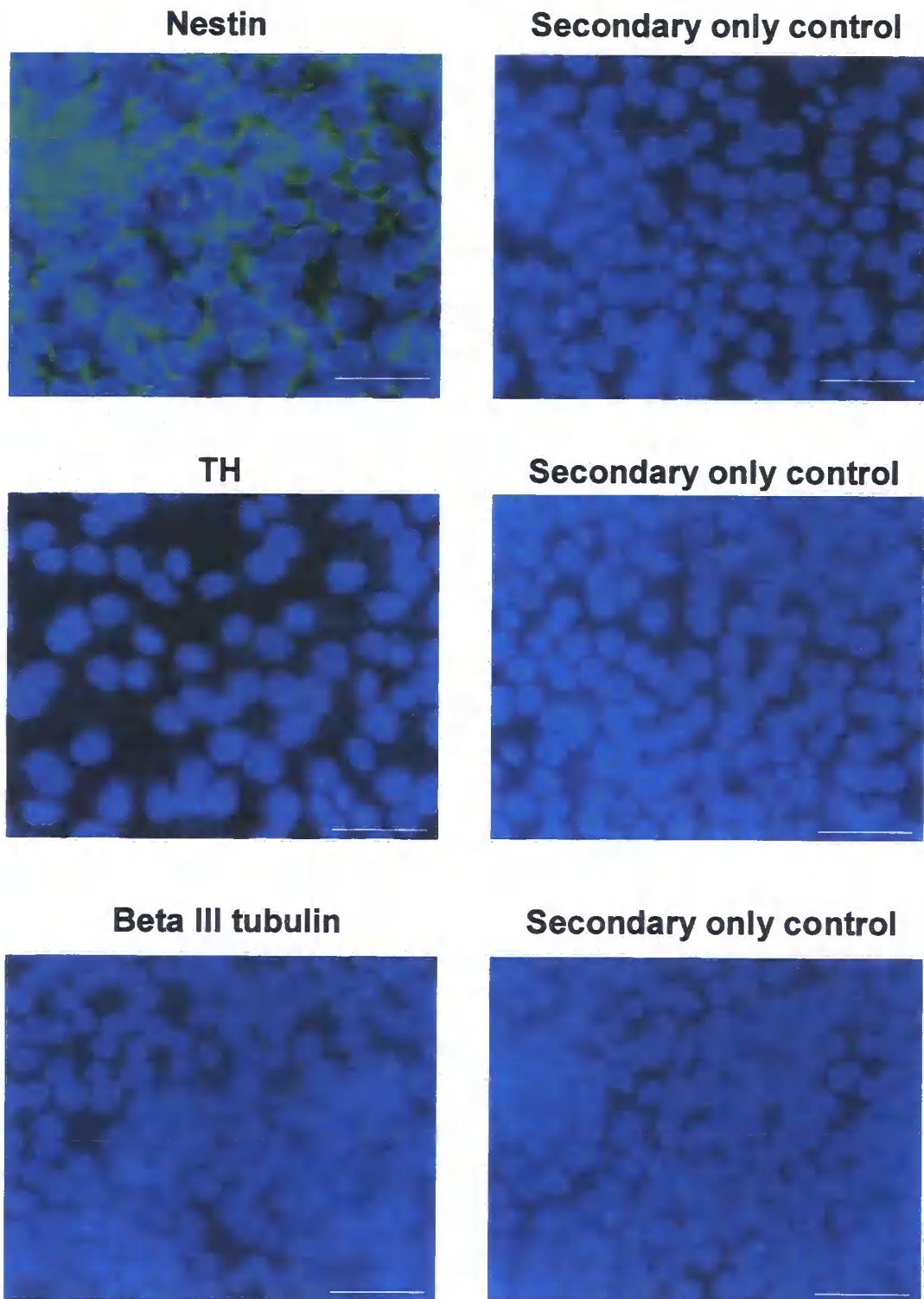


Figure 3.17: Immunostaining of confluent Tera2.cl.SP12 cultures for Nestin, TH and Tuj1. Hoescht nuclear stain was used to confirm the presence of cells by staining their nuclei. Secondary only controls received no primary antibody treatment just the vehicle that primary antibodies were diluted in for the test conditions. All images were merged using Adobe Photoshop (scale bars represent 50 μ m). In all experiments n=3. Nestin is clearly detectable in EC cells. However TH and Beta III tubulin are only detectable as a weak haze on the image that is barely observable. This background level of detection is almost too faint to discern and may arise from a degree of non specific binding of the primary antibodies used. This background detection is slightly more apparent for TH than Beta III tubulin, which is consistent with the TH antibody detecting multiple bands on the Western blot in Figure 3.14.

Figure 3.18: Immunostaining of 4 week R.A. differentiated EC cells

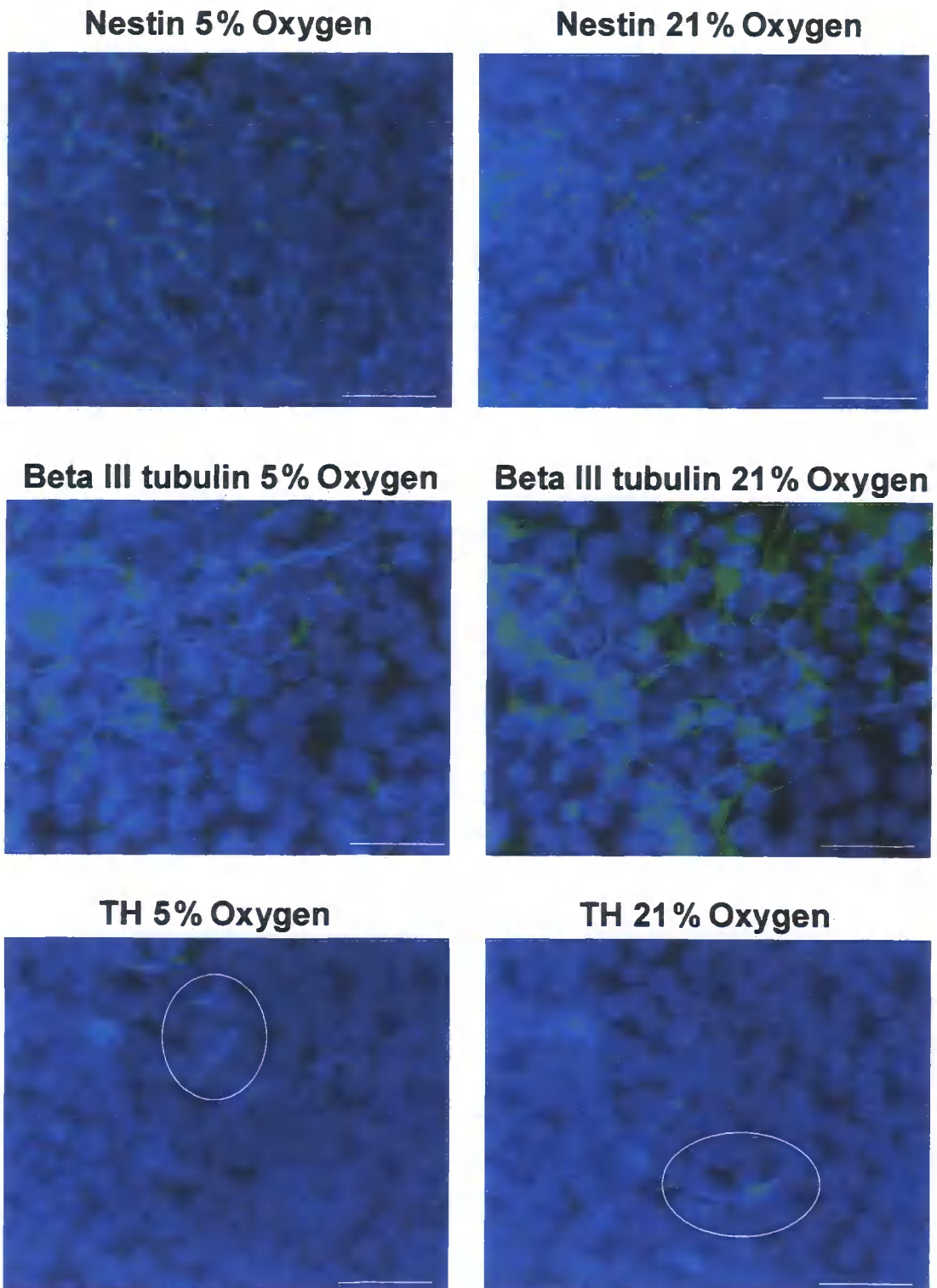


Figure 3.18: Immunostaining for Nestin, TH and Tuj1 of Tera2.cl.SP12 cultures treated with 10 μ M retinoic acid for 4 weeks in either 5% or normal (21%) oxygen conditions. All images were merged using Adobe Photoshop, Hoescht was used to stain cell nuclei (scale bars represent 50 μ m). In all experiments n=3. Nestin is still detectable in EC cell cultures after 4 weeks of exposure to retinoic acid. Beta III tubulin is detectable in structures resembling neuronal cell bodies and processes. TH is in areas such as those highlighted with circles present in small process like structures associated with potential cell bodies. This staining although dominated by the blue Hoescht stain is stronger than the background haze observed in Figures 3.16 and 3.17 and may indicate that there are a very small number of cells that are tyrosine hydroxylase positive that possess a morphology characteristic of what might be expected for a TH positive neurite in culture.

3.4 Discussion

In this Chapter the core objective was to gain an understanding of the human Tera2.cl.SP12 embryonal carcinoma stem cell line as a model to study dopaminergic neurogenesis. A number of hypotheses based on the literature outlined in the introductory Section 3.1 were specifically tested, in addition to a broader characterisation of the system being undertaken. The most basic hypothesis to be tested was whether retinoic acid can be used to induce expression of dopaminergic markers over time and also do embryonal carcinoma stem cells express any of these markers? From the results obtained it can be seen that Tera2.cl.SP12 cells do express often low but detectable levels of (at both the mRNA and protein level) a range of factors associated with a dopaminergic phenotype. However in some cases for proteins this may be questionable due to possible non specific binding of the immunological reagents used, but in terms of detection of the mRNA although detectable expression is often weak, this is a coherent finding in the context of embryonal carcinoma cells generally showing a propensity for neural differentiation. It appears that although the Tera2.cl.SP12 embryonal carcinoma cells express markers of pluripotency in a similar fashion to embryonic stem cells for which they are an apposite model system, they also differ in that they appear to be primed for neural differentiation and possibly exist in a state between an embryonic stem cell and a neuroprogenitor based on the marker expression data obtained (Figure 3.19) (Abeliovich & Hammond, 2007)

Figure 3.19: Midbrain dopaminergic neuron development (based on Abeliovich & Hammond, 2007)

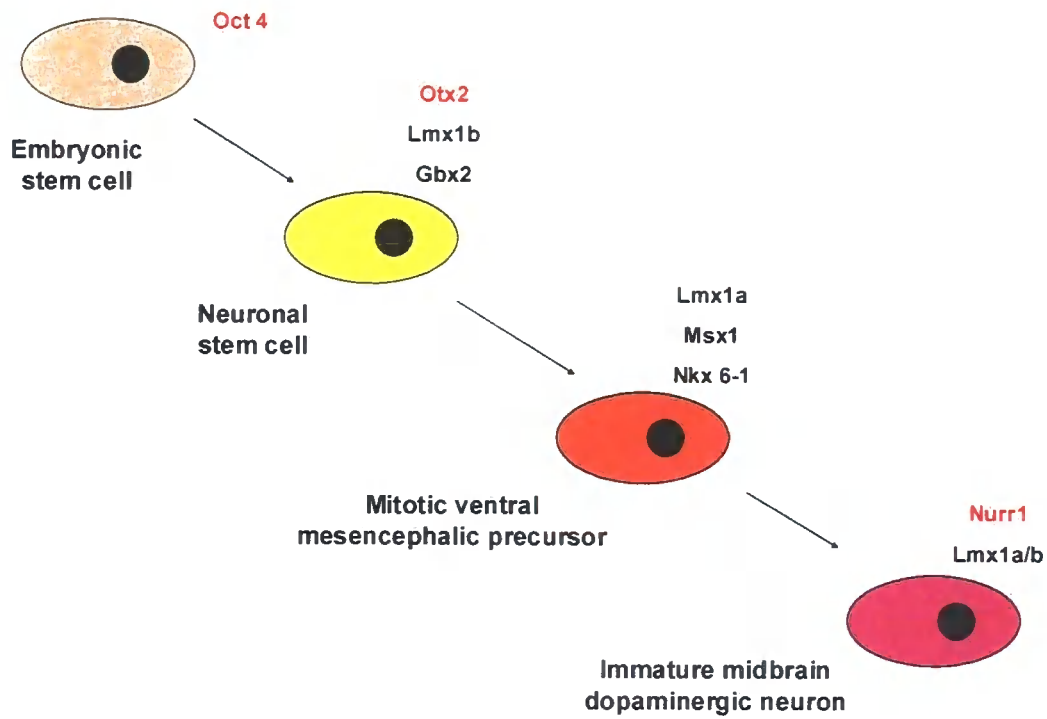


Figure 3.19: Changes in marker expression during dopaminergic development. Markers expressed by embryonal carcinoma cells are shown in red. The fact that such cells express markers associated with a range of developmental stages makes it difficult to assess where they lie in terms of them acting as a developmental model. It may be that it is the amount of certain markers present at any given time that is critical to their effect, based on Abeliovich & Hammond, 2007.

It is also clear from the findings presented in Section 3.3 that retinoic acid can induce and generally up regulate a number of factors associated with a dopaminergic phenotype. However there are differences in the patterns of regulation seen. Sox1 mRNA expression for example is controlled in an almost linear, highly predictable manner following retinoic acid treatment. In contrast Otx2 mRNA levels are driven to almost zero after just one day of exposure to retinoic acid, in an on/off type response. Some determinants of a dopaminergic phenotype like Nurr1 are seemingly expressed throughout the differentiation process, as has been observed elsewhere (Misiuta et al., 2003), with possible peaks such as that seen for the mRNA at one day. In addition other markers like tyrosine hydroxylase and Beta III tubulin show a differential regulation with peaks and troughs, such as the peak in Beta III tubulin expression after one day of retinoic acid

induction, the level of these factors though ultimately tends to increase with retinoic acid exposure time.

There also appears to be some correlation between mRNA and protein expression levels. This could indicate that a high level of mRNA can rapidly be processed to yield a comparably large amount of protein. If this is the case then the peak seen in Nurr1 mRNA expression after exposure to retinoic acid for one day, that mirrors the peak seen in Nurr1 protein expression in the work of Misiuta et al., 2006, where the sister Ntera2.D1 cell line is utilised, could correlate with the statistically significant increase observed in Beta III tubulin protein expression at this time point. Hara et al., 2007 were able to show Ntera2 cells over expressing Nurr1 were nestin immunonegative which could indicate Nurr1 may trigger an early commitment towards the neuronal lineage. If this simple correlation exists between mRNA and protein levels and the processing of mRNA to protein is rapid then the results obtained here for Nurr1 mRNA and Beta III tubulin protein may be related, with Nurr1 possibly having an effect on the levels of Beta III tubulin present. This possible interaction is extremely unlikely to be instantaneous prior to the time of sample collection but could feasibly occur in the first day of differentiation. Given retinoic acid treatment of Tera2.cl.SP12 cultures results in a mix of cell types, it may be that the neuronal lineage is specified early via a mechanism involving Nurr1. However there may not be such a simple correlation between mRNA and protein levels. If this is the case then the finding that Nurr1 is expressed in the Tera2.cl.SP12 cells themselves may be at least part of the reason why this rise in Beta III tubulin after one day of retinoic acid exposure is observed. This is in keeping with the concept that the embryonal carcinoma cells are primed for neural and possibly particularly neuronal differentiation. If either of these scenarios is true and the early rise in neuronal marker expression is not due to chance or another set of factors not investigated here then there is the suggestion that the Tera2.cl.SP12 cell line might be a suitable vehicle for studying dopaminergic neurogenesis.

Oxygen pressure however appears to be unable to exert any effect on the acquisition of a dopaminergic phenotype by Tera2.cl.SP12 cells. In the light of the work of Studer et al., 2000 who showed that this variable can strongly influence the production of dopaminergic neurons from rat mesencephalic precursors, the absence of any real difference in the Tera2.cl.SP12 system could be viewed as surprising. It may even

suggest that the embryonal carcinoma cells are perhaps unsuitable for use or incapable of responding in a similar fashion to the rat midbrain cells to oxygen. This however might be expected given that rat mesencephalic precursors are very disparate from human embryonal carcinoma stem cells, not just in the fact they come from different species but also different locations. A midbrain cell may be more responsive to *in vitro* replication of its *in vivo* environment, where as an embryonal carcinoma stem cell may exhibit no preference, as it would not naturally be found in the brain. This is a negative in that embryonal carcinoma cells are unresponsive to one factor that may increase the production of dopaminergic neurons. It could though be viewed as a positive in that they offer a robust system in which other factors can be studied without the variable of oxygen having an effect on any findings. Also during the specification of a dopaminergic phenotype, a cell bound for this fate may experience a range of oxygen pressures during its development, so although the embryonal carcinoma system may appear to simplify the possible level of understanding that can be obtained, it may actually in the light of current knowledge act as a more realistic base from which to develop. In addition a study by Kim et al., 2008 showed only a 1.34 fold increase in the number of tyrosine hydroxylase positive cells when a lowered (3.5%) oxygen environment was used to try to enhance the dopaminergic differentiation of murine embryonic stem cells. This again supports the notion that lowered oxygen may be best reserved for work using mesencephalic cultures, where it has a markedly greater effect both alone as in Studer et al., 2000 and in concert with other inductive factors (Maciaczyk et al., 2008) . Given the observations in this Chapter the decision was made that all future work was to be carried out under standard culture conditions.

Two other observations of interest that may also influence future work are those relating to the expression of the mRNA for Sox1 and Otx2. First of all it appears that if Sonic hedgehog and Fibroblast growth factor 8 are to be used then as shown for embryonic stem cells in Yan et al., 2005 and in the light of the findings of Stull and Iaccovitti, 2001, it is highly probable that they need to be applied reasonably early in the differentiation process to try and maximise their effect. Given the steady rise in Sox1, it is likely that no more than seven days of retinoic acid treatment will be required. However given that the most significant effects of this molecule appear to be at one day, the combined use of retinoic acid, Sonic hedgehog and Fibroblast growth factor 8 could be highly optimal, especially in a temporal sense.

The observation though that retinoic acid strongly down regulates the level of Otx2 mRNA appears to be a negative indicator in terms of the suitability for use of the Tera2.cl.SP12 system in the context of studying dopaminergic differentiation. The counter balance to this is the question of whether Wnt1 or another means could be used to rescue Otx2 expression. Wnt1 has also been shown to possess a range of other effects (shown in Figure 3.20), of particular interest are its ability to increase the number of Nurr1 positive precursors and the number of neurons produced by rat midbrain precursor cultures (Castelo Branco et al., 2003). If these possible effects could augment those of retinoic acid on Nurr1 and Beta III tubulin expression already observed then this could be one route to enriching the number of neurons particularly those of a dopaminergic phenotype produced from Tera2.cl.SP12 cultures and for this reason the effects of Wnt1 were decided upon as the next factor for investigation.

Figure 3.20: Investigating the potential effects of Wnt1 on the dopaminergic differentiation potential of Tera2.cl.SP12 human EC stem cells

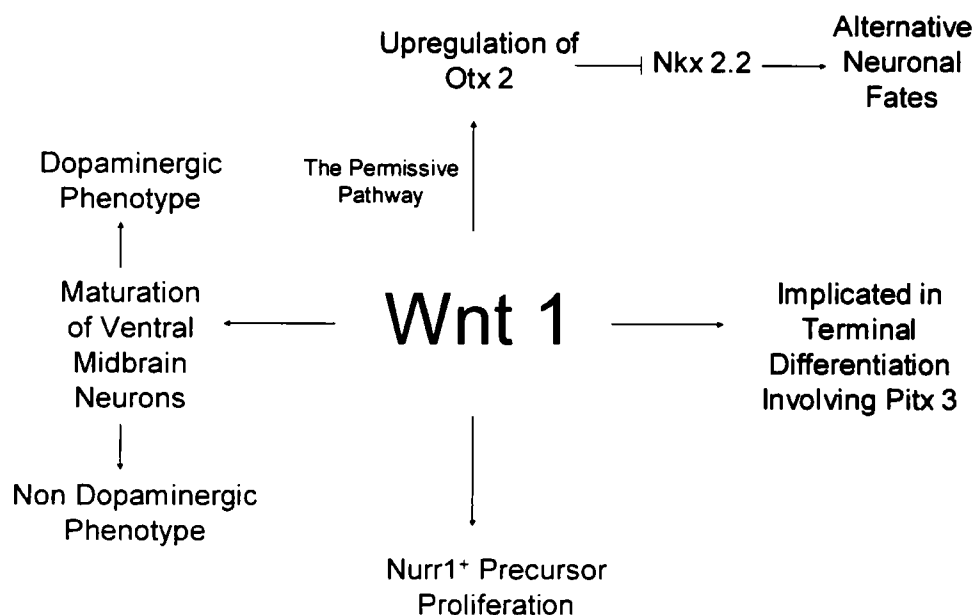


Figure 3.20: Potential effects of Wnt1 that could possibly be exploited in the Tera2.cl.SP12 cell system, based on the *in vitro* work of Castelo Branco et al., 2003 (red text), and the *in vivo* work of Prakash et al., 2006 (blue text).

Chapter 4

The effects of Wnt1 on the dopaminergic differentiation of the Tera2.cl.SP12 cell line

4.1 Introduction

Wnts are a family of proteins consisting of nineteen members that are typically 350 to 500 amino acids in length (Castelo-Branco & Arenas, 2006). They are subject to post transcriptional modifications such as glycosylation (Nusse & Varmus, 1992), and palmitoylation (Willert et al., 2003) and it is these alterations that account for their poor solubility in aqueous solutions, hydrophobic nature and preference for being located in cell membranes and the extracellular matrix. These properties may indicate that Wnts are likely to possess an action mainly at short range; however they are also able to exert an activity on targets at a distance (Zecca et al., 1996). As a family they are known to regulate a broad range of developmental processes including proliferation (Taipale & Beachy, 2001), stem cell self renewal (Rega et al., 2003), fate specification (Dorsky et al., 1998) and differentiation in neural cells (Rosso et al., 2005).

Wnts bind to and can activate two different types of receptor. The first of these are the seven pass transmembrane protein frizzled receptor family which comprises ten members. In addition Wnts may also interact with two low density lipoprotein related receptor proteins, LRP5 and LRP6. Generally Wnt family proteins are classified as either canonical or non canonical in relation to the means by which they relay their signals. Wnt1 is a classical example of a canonical Wnt that transduces its signal intracellularly by causing the stabilisation of beta-catenin, with a subsequent activation at the transcriptional level of T-cell factor/lymphoid enhancer elements (Figure 4.1) (Logan & Nusse, 2004). Non canonical Wnts like Wnt5a may signal in a variety of ways, for example, via activation of small GTPases, by activation of the c-Jun N-terminal kinase/planar cell polarity pathway or through increases in the amount of intracellular calcium (Veeman et al., 2003). A number of different processes from the early to late stages of neural development are regulated by members of the Wnt family (Castelo-Branco & Arenas, 2006), making it unsurprising that the effects of one such protein were the subject of investigation in this work. Wnt1 was chosen as the specific target to be studied for a number of reasons, the background to which is detailed below.

Figure 4.1: Wnt Signalling Pathways (redrawn from Arenas, 2005)

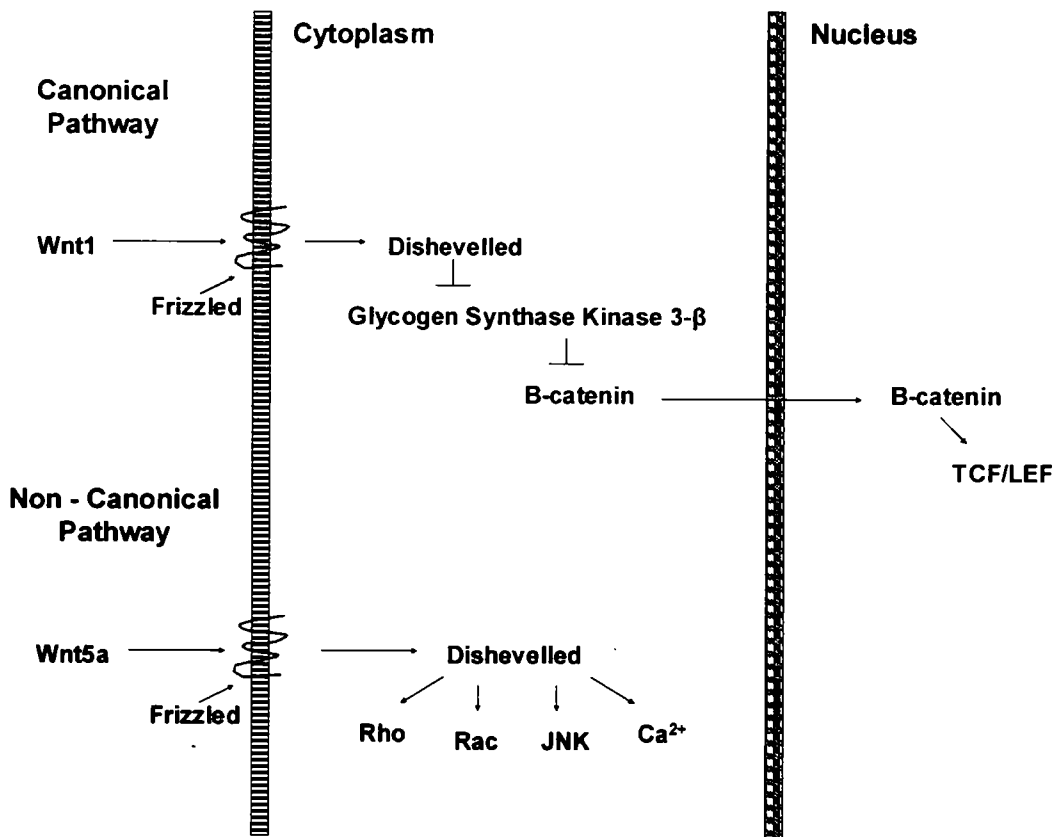


Figure 4.1: Canonical and non canonical Wnt signalling pathways, based on Arenas, 2005.

To begin, Wnt1 is expressed in the midbrain/hindbrain boundary by E8 and by E10.5 the expression of it has extended specifically to the domain in the ventral midbrain where dopaminergic neurons are formed. Wnt1 null mutant mice display a deletion of the posterior midbrain that includes the area where dopaminergic neurons are located, as well as an absence of the anterior hindbrain (McMahon & Bradley, 1990; Thomas & Kapecchi, 1990). Mice mutant for the receptor LRP6 have been reported to display a similar phenotype (Pinson et al., 2000). These results together indicate that Wnt1 is likely to play a key role in the processes specifying neural precursor cells in specific brain regions. Theoretically it may exert an effect either on their proliferation, their differentiation or possibly even elicit a response based on a combination of both factors (Arenas, 2005). Interestingly when Wnt1 is over expressed in transgenic animals under the control of the promoter for engrailed 1, the result via a mechanism that involves proliferation and differentiation based effects is an increase in the cell

numbers and size of the midbrain/hindbrain structures (Panhuysen et al., 2004). All of this evidence is indicative of Wnt1 possessing a key role in neural development. Further specific effects of this signalling molecule have also been identified in other *in vivo* as well as in, *in vitro* systems.

In a series of experiments using a murine model and explant cultures, it was found that Wnt1 exerts two clear effects. First of all it is implicated in the formation of the mesencephalic dopaminergic progenitor domain. It carries out this function via its ability to maintain expression of Otx2 in the ventral mesencephalon which subsequently is involved in repressing the expression of Nkx2-2 in this area. If Nkx2-2 is not repressed in the midbrain floor plate and basal plate, this results in the production of serotonergic neurons rather than their dopaminergic counterparts (Prakash et al., 2006). Given the observation in Chapter 3 that retinoic acid causes a rapid decrease in Otx2 mRNA expression relative to that seen in the Tera2.cl.SP12 cells themselves. It appeared prudent to investigate the potential ability of Wnt1 to rescue this effect or override the retinoic acid induced down regulation via its facility to maintain Otx2 expression.

Figure 4.2: Possible Signalling Pathways Implicated in Dopaminergic Neurogenesis (Adapted from Abeliovich & Hammond, 2007)

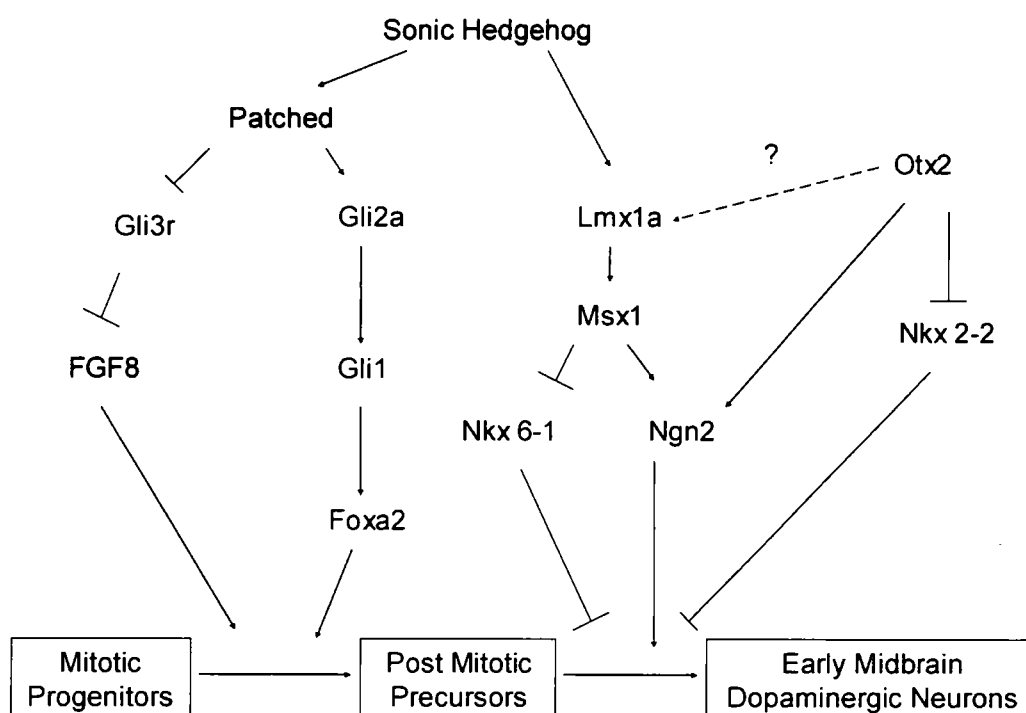


Figure 4.2: Transcription factors are in black text and soluble factors are in blue text. In the context of this work the question is; does Otx2 effect Lmx1a expression in the Tera2.cl.SP12 cell line in the presence of Wnt1 and retinoic acid?

In addition in a fate mapping study that utilised fluorescence activated cell sorting involving corin, a floor plate specific cell surface marker. Ono et al., 2007, were able to demonstrate that mesencephalic floor plate cells possess a neurogenic activity and are able to form midbrain dopaminergic neurons *in vitro*, but that caudal floor plate cells lack this neurogenic potential. By carrying out further work in *dreher* mutant mice that contained a mutation in the locus for Lmx1a and via the use of transgenic mice that displayed ectopic expression of Otx2 in caudal floor plate cells, Ono et al., 2007 were able to illustrate that Otx2 establishes an anterior identity that imparts neurogenic activity to cells of the floor plate. In turn defining a mesencephalic dopaminergic fate at least to some degree via induction of Lmx1a (Ono et al., 2007). Given the concept that Wnt1 may create a permissive environment in which inductive cues such as Sonic hedgehog and Fibroblast growth factor 8 can operate to specify the

formation of dopaminergic neurons (Burbach & Smidt, 2006) and the finding that Wnt1 can maintain expression of Otx2 which itself may help specify a dopaminergic fate at least in part by a mechanism involving Lmx1a. This poses the question of whether Wnt1 could not only rescue or maintain Otx2 expression in itself but could it also display positive effects on the expression of Lmx1a and other downstream determinants (Figure 4.2) of a dopaminergic phenotype? Also Sonic hedgehog pre treated mouse embryonic stem cells that have had the Lmx1a gene introduced into them show a strong capacity for dopaminergic differentiation (Andersson et al., 2006). This opens up the possibility if Wnt1 is able to exert a positive effect in the Tera2.cl.SP12 system that the next investigations to be undertaken could coherently link the Wnt1 studies into new work involving Sonic hedgehog and Fibroblast growth factor 8, based on the model presented in Burbach & Smidt, 2006 (Figure 4.3).

Figure 4.3: Factors & Signals Involved in The Specification of a Dopaminergic Neuronal Fate from Neuroepithelial Stem Cells (based on Burbach & Smidt, 2006)

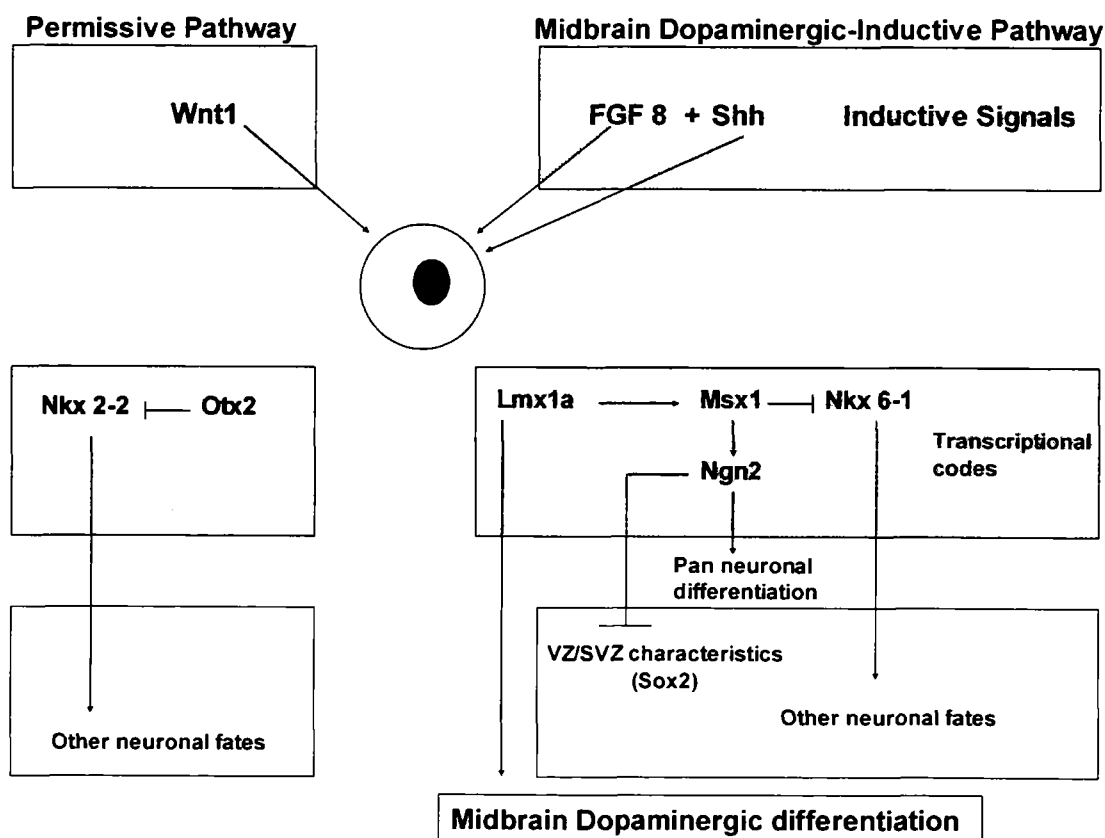


Figure 4.3: Wnt1 may create a permissive environment in which Sonic hedgehog and Fibroblast growth factor 8 can function to drive the sequential development of dopaminergic neurons (based on Burbach & Smidt, 2006).

On top of the possibilities for investigation that Wnt1 offers based on the first clear effect seen in Prakash et al., 2006, in this work they were also able to demonstrate that this glycoprotein has a secondary activity that is seen later in the process of dopaminergic fate specification. It appears that Wnt1 is required for the terminal differentiation of midbrain dopaminergic precursors into mature dopaminergic neurons (Prakash et al., 2006).

In an *in vitro* system using rat mesencephalic cultures Wnt1 was likewise able to exert two effects. In a similar fashion to Wnt3a, it was able to promote neurogenesis by a mechanism involving proliferation of Nurr1 positive precursors. In a comparable manner but to a lesser extent than Wnt5a, it was able to influence the differentiation of such precursors into mature dopaminergic neurons. The general ability of Wnt1 to increase the number of Tuj1 positive cells by two fold but unfortunately not the proportion of tyrosine hydroxylase positive cells within this subpopulation (Castelo Branco et al., 2003) was another desirable effect to pursue, as it offered the prospect of at least producing a more heavily neuronal, less mixed culture than those obtained using only retinoic acid in conjunction with the Tera2.cl.SP12 embryonal carcinoma stem cell system. In the light of the sharp rise in Nurr1 expression seen in this retinoic acid only condition after just 1 day of exposure to it, and given the effects of Wnt1 on Nurr1 positive precursor cells, this too opened up the possibility of whether Wnt1 could augment or cause some sort of additional complimentary effect to that already observed. Therefore given that it may exert two effects rather than just one as appeared to be the case for Wnt3a and Wnt5a, and that any positive potentially demonstrable effects may not only tie in with previous work but also facilitate further study possibly involving the application of Sonic hedgehog and Fibroblast growth factor 8, Wnt1 seemed a prime factor for investigation.

The fact that it is a classical example of a canonical Wnt added further to this argument as over expression of beta-catenin in neural stem cells can both enhance their proliferation when Fibroblast growth factor 2 is present, as well as helping to induce neuronal differentiation when it is absent. As a canonical Wnt, it would be expected that Wnt1 would stabilise expression of beta-catenin and thus possibly exert favourable effects in terms of achieving production of a dopaminergic phenotype from Tera2.cl.SP12 cells. Further work in rat E14.5 ventral midbrain precursor

cultures gave extra support to this idea. In this study the focus was on glycogen synthase kinase - 3β a key part of the canonical signalling pathway. By applying two competitive inhibitors of glycogen synthase kinase - 3β to the precursors, Castelo Branco et al., 2004 were able to show in a concentration dependent manner an increase in the number of tyrosine hydroxylase positive cells. Beta-catenin over expression in such cultures was also capable of causing an increase in tyrosine hydroxylase positive cell numbers. Interestingly both inhibitors increased the number of neurons present as well as the number of tyrosine hydroxylase positive cells within the neuronal population (Castelo Branco et al., 2004). One of the inhibitors, Kenpauillone, was able to raise the number of Nurr1 positive precursors gaining a tyrosine hydroxylase positive phenotype, as well as causing an increase in expression of the dopaminergic marker c-ret. This compound was then applied to the dopaminergic cell line MN9D causing significant neurite outgrowth but exerting no effect on the proliferation rate of such cultures. Overall these observations suggest that the effects of Wnts on dopaminergic differentiation may be mimicked by inhibition of glycogen synthase kinase - 3β (Castelo Branco & Arenas, 2006). As described in Castelo Branco & Arenas, 2006, it is of interest that inhibition of glycogen synthase kinase - 3β can be achieved via stimulation of other pathways, for example by Sonic hedgehog, giving a possible synergy with beta-catenin signalling (Mill et al., 2005). This raises the question as to whether dopaminergic neurogenesis is regulated at key junctions where signals from different pathways intersect and are integrated (Castelo Branco & Arenas, 2006).

All of this evidence points to Wnt1 potentially possessing a number of effects that could be effectively harnessed to influence the dopaminergic differentiation of the Tera2.cl.SP12 embryonal carcinoma stem cell line. In summary the main hypotheses to be tested are; can Otx2 expression be rescued and if so has this any effect on the expression of Lmx1a and its downstream partners? Can Wnt1 exert any additional effect on Nurr1 expression in comparison to that already observed using retinoic acid in Chapter 3? Can any other dopaminergic markers be up regulated in their expression and is it possible to achieve an enhanced yield of neurons? Also if any of these effects were achieved might they occur via a mechanism involving regulation of beta-catenin? The methods used to test these theories follow in the next section and the experimental results are described in section 4.3.

4.2 Cell Culture Methods

Treatment of EC cell cultures with Retinoic acid and Wnt1

Cultures of Tera2.cl.SP12 EC cells were grown to confluency, the media aspirated from them, before being washed with 1x PBS, prior to treatment for 5 minutes with 0.25% (w/v) Trypsin/0.1% (w/v) EDTA (in HBSS) (Cambrex), to release the cells from the surface of the culture flask. The Trypsin was then neutralized by addition of 4ml of DMEMFG and any cells remaining loosely bound washed off the surface of the flask by pipetting the DMEMFG/Trypsin-EDTA mix against the side of the flask where cell attachment occurs. A further wash with 1x PBS was carried out to optimize the retrieval of EC cells for use. The cell suspension was then transferred to a 15ml Falcon tube and spun in a centrifuge (Eppendorf 5810R (swing bucket rotor A-4-62)) at 800rpm, 4°C, for 2 minutes. The excess fluid was removed to leave a pellet. This was resuspended in DMEMFG and cell number determined by using a haemocytometer. Once the cell number was known the EC cells were subsequently seeded out at the following densities depending on the culture ware used:

- For a T25 – 0.5×10^6 cells per flask,
- For a single well of a 6 well plate containing a 22mm poly-D-lysine coated glass coverslip – 0.2×10^5 cells per well.

Once cells were seeded out at an appropriate density for the culture vessel, they were exposed to one of the following 3 conditions for varying periods of time to attempt to induce differentiation. The 3 treatment regimes were exposure to a final concentration of $10 \mu\text{M}$ all trans retinoic acid (Sigma). Exposure to a final concentration of $10 \mu\text{M}$ all trans retinoic acid (Sigma) along with Wnt1 (Peprotech) (made up in sterile autoclaved distilled water) at a final concentration of 2ng/ml, and finally exposure to a final concentration of 2ng/ml of Wnt1. Media was changed every 2 - 4 days. All cells were maintained in standard 37°C, 5% CO₂ incubators (Sanyo).

4.3 Results

Prior to investigating the potential effects of Wnt1, a small control experiment was carried out to try and assess if the commercially available compound that had been purchased for use was able to function and exert an effect on the Tera2.cl.SP12 system. In addition as the Wnt1 that was to be applied exogenously was dissolved in water before use, a vehicle control containing only water was included in the analysis to see if the vehicle was able to cause any effect. In theory if the vehicle caused little or no effect then any results observed were likely to be due to the presence of Wnt1. Real time PCR was used to assess expression of the mRNA for Oct4 and Sox1 as these two markers had previously been shown to be regulated in a predictable manner. Figure 4.4A shows that for Oct4, expression is high in the embryonal carcinoma cells and is subsequently down regulated following exposure of them to retinoic acid. Exposure in the Wnt1 only condition is almost identical to that in the starting material, which may suggest Wnt1 can maintain expression of this stem cell marker or more realistically may indicate that Wnt1 alone is insufficient to gain control of the highly proliferative embryonal carcinoma cell cultures. The vehicle control appears to have no real effect and the embryonal carcinoma cells in practice just become very confluent and display continued Oct4 expression. The combined use of retinoic acid and Wnt1 shows little difference to the effect observed using retinoic acid alone. This may suggest Wnt1 has no effect in this instance or that retinoic acid is predominant in down regulating expression of markers of pluripotent stem cells and as such more responsible for exercising control over the cell system possibly by driving it towards a more differentiated state as was broadly seen in Chapter 3.

Figure 4.4B shows that Sox1 is up regulated as expected following 7 days of retinoic acid treatment of the Tera2.cl.SP12 cell line in which it is only expressed at a low level. The Wnt1 only and vehicle control conditions show a highly similar result. This could indicate that the Wnt1 is not able to exert a detectable effect. However it may indicate that at the dose used Wnt1 alone is unable to exert any control over Sox1 mRNA expression in the embryonal carcinoma cells. This latter option appears more likely as in the retinoic acid and Wnt1 condition there is a greater expression of Sox1 mRNA than that seen in the retinoic acid only sample. This would suggest therefore

that Wnt1 in conjunction with retinoic acid can function and possibly influence the differentiation of the Tera2.cl.SP12 cell line. It also underpins the concept that retinoic acid may be necessary to gain control over the proliferative embryonal carcinoma stem cell starting material, by acting as an inducer of differentiation.

Figure 4.4: Testing for an effect due to the exogenously applied Wnt1

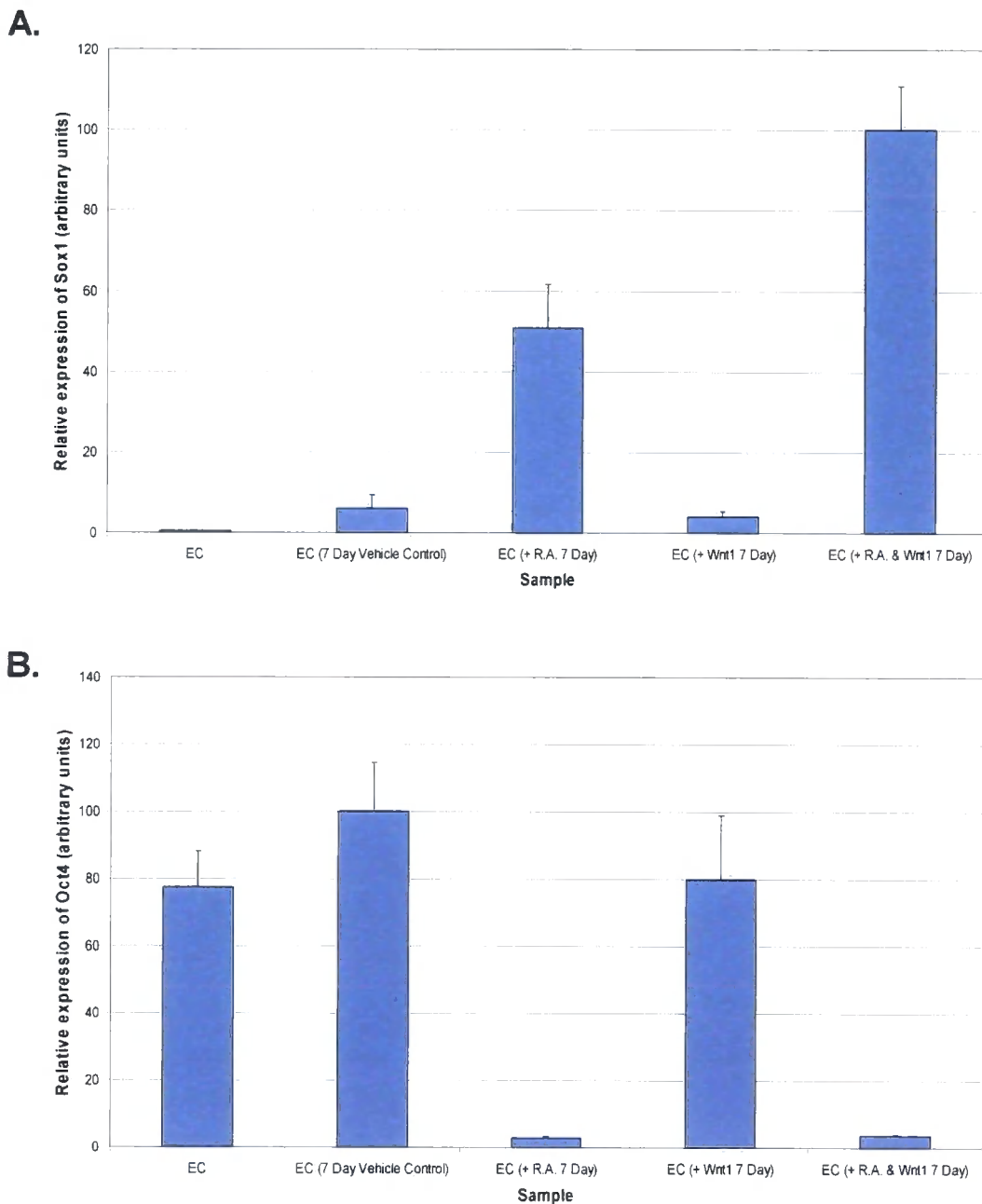


Figure 4.4A: Real time PCR analysis of Sox1 expression in Tera2.cl.SP12 cultures under a range of treatment conditions involving the application for 7 days of 10 μ M retinoic acid, 2ng/ml Wnt1 or water in the vehicle control. **B:** Real time PCR analysis of Oct4 expression in Tera2.cl.SP12 cultures under a range of treatment conditions involving the application for 7 days of 10 μ M retinoic acid, 2ng/ml Wnt1 or water in the vehicle control. Error bars represent the S.D. of the mean.

Figure 4.5 shows the results of an MTS assay used to assess cell proliferation. After seven days in culture the Wnt1 only condition is nearly identical to that for the embryonal carcinoma cell only control. This suggests that Wnt1 alone has no real tangible effect on the cell system. Retinoic acid alone and in combination with Wnt1 does however show a clear effect after seven days as would be predicted. There is though no statistically significant difference between the retinoic acid only and retinoic acid plus Wnt1 conditions. Both these sample sets do display statistically significant differences from the Wnt1 only condition, thus it appears that retinoic acid as suspected is likely to be required in some capacity to induce differentiation and gain a basic level of control when trying to utilise Wnt1 at the concentration tested in this cell system. As a result most subsequent analyses in this Chapter do not include Wnt1 only samples. When such a condition is included it is just as a small scale screen to see if Wnt1 alone can cause a result that may be detectable by one of the range of techniques used.

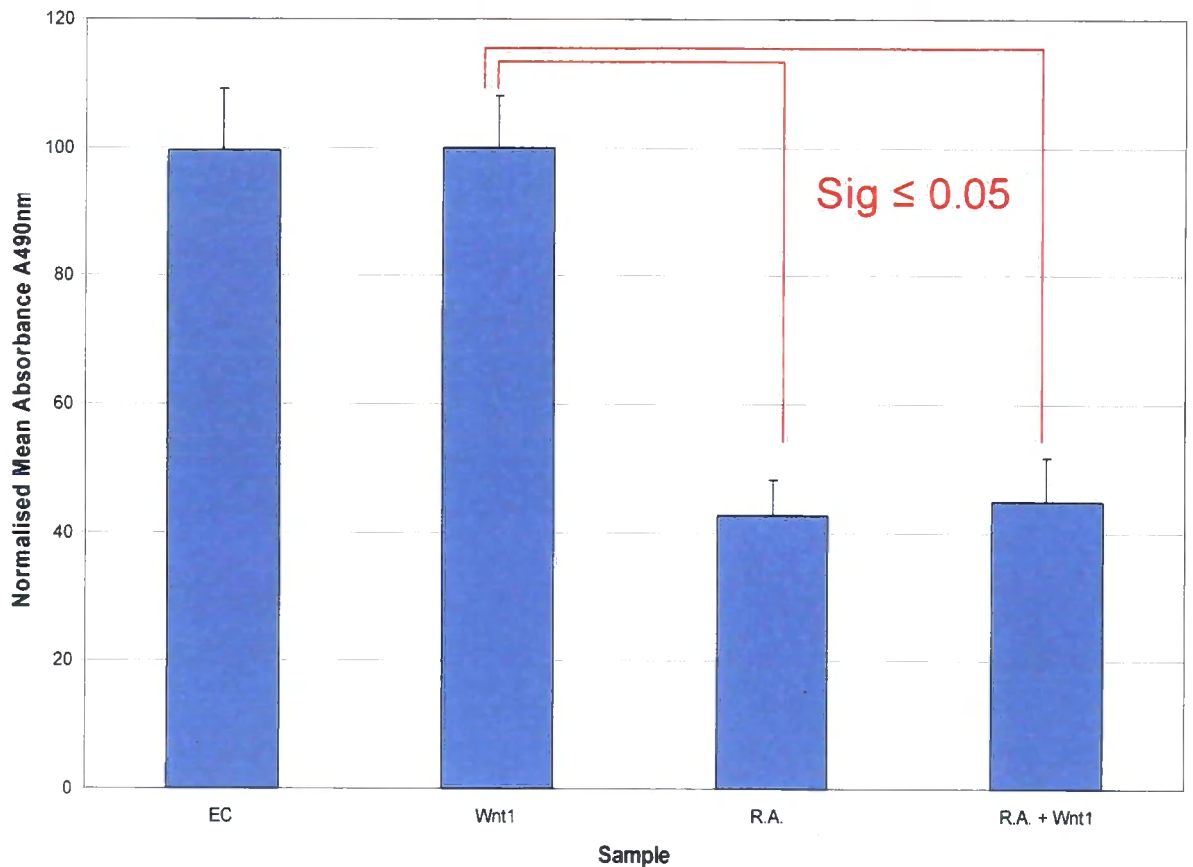
Figure 4.5: Proliferation of Tera2.cl.SP12 cultures treated with R.A. and Wnt1

Figure 4.5: MTS assay data showing the differences in cell proliferation between untreated Tera2.cl.SP12 cells cultured for 7 days and their counterparts following treatment with 2ng/ml Wnt1 alone, 10 μ M retinoic acid alone, or a combination of both reagents for the same time span. In all experiments $n=3$ and error bars represent the S.D. of the mean. The one way anova output indicates that the Wnt1 only condition differs significantly from the retinoic acid only and combined Wnt1 plus retinoic acid samples, but these data sets do not differ significantly from each other.

Although Wnt1 could seemingly exert an effect when used alongside retinoic acid, if it were to do so it would be expected to act via an appropriate receptor and by a canonical signalling mechanism. The pattern of expression of Frizzled 8, the receptor for Wnt1, was assessed using real time PCR (Figure 4.6). This confirmed the presence of the mRNA for it and was therefore indicative that Wnt1 may be able to apply an effect on Tera2.cl.SP12 cultures. Receptor expression was also assessed in 7, 14 and 21 day old cultures treated with retinoic acid alone or in combination with Wnt1. There was a generally variable expression pattern, however at 7 days in the presence of both reagents there was almost double the mean expression level seen in the retinoic acid only samples. In other work presented later in this Chapter a similar effect is apparent at this time point. This may suggest that Wnt1 in conjunction with retinoic acid may regulate its own activity to some degree, possibly by up regulating expression of the receptor for it. The difference though between the retinoic acid only and retinoic acid plus Wnt1 conditions as indicated by the one way anova output, is not statistically significant, nor do the levels of mRNA expression in either test condition at seven days differ to a statistically significant degree from those seen in the embryonal carcinoma stem cells. Interestingly after three weeks of exposure to retinoic acid there is a noticeable peak in Frizzled 8 expression, this could be due to the system being primed at this stage for further neuronal differentiation or maturation possibly driven by Wnt1. Alternatively it may be due to a difference in expression of the house keeping gene used, however this is not that likely as the same trend is observed across two different house keeping genes, which serve different cellular functions. Therefore it may reflect that after three weeks of retinoic acid treatment Tera2.cl.SP12 cells are potentially open to manipulation by Wnt1. It may be that such cultures are themselves producing this molecule in this condition at this time. This would be in line theoretically with the notion that Wnt1 may be able to self regulate its activity.

Figure 4.6: The effects of Wnt1 and R.A. on Frizzled 8 mRNA expression

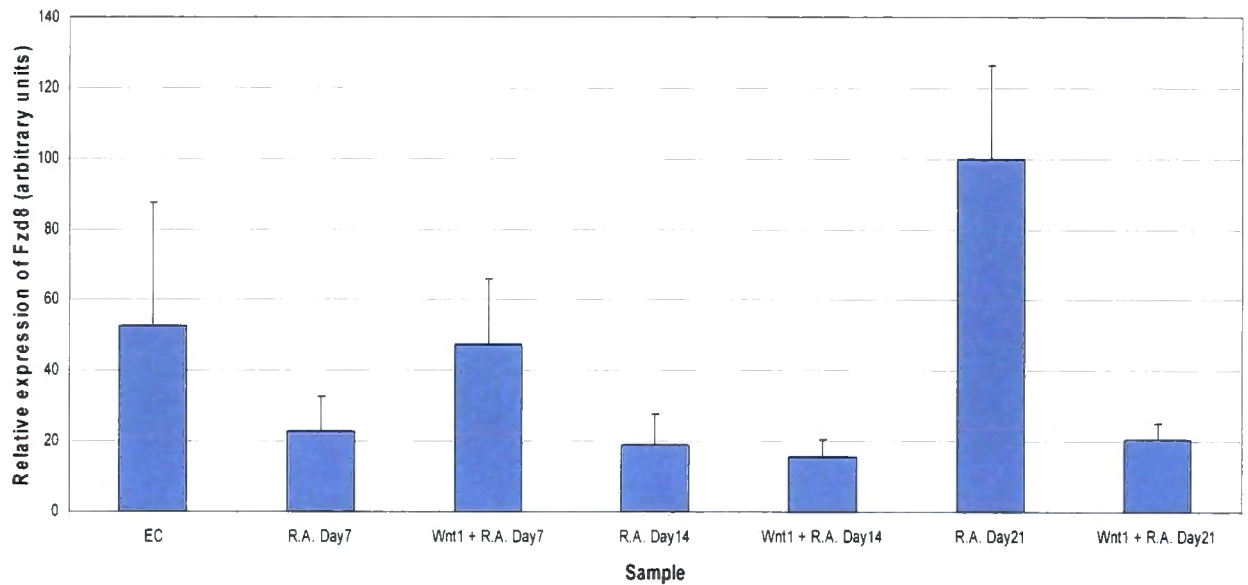


Figure 4.6: The relative expression of Frizzled 8 mRNA in Tera2.cl.SP12 cells and their derivatives treated with 10µM retinoic acid only, or 10µM retinoic acid plus 2ng/ml Wnt1 for 7, 14 and 21 days. The one way anova output indicates there is no statistically significant difference between the two test conditions at 7 days and that these samples do not differ to a statistically significant level from the embryonal carcinoma stem cell starting material. In all experiments n=3 and error bars represent the standard deviation of the mean.

Figure 4.7: The effects of Wnt1 and R.A. on Beta-catenin mRNA expression

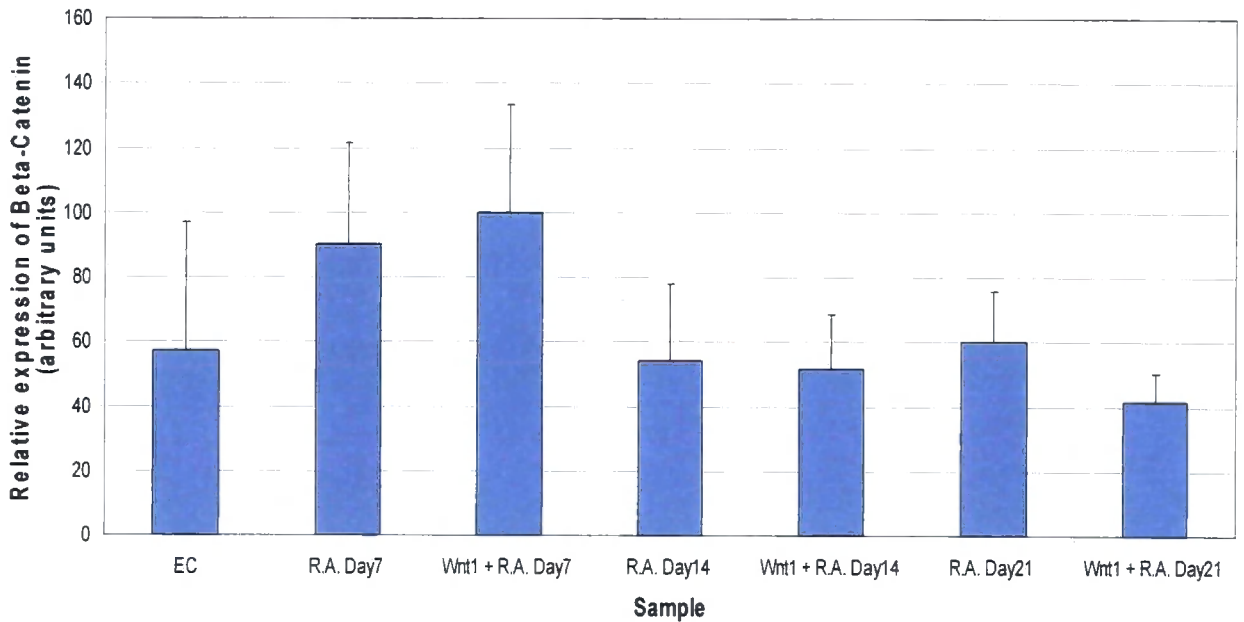


Figure 4.7: The relative expression of Beta-catenin mRNA in Tera2.cl.SP12 cells and their derivatives treated with 10µM retinoic acid only, or 10µM retinoic acid plus 2ng/ml Wnt1 for 7, 14 and 21 days. The one way anova output indicates there is no statistically significant difference between the two test conditions at 7 days and that these samples do not differ to a statistically significant level from the embryonal carcinoma stem cell starting material. In all experiments n=3 and error bars represent the standard deviation of the mean.

If Wnt1 is having an effect it would be expected to do so via a canonical route, involving stabilisation of beta-catenin. Although there is no statistically significant difference in the expression of beta-catenin mRNA between the two seven day conditions tested and the embryonal carcinoma cell starting material, a similar trend as is seen for Frizzled 8 expression is observed (Figure 4.7). In the presence of Wnt1 and retinoic acid at seven days there appears to be a greater expression of beta-catenin, suggesting Wnt1 may be exerting any possible effects at this time via a canonical signalling mechanism. Beyond seven days though there is little difference between samples. Ideally if possible it would be worthwhile to test for differences in protein expression of this molecule as it is the protein that would be responsible for any tangible effect. The mRNA data though imply that there may be positive effects due to Wnt1 that could be mediated by increases in beta-catenin mRNA levels, that may logically in theory be related to stabilisation of this protein.

In the light of these initial findings it appears that Wnt1 may be able to influence the dopaminergic differentiation of Tera2.cl.SP12 embryonal carcinoma cells in the presence of retinoic acid. The next step therefore was to try and test the hypotheses outlined in the introductory section 4.1. Given the positive effects on Nurr1 mRNA expression seen using retinoic acid in Chapter 3 and the work of Castelo Branco et al., 2003, who were able to demonstrate that Wnt1 could increase the number of Nurr1 positive precursors in rat mesencephalic cultures, this was the first factor to be investigated. Figure 4.8 shows that at the mRNA level there is hardly any difference in the expression pattern of this transcription factor. Wnt1 may slightly augment the relative level of it at 1 day but given the variation in the results, there is no real difference. The Wnt1 only condition was also included in this analysis and shows a highly variable expression profile, supporting the view that Wnt1 alone is insufficient to direct the differentiation of Tera2.cl.SP12 embryonal carcinoma stem cells. It appears in this instance that even in the presence of retinoic acid; Wnt1 is unable to or does not play a role in the regulation of Nurr1, a key determinant of a dopaminergic phenotype.

Figure 4.8: The effects of Wnt1 and R.A. on Nurr1 mRNA expression

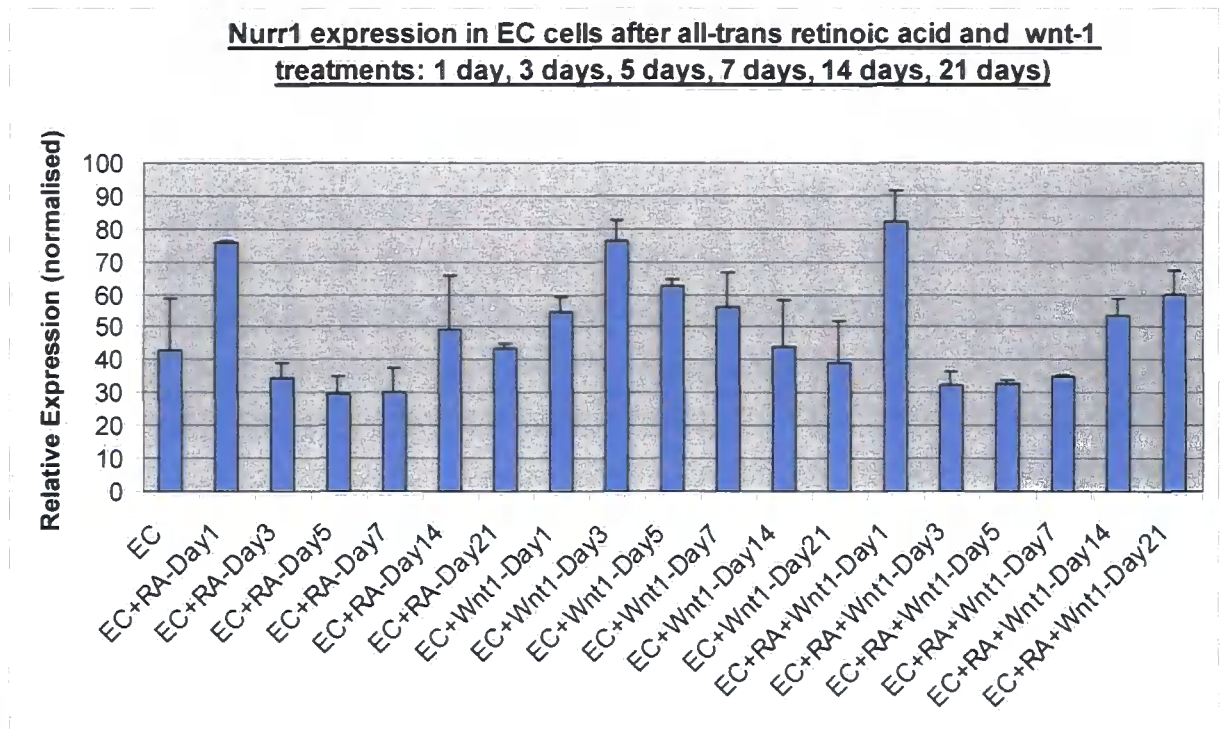


Figure 4.8: The relative expression of Nurr1 mRNA in Tera2.cl.SP12 cells and their derivatives treated with 10 μ M retinoic acid only, 2ng/ml Wnt1 only, or 10 μ M retinoic acid plus 2ng/ml Wnt1 for 1, 3, 5, 7, 14 and 21 days. In all experiments n=3, error bars represent the S.D. of the mean.

Flow cytometry was used to try and assess if there was any detectable difference between test conditions at the protein level. However Figure 4.9 shows that as in Chapter 3 technical problems with the antibody used, essentially led to low intensity expression that was highly variable between cultures and gave no results on which meaningful conclusions could be made.

Figure 4.9: Nurr1 protein expression in EC cells treated with Wnt1 and R.A.

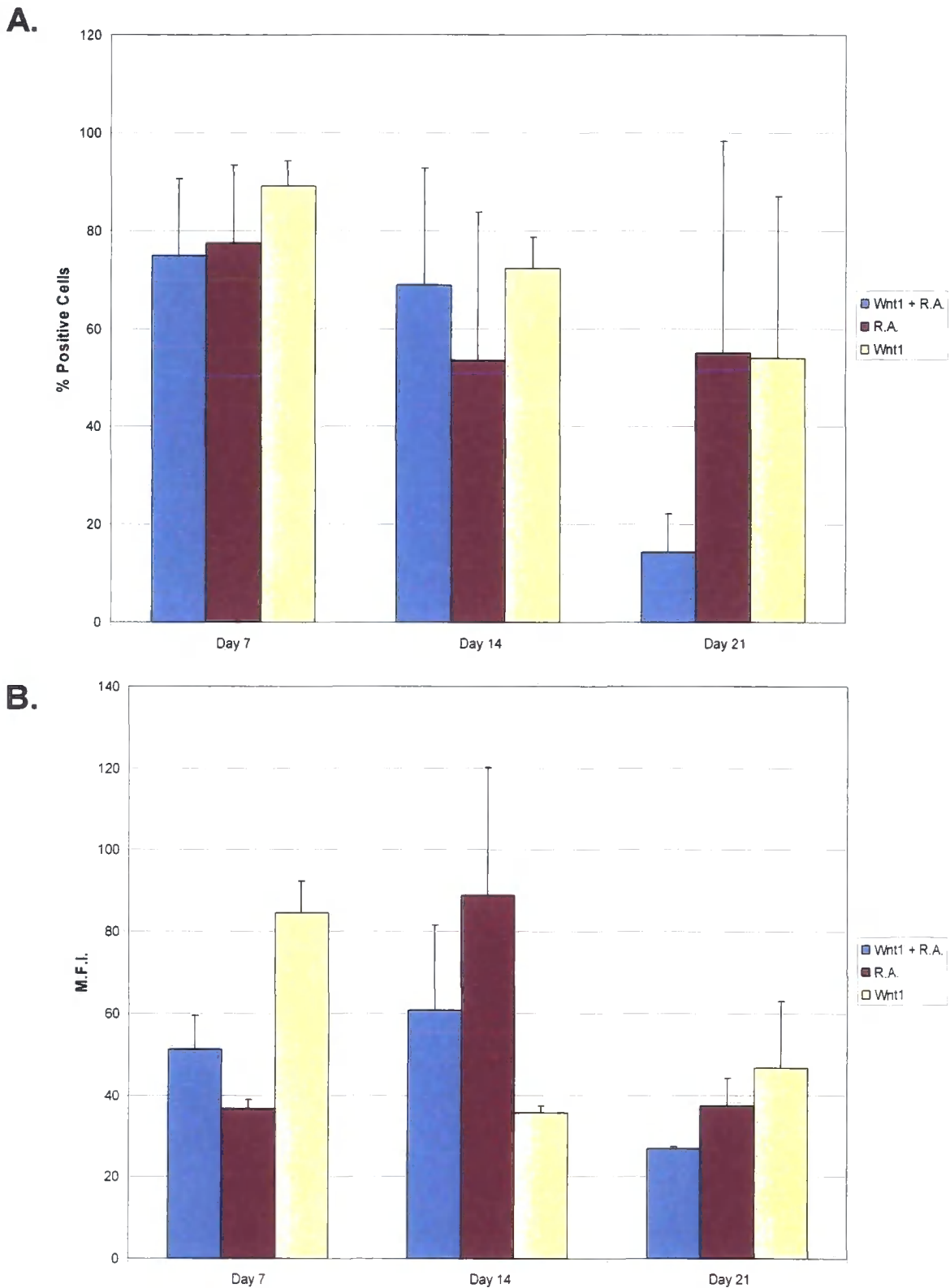


Figure 4.9A: The number of Nurr1 antigen positive cells in Tera2.cl.SP12 cultures treated with 10 μ M retinoic acid only, 2ng/ml Wnt1 only, or a combination of both reagents after 7, 14 and 21 days. Error bars represent the S.D. of the mean. **B:** The intensity of Nurr1 expression in the same culture conditions outlined above. Error bars represent the standard error of the mean. In all experiments n=3. The results from this analysis as discussed in the text are essentially meaningless due to the possibility of non specific binding of the antibody and technical problems with its use.

These results offered little prospect of providing a route forward, so the focus of study was switched to other variables that may influence the acquisition of a dopaminergic cell fate, starting with Otx2. Wnt1 can maintain expression of this molecule in other systems (Prakash et al., 2006) and as such it was hypothesised that it may rescue the rapid drop in expression of Otx2 shown following retinoic acid treatment of Tera2.cl.SP12 embryonal carcinoma stem cells in Chapter 3. Figure 4.10 displays a similar trend to that recorded previously whereby Otx2 mRNA expression drops rapidly upon retinoic acid induction in the first five days of culture. However at one and three days in the presence of Wnt1 and retinoic acid there is a small rescue effect that appears to be due to Wnt1. By the fifth day though expression is almost zero in both treatment regimes. The one way anova comparing the two 1 day test conditions to each other as well as the embryonal carcinoma cell starting material, demonstrates that there is a statistically significant drop in Otx2 mRNA expression in the embryonal carcinoma cells after 1 day of treatment with retinoic acid alone and with retinoic acid in combination with Wnt1. However there is no statistically significant difference between the retinoic acid only and retinoic acid plus Wnt1 condition at 1 day. Therefore it appears that although the trend at 1 and 3 days indicates Wnt1 may be able to cause a rescue of Otx2 expression, this effect is small in magnitude, is not statistically significant (at 1 day) and given the almost total absence of expression at 5 days in either test sample set, short lived. That is not to say that this effect although small and insignificant may not be able to cause a real difference. A minor change as is observed may be sufficient to instigate more considerable developments downstream of it. As a result the mRNA expression patterns of Lmx1a, Msx1, Ngn2 and Nkx 6-1 were assessed using real time PCR based on the scheme in Figure 4.2 showing the possible pathway that Otx2 may influence.

First of all Lmx1a appears to be up regulated in its expression by increasing the duration of retinoic acid exposure up to 3 weeks (Figure 4.11). Of perhaps greater interest is the difference in expression between the retinoic acid only and retinoic acid plus Wnt1 samples at 7 days. Although the one way anova output indicates there is no statistically significant difference between these two conditions or between them and the embryonal carcinoma cell starting material, the trend of Wnt1 augmenting expression of Lmx1a in the presence of retinoic acid is comparable to that seen for Frizzled 8 in Figure 4.6 and that seen for beta-catenin (Figure 4.7) as well. The lack

of a statistically significant induction at 7 days is consistent with the findings of analyses carried out in collaboration with Dr. V. Lakics mentioned in Chapter 3, whereby expression of dopaminergic markers is generally low in this early time period. This lower level of expression may contribute to the lack of a statistically significant difference as the error is often greater when making small measurements, thus making it harder to observe an effect. The trend though of Wnt1 in combination with retinoic acid having a favourable effect at 7 days is reinforced. Figure 4.12 indicates that for Msx1 there is little difference between the retinoic acid only and retinoic acid plus Wnt1 conditions. Although as for Nurr1 the retinoic acid plus Wnt1 samples show a slightly higher level of expression at 7 days than their retinoic acid only counterparts. The same almost identical trend is seen for Ngn2 expression in Figure 4.13 and similarly for Nkx 6-1 in Figure 4.14. For all three of these markers the Wnt1 only condition shows a highly variable expression supporting the notion that it is not suitable for use as no control over the process of differentiation is possible.

These findings therefore suggest that the small amount of Otx2 rescue by Wnt1 is not having a larger effect on the initial downstream targets its expression may influence. However at 7 days Wnt1 in conjunction with retinoic acid may cause small increases in expression of these markers associated with a dopaminergic fate, or in the case of Nkx 6-1, a ventral midbrain phenotype. Also retinoic acid alone over the time course investigated tends to up regulate expression of all these markers with increasing duration of exposure to it. This is coherent with the observations in Chapter 3 for the other markers of a dopaminergic phenotype studied there. Given the trend seen at 7 days further analysis was carried out to assess the combined effects of Wnt1 and retinoic acid on some of these as well as additional factors in the Tera2.cl.SP12 system.

Figure 4.10: The effects of Wnt1 and R.A. on Otx2 mRNA expression

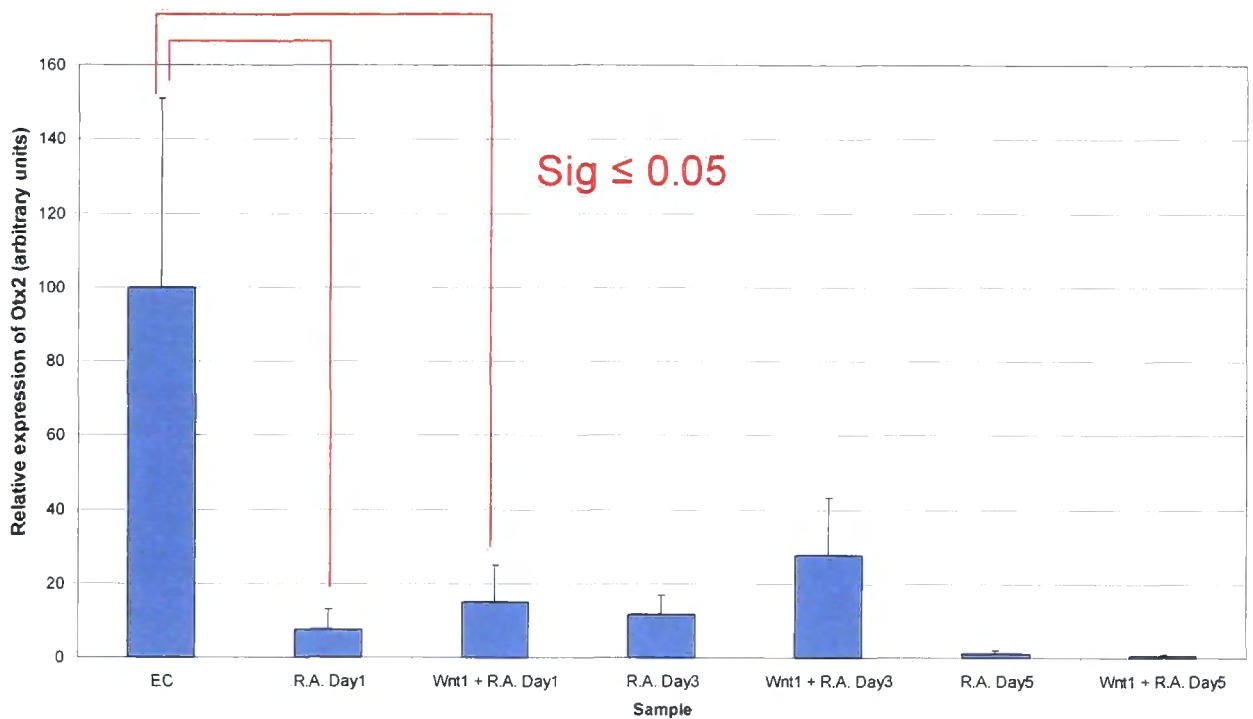


Figure 4.10: The relative expression of Otx2 mRNA in Tera2.cl.SP12 cells and their derivatives treated with 10 μ M retinoic acid only, or 10 μ M retinoic acid plus 2ng/ml Wnt1 for 1, 3 and 5 days. The one way anova output indicates that although there is no statistically significant difference between the two test conditions at 7 days, both of these samples do differ to a statistically significant level from the embryonal carcinoma stem cell starting material. In all experiments n=3 and error bars represent the standard deviation of the mean.

Figure 4.11: The effects of Wnt1 and R.A. on Lmx1a mRNA expression

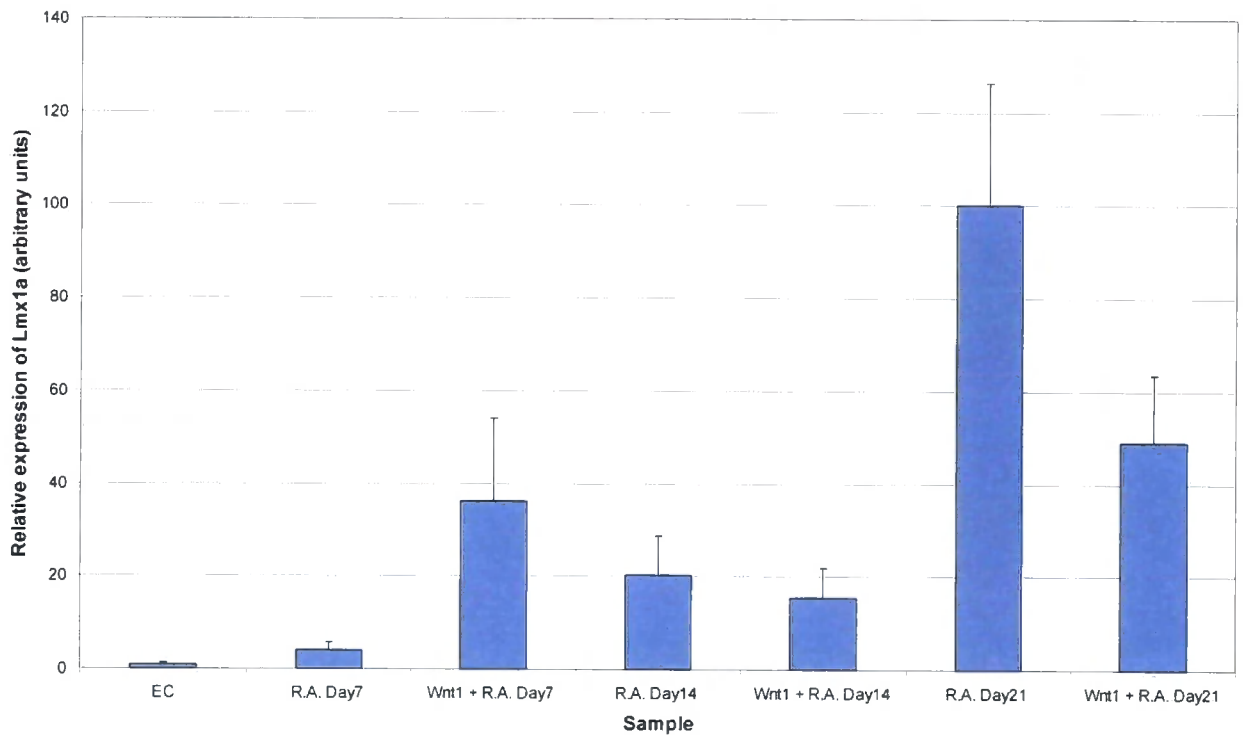


Figure 4.11: The relative expression of Lmx1a mRNA in Tera2.cl.SP12 cells and their derivatives treated with 10 μ M retinoic acid only, or 10 μ M retinoic acid plus 2ng/ml Wnt1 for 7, 14 and 21 days. The one way anova output indicates there is no statistically significant difference between the two test conditions at 7 days and that these samples do not differ to a statistically significant level from the embryonal carcinoma stem cell starting material. In all experiments n=3 and error bars represent the standard deviation of the mean.

Figure 4.12: The effects of Wnt1 and R.A. on Msx1 mRNA expression

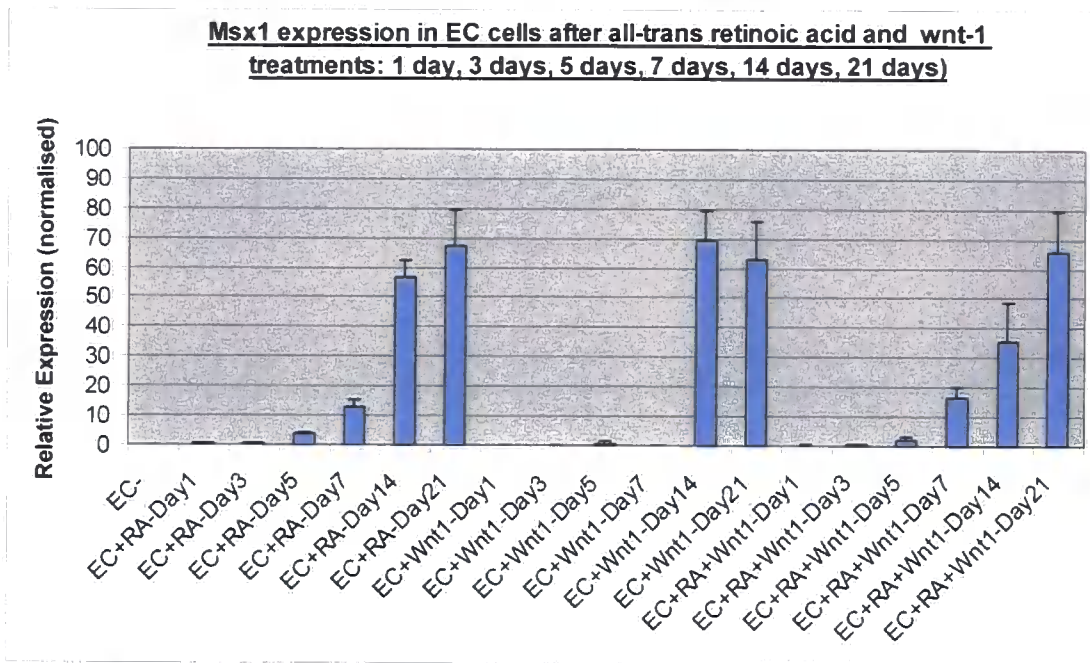


Figure 4.12: The relative expression of Msx1 mRNA in Tera2.cl.SP12 cells and their derivatives treated with 10 μ M retinoic acid only, 2ng/ml Wnt1 only, or 10 μ M retinoic acid plus 2ng/ml Wnt1 for 1, 3, 5, 7, 14 and 21 days. In all experiments n=3, error bars represent the S.D. of the mean.

Figure 4.13: The effects of Wnt1 and R.A. on Ngn2 mRNA expression

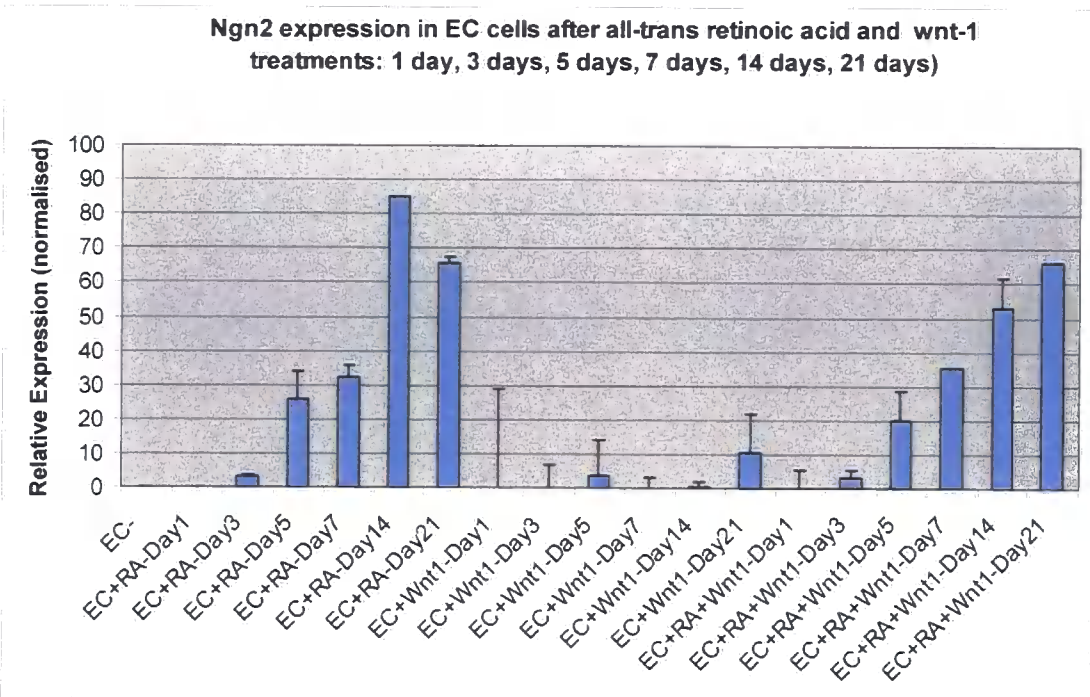


Figure 4.13: The relative expression of Ngn2 mRNA in Tera2.cl.SP12 cells and their derivatives treated with 10 μ M retinoic acid only, 2ng/ml Wnt1 only, or 10 μ M retinoic acid plus 2ng/ml Wnt1 for 1, 3, 5, 7, 14 and 21 days. In all experiments n=3, error bars represent the S.D. of the mean.

Figure 4.14: The effects of Wnt1 and R.A. on Nkx 6-1 mRNA expression

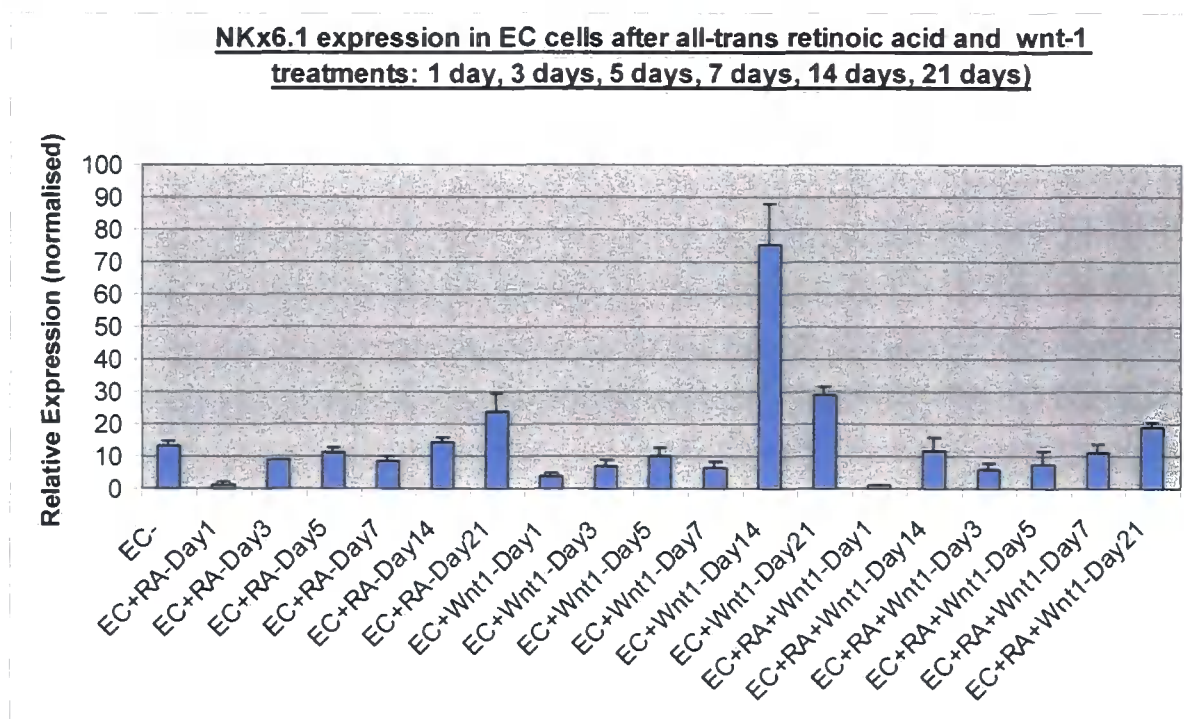


Figure 4.14: The relative expression of Nkx 6-1 mRNA in Tera2.cl.SP12 cells and their derivatives treated with 10 μ M retinoic acid only, 2ng/ml Wnt1 only, or 10 μ M retinoic acid plus 2ng/ml Wnt1 for 1, 3, 5, 7, 14 and 21 days. In all experiments n=3, error bars represent the S.D. of the mean.

Before any markers associated specifically with a dopaminergic phenotype were investigated, the expression of beta III tubulin, a classical indicator of the presence of neurons, was assayed (Figure 4.15). In the presence of both retinoic acid and Wnt1 at 7 days there was almost double the level of mRNA present in comparison to the retinoic acid only condition. This difference was not statistically significant and neither test condition at this time showed any statistically relevant difference in relation to the embryonal carcinoma cell starting material. The trend though was as observed previously, supporting the notion that Wnt1 may exert an effect early in the process of differentiation. Interestingly the expression seen in the embryonal carcinoma cells of this marker is quite high and variable, perhaps indicating that Tera2.cl.SP12 cells are primed for neural differentiation, or actually slightly more differentiated along a neural pathway than might be expected for a cell line that acts as a model for embryonic stem cells. As seen in Chapter 3, in the flow cytometry data for Beta III tubulin, there is a lull in expression at 2 weeks in the retinoic acid only

samples before a subsequent rise as time progresses. This suggests that at both the mRNA and protein level there is a non linear regulation of neuronal differentiation in this cell system, under the conditions tested. This may be due to the involvement of different signalling pathways at different times during the process of differentiation. Also it may be that retinoic acid provides the initial signal but that this causes the embryonal carcinoma cells to produce factors that subsequently continue to drive the cultures towards the acquisition of neural phenotypes.

Beyond 7 days it is also noticeable that Wnt1 appears to exert no effect on the mRNA expression levels of beta III tubulin. This is indicative that in the Tera2.cl.SP12 system Wnt1 has an early effect on differentiation but up to 3 weeks of culture may not cause any subsequent effects. It may be however that beyond 3 weeks it can have an impact; this is a potential area for further work.

When a panel of dopaminergic markers had their expression patterns assessed, a very strong conserved trend was found. First of all two components of the biosynthetic machinery involved in the production of dopamine were discovered to display almost identical tendencies in the conditions tested. Figure 4.16 demonstrates that for tyrosine hydroxylase in the presence of retinoic acid alone, expression starts low and rises over time as observed in Chapter 3. However when Wnt1 is included with retinoic acid, after 7 days there is approximately double the mRNA expression of this marker. Beyond this time point though, in the combined Wnt1 plus retinoic acid condition up to 3 weeks there is a plateau in the levels of mRNA for tyrosine hydroxylase. A close to identical trend is seen for dopa decarboxylase in Figure 4.17 which is unsurprising given it is involved in the same pathway of dopamine biosynthesis; indeed it acts on the product of the reaction catalysed by tyrosine hydroxylase. In both instances there is no statistically significant difference between the two test conditions at 7 days and neither of them differs significantly from the starting material. However the trend is consistent with other data presented previously and reinforces the notion that Wnt1 may play an early role (at around 7 days) in the differentiation process of Tera2.cl.SP12 cells. The plateau effect seen for these two markers helps support this.

Figure 4.15: The effects of Wnt1 and R.A. on Beta III Tubulin mRNA expression

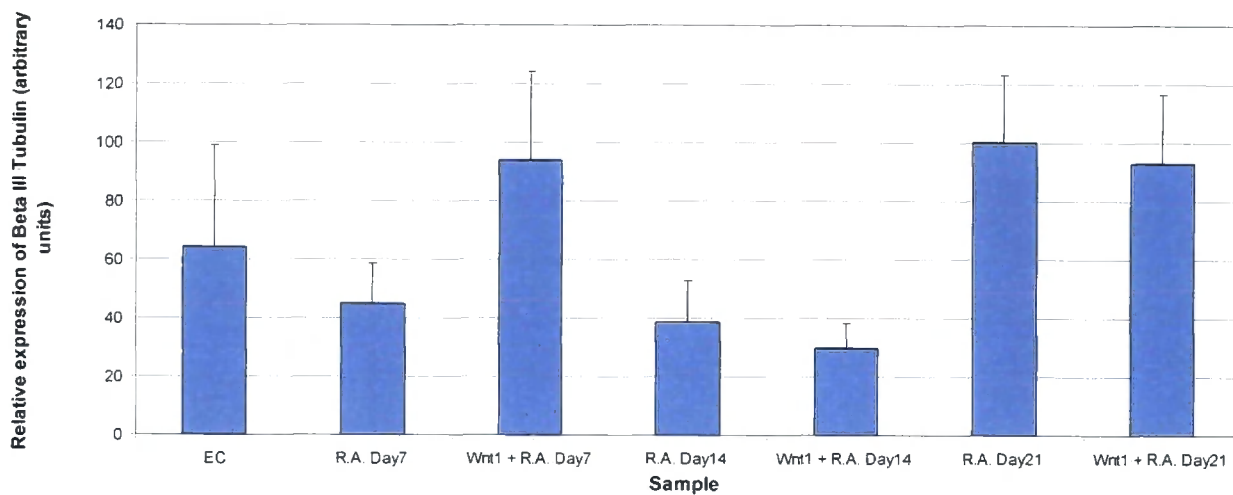


Figure 4.15: The relative expression of beta III tubulin mRNA in Tera2.cl.SP12 cells and their derivatives treated with 10 μ M retinoic acid only, or 10 μ M retinoic acid plus 2ng/ml Wnt1 for 7, 14 and 21 days. The one way anova output indicates there is no statistically significant difference between the two test conditions at 7 days and that these samples do not differ to a statistically significant level from the embryonal carcinoma stem cell starting material. In all experiments n=3 and error bars represent the standard deviation of the mean.

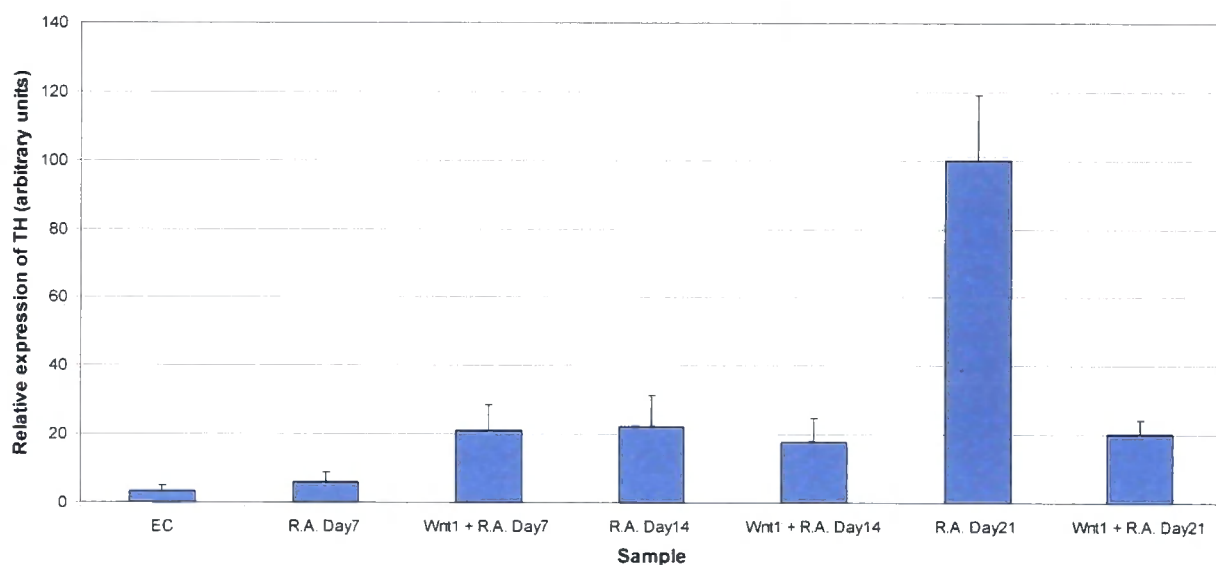
Figure 4.16: The effects of Wnt1 and R.A. on TH mRNA expression

Figure 4.16: The relative expression of tyrosine hydroxylase mRNA in Tera2.cl.SP12 cells and their derivatives treated with 10 μ M retinoic acid only, or 10 μ M retinoic acid plus 2ng/ml Wnt1 for 7, 14 and 21 days. The one way anova output indicates there is no statistically significant difference between the two test conditions at 7 days and that these samples do not differ to a statistically significant level from the embryonal carcinoma stem cell starting material. In all experiments n=3 and error bars represent the standard deviation of the mean.

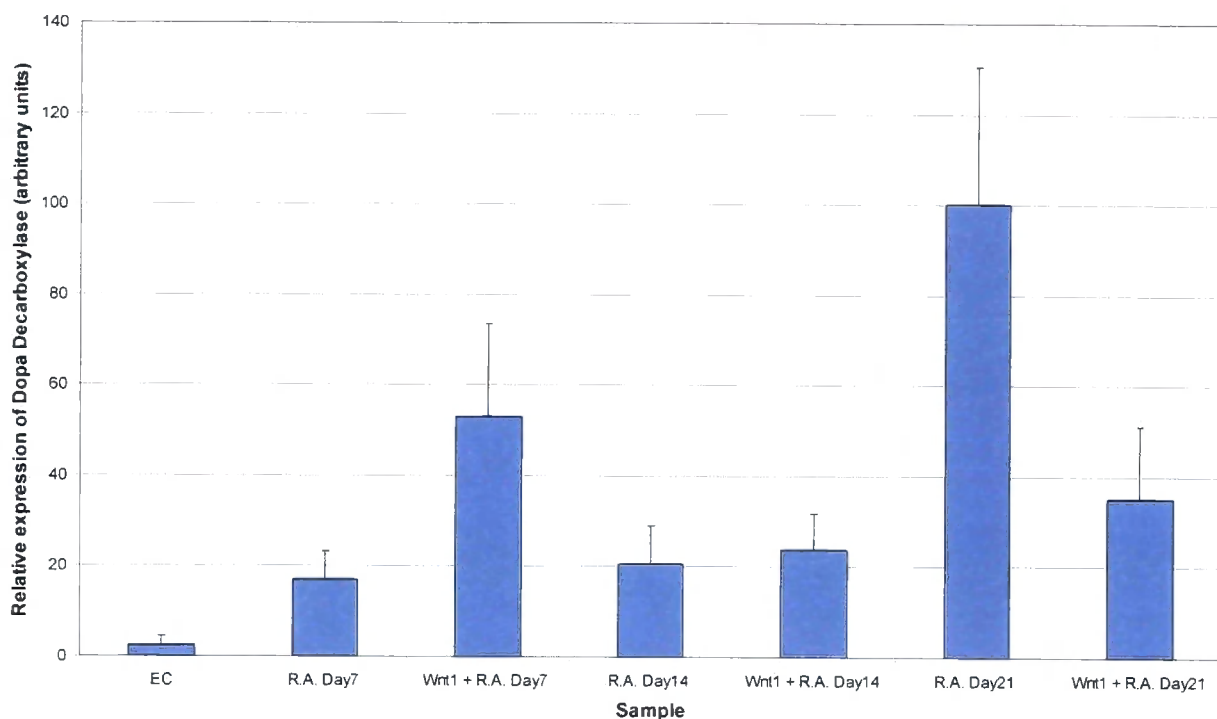
Figure 4.17: The effects of Wnt1 and R.A. on AADC mRNA expression

Figure 4.17: The relative expression of dopa decarboxylase mRNA in Tera2.ci.SP12 cells and their derivatives treated with 10 μ M retinoic acid only, or 10 μ M retinoic acid plus 2ng/ml Wnt1 for 7, 14 and 21 days. The one way anova output indicates there is no statistically significant difference between the two test conditions at 7 days and that these samples do not differ to a statistically significant level from the embryonal carcinoma stem cell starting material. In all experiments n=3 and error bars represent the standard deviation of the mean.

Three other indicators of a dopaminergic phenotype that are involved in a more physical aspect of the dopaminergic system in that they help to detect and respond to dopamine in the case of dopamine receptors 1 and 2, and transport it in the case of the dopamine transporter, also show a comparable expression profile to that seen for tyrosine hydroxylase and dopa decarboxylase. First of all, as can be seen in Figure 4.18, dopamine transporter mRNA is up regulated over time following retinoic acid treatment of Tera2.cl.SP12 cultures. When Wnt1 is added in combination with retinoic acid there is a higher expression at 7 days but then a plateau at subsequent time points up to 3 weeks. Figure 4.19 depicts a similar trend for dopamine receptor 1, although the expression of this marker appeared weaker, as shown by higher cycle threshold values when carrying out the real time PCR. A higher value indicates it has taken more cycles for detection to occur and therefore results tend to be more erroneous, in this case though the trends still hold true to those for other markers. Finally in Figure 4.20 dopamine receptor 2 displays an analogous profile. Although here expression in the embryonal carcinoma cells is highly variable and on average slightly greater than in the 7 day samples treated with only retinoic acid. The trend though between the 7 day retinoic acid only and retinoic acid plus Wnt1 conditions is congruous with that for the other markers tested. Once again for all three of these phenotypic indicators there is no statistically significant difference between the two 7 day conditions and likewise no relevant statistical distinction between either of these two test samples and the embryonal carcinoma cell starting material. However given the trend at 7 days and plateau effect at subsequent time points are repeatedly able to be observed, there is a strong case to suggest that Wnt1 in the presence of retinoic acid exerts an effect on the early stages of the dopaminergic differentiation of Tera2.cl.SP12 embryonal carcinoma cell cultures.

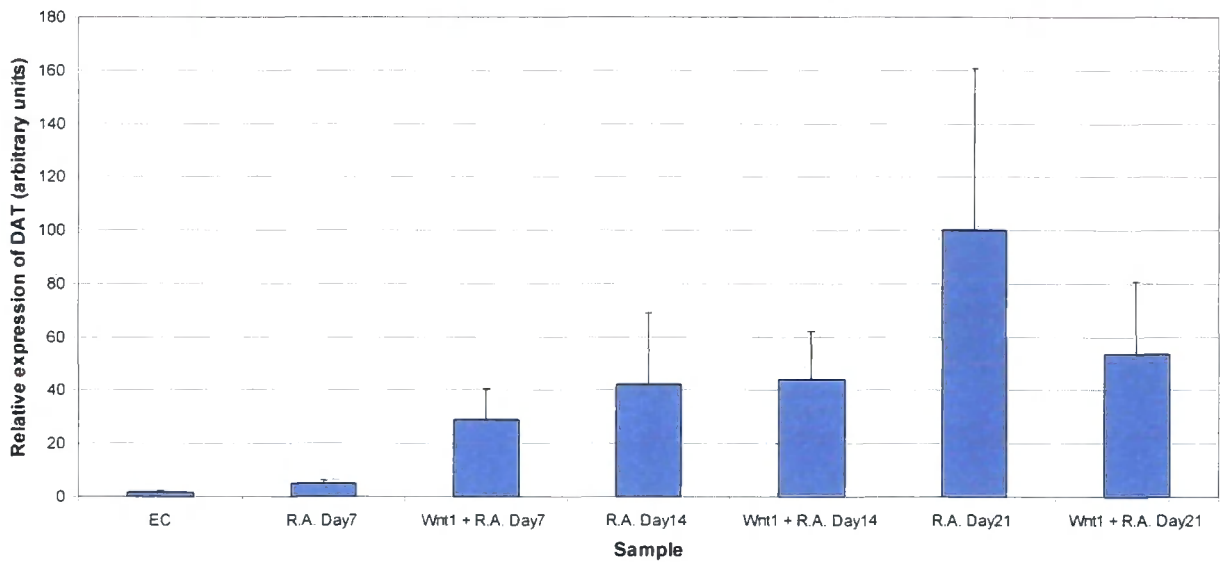
Figure 4.18: The effects of Wnt1 and R.A. on DAT mRNA expression

Figure 4.18: The relative expression of dopamine transporter mRNA in Tera2.cl.SP12 cells and their derivatives treated with 10 μ M retinoic acid only, or 10 μ M retinoic acid plus 2ng/ml Wnt1 for 7, 14 and 21 days. The one way anova output indicates there is no statistically significant difference between the two test conditions at 7 days and that these samples do not differ to a statistically significant level from the embryonal carcinoma stem cell starting material. In all experiments n=3 and error bars represent the standard deviation of the mean.

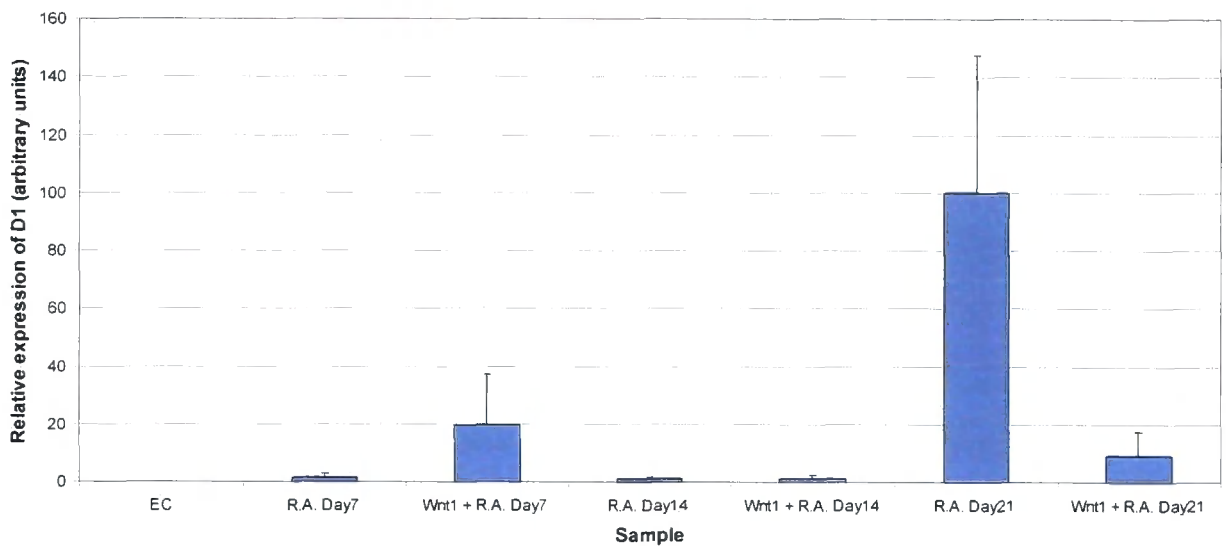
Figure 4.19: The effects of Wnt1 and R.A. on D1 mRNA expression

Figure 4.19: The relative expression of dopamine receptor 1 mRNA in Tera2.cl.SP12 cells and their derivatives treated with 10 μ M retinoic acid only, or 10 μ M retinoic acid plus 2ng/ml Wnt1 for 7, 14 and 21 days. The one way anova output indicates there is no statistically significant difference between the two test conditions at 7 days and that these samples do not differ to a statistically significant level from the embryonal carcinoma stem cell starting material. In all experiments n=3 and error bars represent the standard deviation of the mean.

Figure 4.20: The effects of Wnt1 and R.A. on D2 mRNA expression

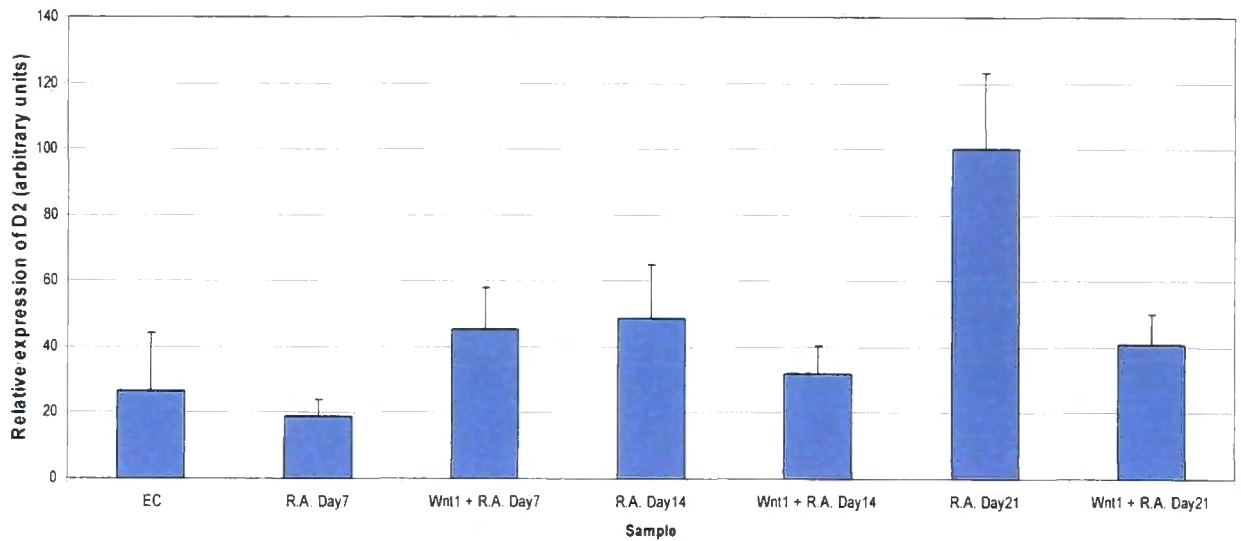
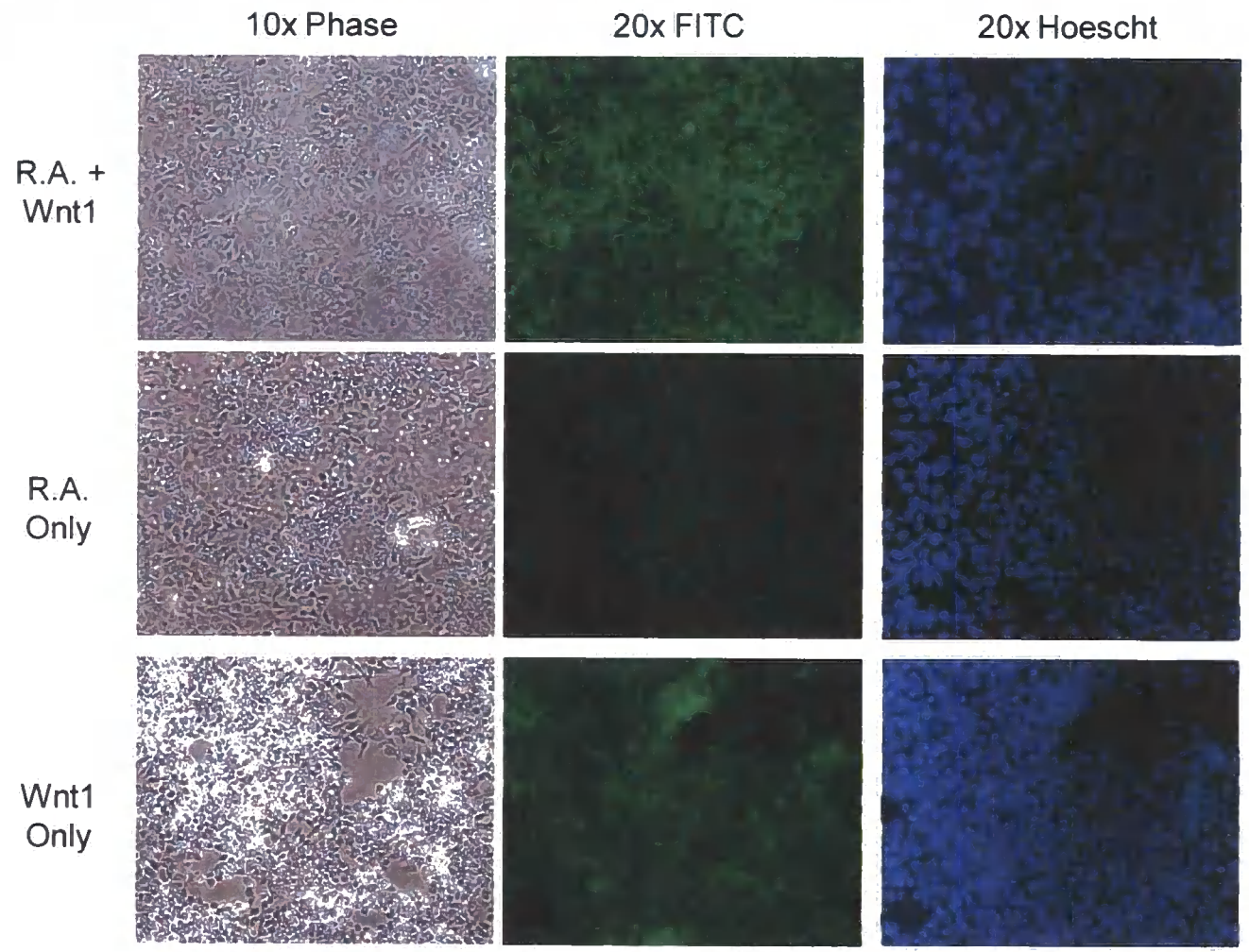


Figure 4.20: The relative expression of dopamine receptor 2 mRNA in Tera2.cl.SP12 cells and their derivatives treated with 10 μ M retinoic acid only, or 10 μ M retinoic acid plus 2ng/ml Wnt1 for 7, 14 and 21 days. The one way anova output indicates there is no statistically significant difference between the two test conditions at 7 days and that these samples do not differ to a statistically significant level from the embryonal carcinoma stem cell starting material. In all experiments n=3 and error bars represent the standard deviation of the mean.

Given that Wnt1 in combination with retinoic acid is seemingly able to influence the expression of the mRNA for neuronal and dopaminergic markers during the process of differentiation of Tera2.cl.SP12 cells under the conditions tested. The next logical step appeared to be to investigate if there were any detectable changes at the morphological and protein level. Immunocytochemistry was employed to assess expression of the neuronal marker Beta III tubulin at 7 days, as from experience staining for this cytoskeletal protein was more readily observable than for cytosolic dopaminergic marker proteins such as tyrosine hydroxylase. Figure 4.21 shows that in the Wnt1 only condition, cultures are overgrown, messy and display no true staining, just patchy blotches where cells are over confluent and physical entrapment of the antibodies used probably occurs, hence the fuzzy detection that is seen. The retinoic acid only condition displays only very faint staining. However the combined Wnt1 plus retinoic acid condition demonstrates an enhanced level of detection relative to the retinoic acid only samples. At this stage though there are few neuritic processes, therefore staining was carried out after 2 weeks. Figure 4.22 shows that at this stage there is some process formation and some positive staining in the retinoic acid only and retinoic acid plus Wnt1 samples. Interestingly as for the mRNA data there appears to be little detectable difference in Beta III tubulin expression at this time. This could be due to the seemingly general lull in expression of this neuronal marker at this particular time point. However when staining is carried out after 3 weeks, there is again an observable difference. Figures 4.23, 4.24 and 4.25 display nestin, Beta III tubulin and tyrosine hydroxylase staining at this stage of differentiation in the Wnt1 only, retinoic acid only and retinoic acid plus Wnt1 conditions. The Wnt1 only condition (Figure 4.23) shows no true staining for Beta III tubulin or tyrosine hydroxylase, there is some nestin staining as might be predicted when using embryonal carcinoma cells. The phase image and Hoescht control panels all indicate that the cultures are very confluent to overgrown. This in essence confirms earlier findings that the Wnt1 only condition is not viable for use. Figure 4.24 shows that when using only retinoic acid after 3 weeks, there is still expression of nestin as expected, tyrosine hydroxylase is detectable but being a cytosolic protein its presence is more difficult to define. There is though clear Beta III tubulin expression with neuronal like cell bodies and neuritic processes being clearly observable. Figure 4.25 however shows there are clearly more Beta III tubulin positive cells present in the combined retinoic acid plus Wnt1 treated cultures at 3

weeks. There are neuronal cell body type structures with clear processes emanating from them. Tyrosine hydroxylase expression is faint but detectable once again and it is hard to tell if there is any difference between conditions for this dopaminergic marker. The non specific binding of the antibody observed in Chapter 3 also makes the tyrosine hydroxylase data questionable as it might be that as in the highly confluent/overgrown Wnt1 only condition there is a degree of entrapment of the antibody or non specific binding of it. Hence the tyrosine hydroxylase staining as it is not clear and not associated with a morphology one might expect of a potential neuron in culture, is viewed as being negative or at least too inconclusive on which to base any conclusions. Nestin is still expressed indicating that there are still some relatively immature cells in these cultures. The presence of nestin may indicate Wnt1 is having less of an effect on the maturation of Tera2.cl.SP12 cells.

Figure 4.21: Beta III tubulin staining of Tera2.cl.SP12 cultures treated with R.A., Wnt1 or a combination of both reagents for 7 days



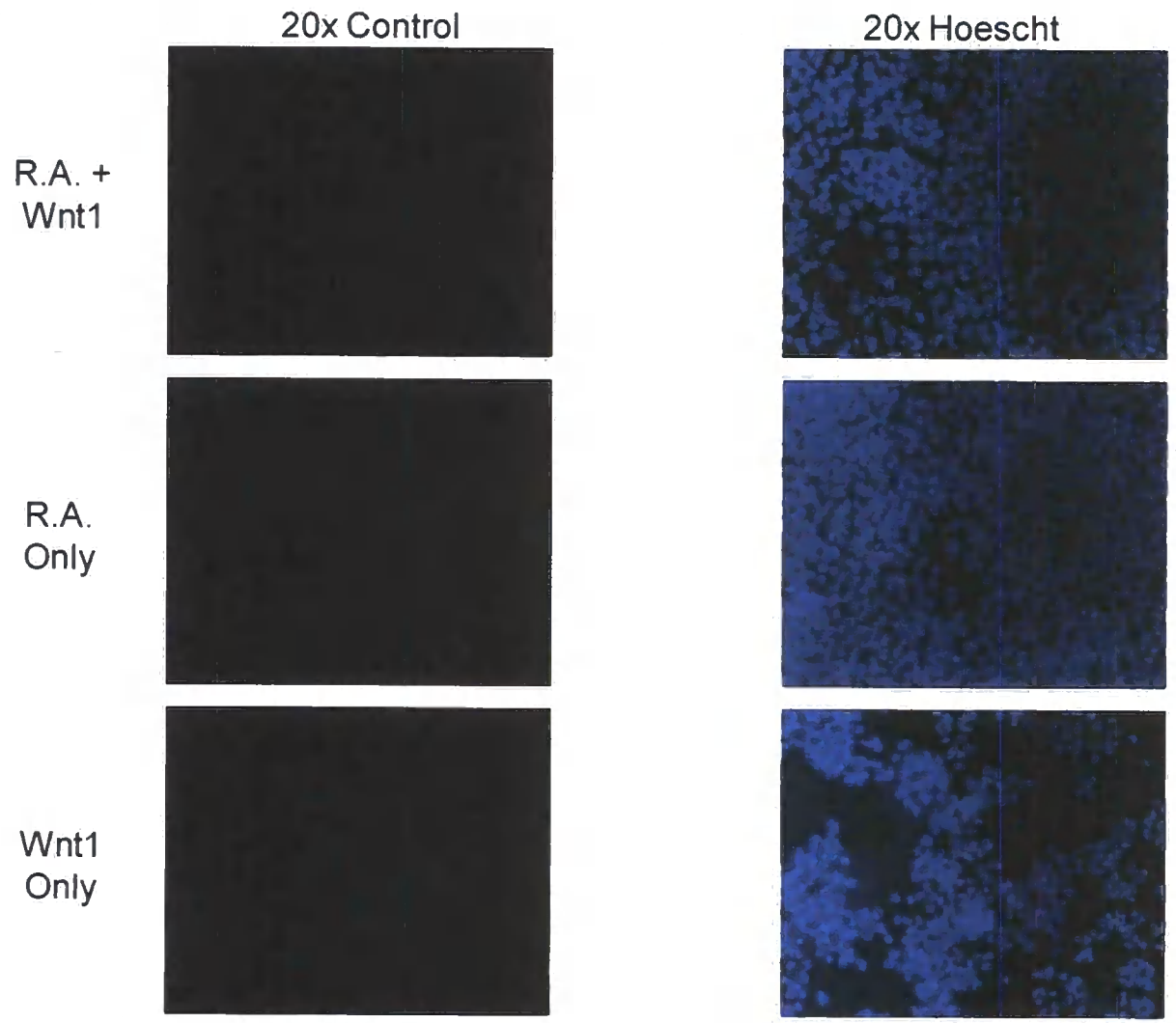
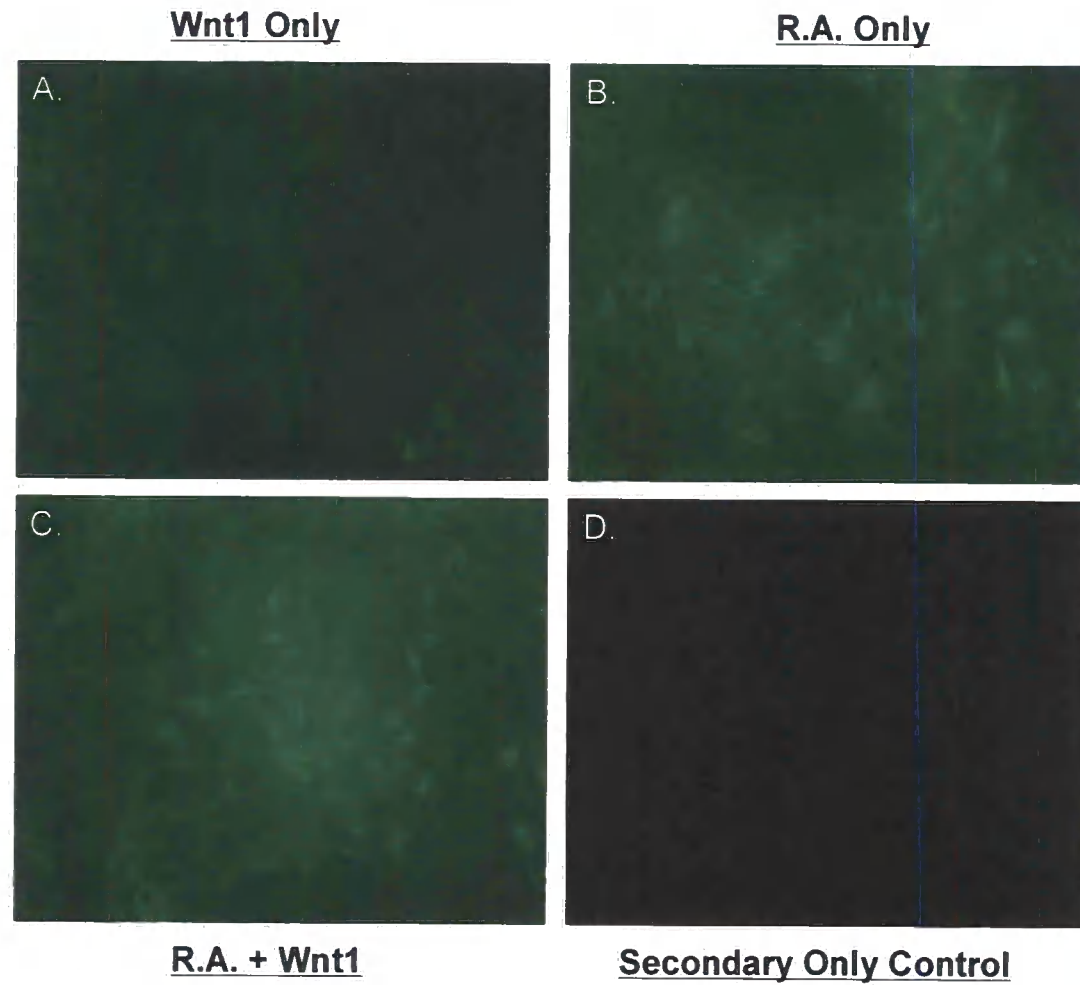


Figure 4.22: Beta III tubulin staining of Tera2.cl.SP12 cultures treated with R.A., Wnt1 or a combination of both reagents for 14 days



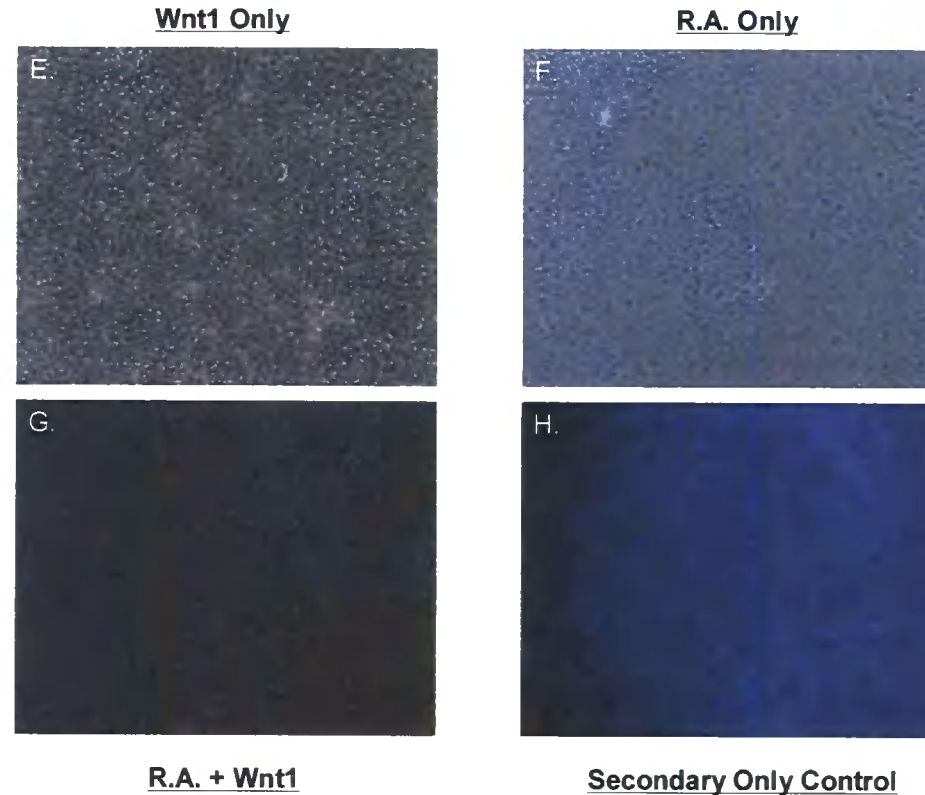


Figure 4.22: A, B, and C show Beta III tubulin staining using a FITC labelled secondary antibody in the three different treatment conditions described individually above or below each image. E, F, and G display phase images of the cultures. D shows a FITC labelled secondary antibody control for a R.A. only treated culture. H represents Hoescht staining of the corresponding R.A. treated culture to confirm the presence of cells by making their nuclei detectable. Wnt1 only and R.A. + Wnt1 cultures were also negative in their secondary only control samples and did have cells present confirmed by Hoescht staining for cell nuclei. These images were not included. In all experiments $n=3$. Scale bars represent $100\mu\text{m}$ in all cases. As in Figure 4.21 not all cells are antigen positive, these images are included solely to bridge the time lag between one and three weeks. They also serve to show that in the retinoic acid only and retinoic acid plus Wnt1 conditions that there are signs of morphological development of the cultures with Beta III tubulin positive cells being detected that appear to be extending possible neuritic type processes. As mentioned previously the Wnt1 only condition is sub optimal and any faint detection is most probably due to non specific binding or physical entrapment of the antibody used.

Figure 4.23: Immunostaining of Tera2.cl.SP12 cultures treated with Wnt1 for 21 days

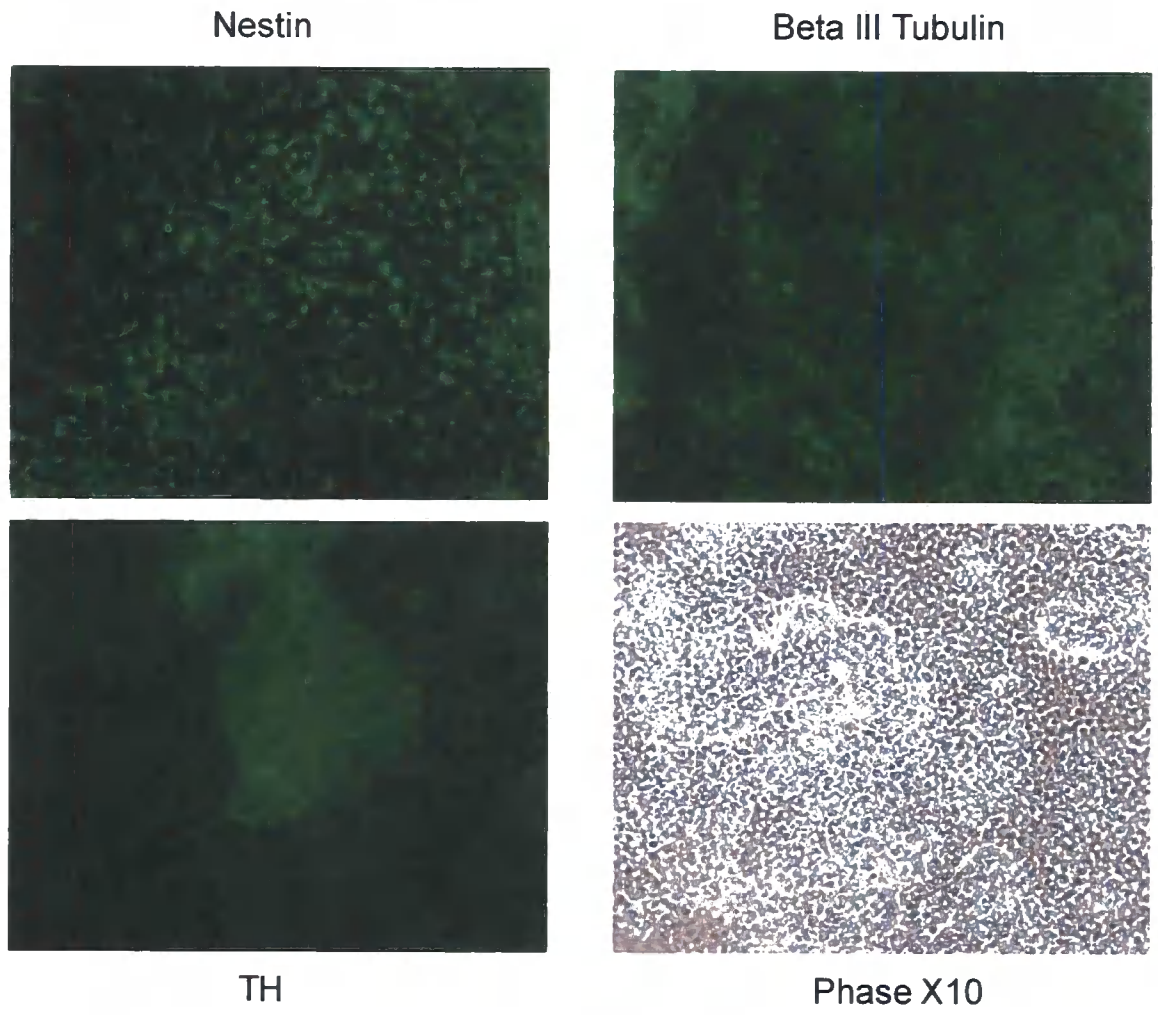
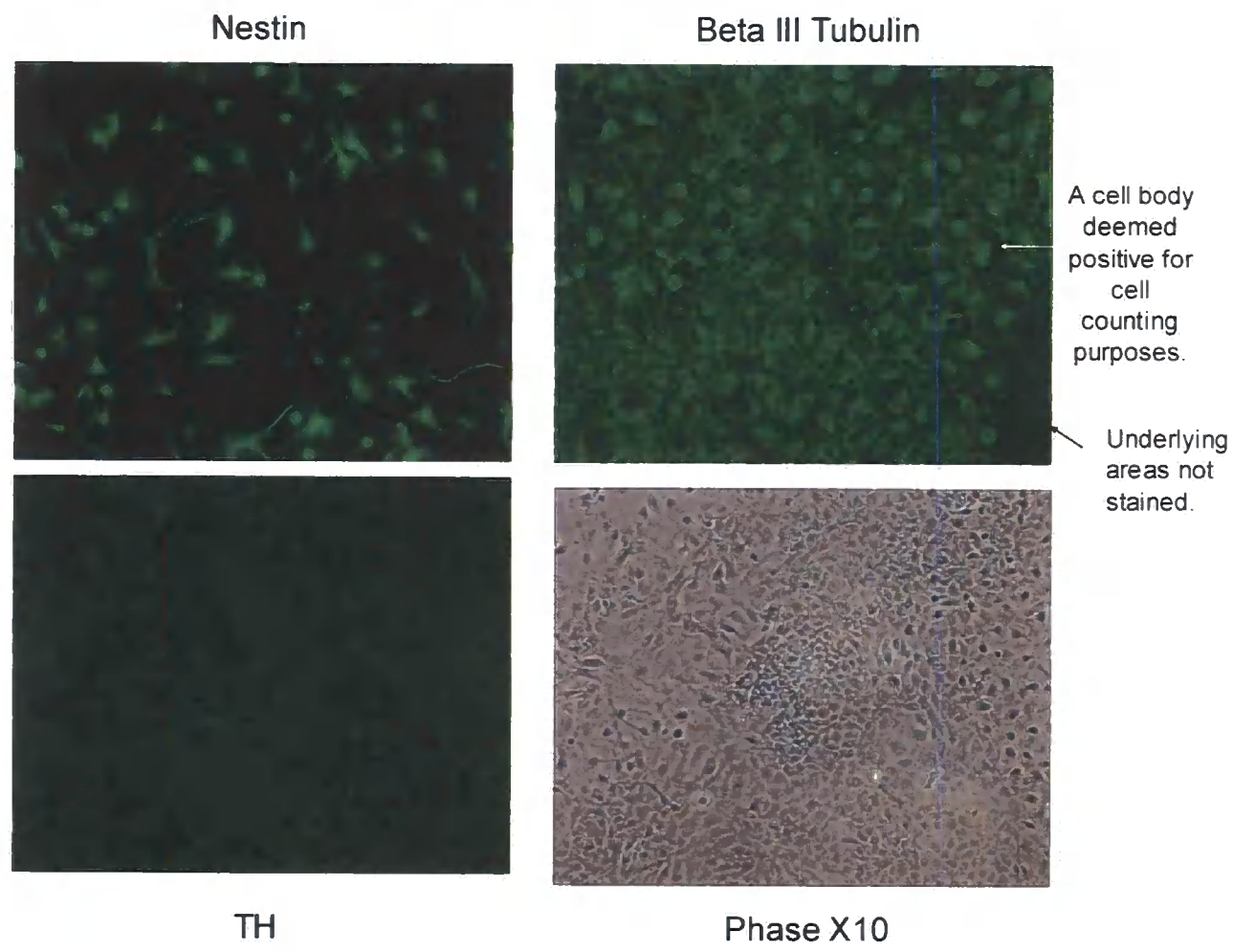




Figure 4.23: Nestin, Beta III tubulin and tyrosine hydroxylase immunostaining using a FITC labelled secondary antibody, of Tera2.cl.SP12 cultures treated with Wnt1 for 3 weeks. The 10x phase image shows how the cultures are very confluent to overgrown. The mouse secondary FITC labelled antibody only control is applicable for the nestin and tyrosine hydroxylase primary antibodies, the rabbit equivalent is for the Beta III tubulin antibody. The corresponding Hoescht control images confirm the presence of cells by staining their nuclei. Scale bars represent 100 μ m in all cases. In all experiments n=3. The Wnt1 only condition shows clear nestin staining suggesting the cultures are relatively immature which would be expected given the overgrown nature of them. This is reflected in the cell count data that follow by a high number of total cells relative to the two conditions that involve the use of retinoic acid. The TH and Beta III tubulin staining is not true staining and as discussed elsewhere for this sub optimal condition, cannot be viewed as positive. It is more likely a product of physical entrapment of the antibody by the overgrown cultures or a result of non specific binding of the antibodies used.

Figure 4.24: Immunostaining of Tera2.cl.SP12 cultures treated with retinoic acid for 21 days



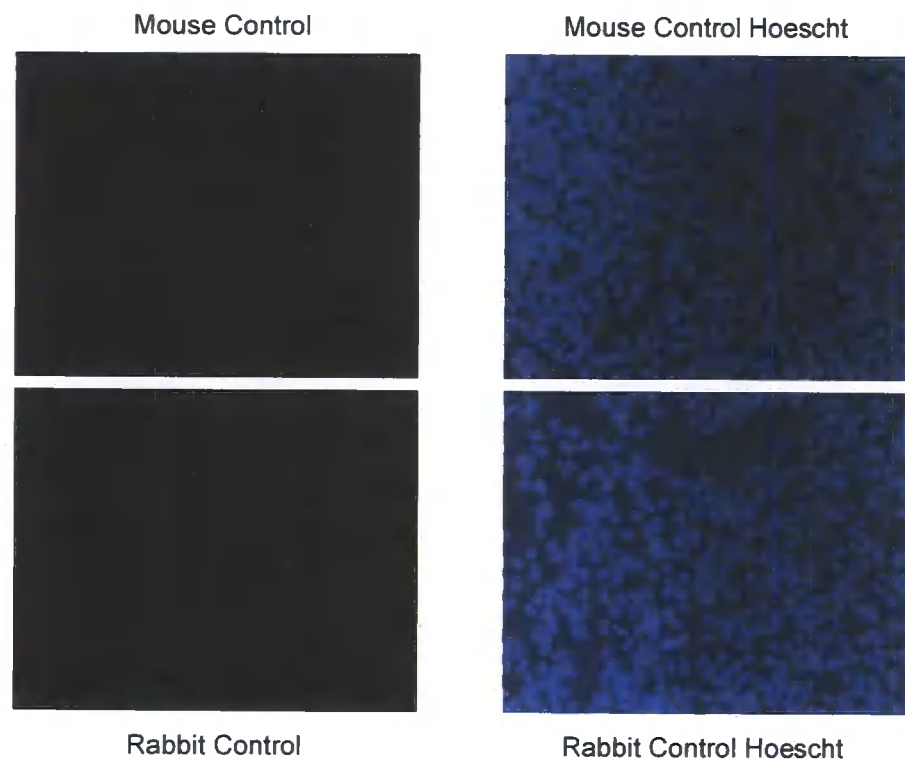
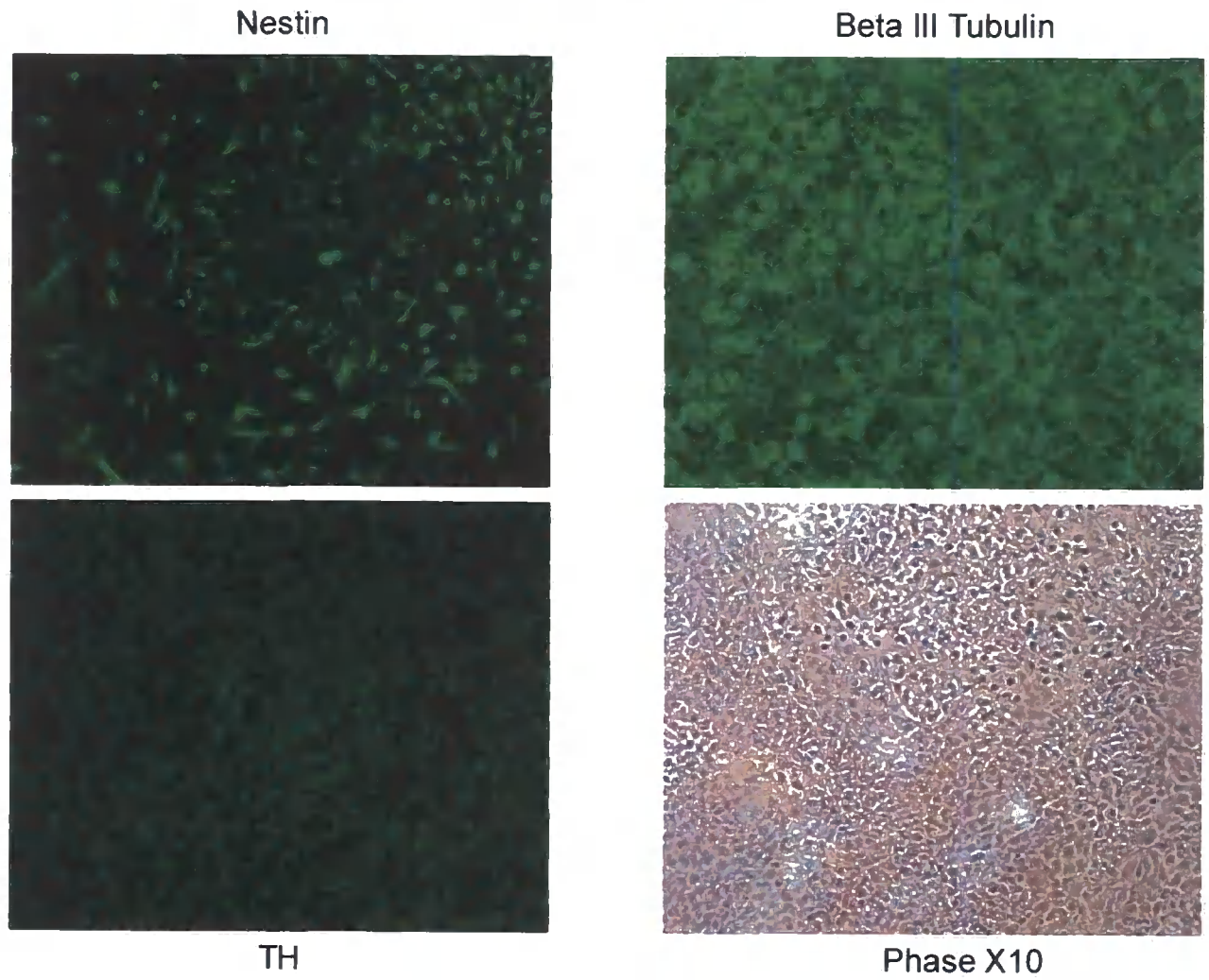


Figure 4.24: Nestin, Beta III tubulin and tyrosine hydroxylase immunostaining using a FITC labelled secondary antibody, of Tera2.cl.SP12 cultures treated with retinoic acid for 3 weeks. The 10x phase image shows that the cultures display a mix of cell morphologies. The mouse secondary FITC labelled antibody only control is applicable for the nestin and tyrosine hydroxylase primary antibodies, the rabbit equivalent is for the Beta III tubulin antibody. The corresponding Hoescht control images confirm the presence of cells by staining their nuclei. Scale bars represent 100 μ m in all cases. In all experiments n=3. Appendix D supports this data and that in Figure 4.25, it shows merged images indicating the Beta III tubulin positive cells against a background of the total number of cells detected by Hoescht staining of cell nuclei. These images were used to produce the total cell number and Beta III tubulin positive cell counts in the data that follow (Figures 4.28 and 4.29). Although there are a number of Beta III tubulin positive cells in the image in this figure, clearly from Appendix D and the cell count data it is apparent not all cells are positive for the antigen of interest. Nestin expression was detectable but this staining was not carried out for quantifiable purposes and is merely an indicator that the cultures still possess some relatively immature cells after this period of differentiation. In further work sorting out of such cells may be desirable. The TH staining is diffuse relatively hard to detect and not associated with cells of any kind of morphology that might be expected of a potential neuritic cell in culture. Given the possible non specific binding of this antibody observed in Chapter 3 this TH staining is highly inconclusive.

Figure 4.25: Immunostaining of Tera2.cl.SP12 cultures treated with Wnt1 plus retinoic acid for 21 days



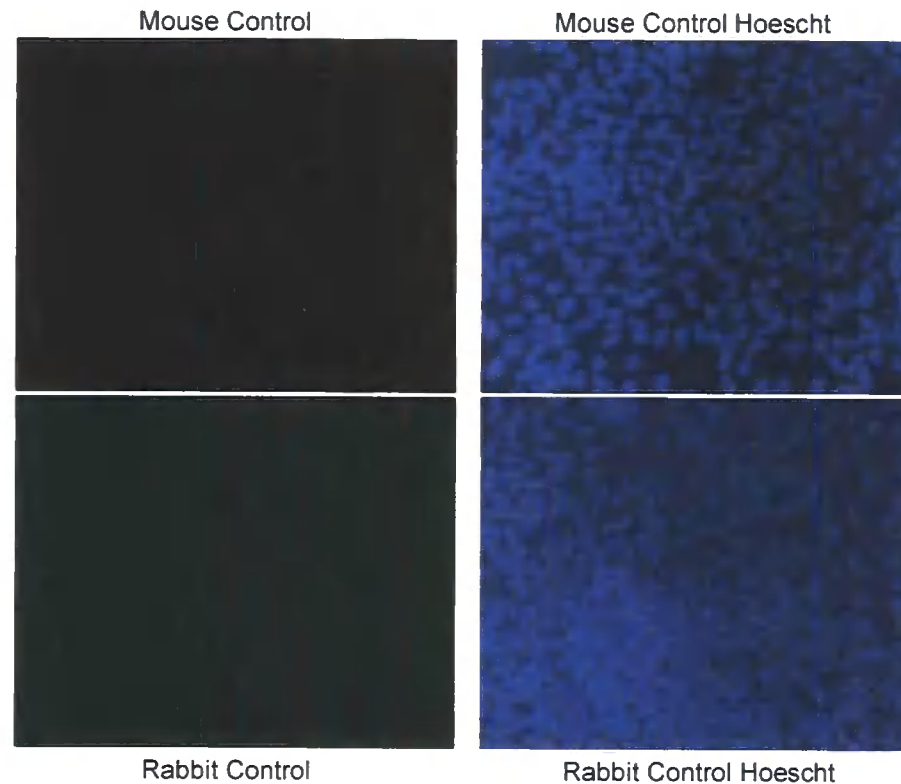


Figure 4.25: Nestin, Beta III tubulin and tyrosine hydroxylase immunostaining using a FITC labelled secondary antibody, of Tera2.cl.SP12 cultures treated with Wnt1 plus retinoic acid for 3 weeks. The 10x phase image shows that the cultures display a mix of cell morphologies; however the Tuj1 staining shows enhanced levels of this marker being present relative to retinoic acid only samples. The mouse secondary FITC labelled antibody only control is applicable for the nestin and tyrosine hydroxylase primary antibodies, the rabbit equivalent is for the Beta III tubulin antibody. The corresponding Hoescht control images confirm the presence of cells by staining their nuclei. Scale bars represent 100 μ m in all cases. In all experiments n=3. Appendix D supports this data and that in Figure 4.24, it shows merged images indicating the Beta III tubulin positive cells against a background of the total number of cells detected by Hoescht staining of cell nuclei. These images were used to produce the total cell number and Beta III tubulin positive cell counts in the data that follow (Figures 4.28 and 4.29). Although there are a number of Beta III tubulin positive cells in the image in this figure, clearly from Appendix D and the cell count data it is apparent not all cells are positive for the antigen of interest. Nestin expression was detectable but this staining was not carried out for quantifiable purposes and is merely an indicator that the cultures still possess some relatively immature cells after this period of differentiation in this experimental condition. In further work sorting out of such cells may be desirable. The TH staining is diffuse relatively hard to detect and not associated with cells of any kind of morphology that might be expected of a potential neuritic cell in culture. Given the possible non specific binding of this antibody observed in Chapter 3 this TH staining is highly inconclusive.

Flow cytometry was used to try and assess if there were any differences in the expression of tyrosine hydroxylase and nestin between the test conditions. Figure 4.26 shows that for tyrosine hydroxylase given the variability in the results there is essentially no real difference in the expression of it between the viable for use retinoic acid only and retinoic acid plus Wnt1 conditions at 7, 14 and 21 days. Interestingly though the greatest level of expression is at 7 days perhaps supporting the notion that at this time point there is an effect. However the retinoic acid only and retinoic acid plus Wnt1 samples at this point show little variation and the results are generally highly variable so no real conclusion can be made based on the flow cytometry data, especially given the possible non specific binding of the antibody discussed previously. Figure 4.27 shows that nestin levels initially rise from 7 to 14 days and then fall off by 3 weeks. Given the deviation in the sample readings there appears to be little difference between the retinoic acid only and retinoic acid plus Wnt1 conditions. The embryonal carcinoma cells express nestin at lower levels on average (68.44 ± 5.96 (S.E.M.) % positive cells, and 79.58 ± 8.24 (S.E.M.) mean fluorescence intensity) than their retinoic acid and retinoic acid plus Wnt1 treated counterparts, which given the small differences between these samples indicates that as might be predicted it is mainly retinoic acid that is responsible for the differential regulation of nestin expression observed.

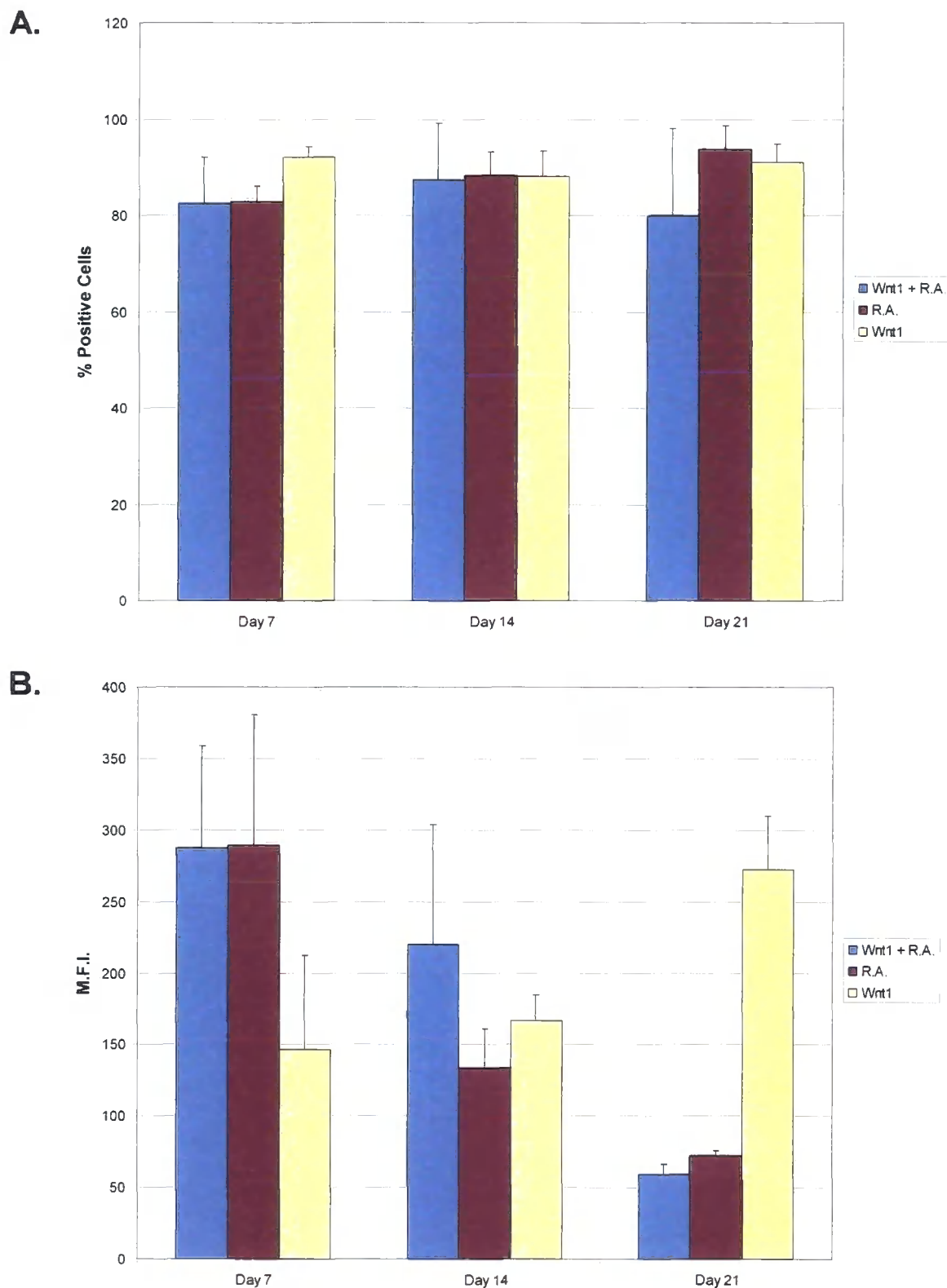
Figure 4.26: TH protein expression in EC cells treated with Wnt1 and R.A.

Figure 4.26A: The number of tyrosine hydroxylase antigen positive cells in Tera2.cl.SP12 cultures treated with 10 μ M retinoic acid only, 2ng/ml Wnt1 only, or a combination of both reagents after 7, 14 and 21 days. Error bars represent the S.D. of the mean. **B:** The intensity of tyrosine hydroxylase expression in the same culture conditions outlined above. Error bars represent the standard error of the mean. In all experiments n=3. The data show no real difference in TH expression, this may be due to no such difference existing, or as for the TH immunostaining shown previously it may be that the antibody is potentially binding in a non specific manner as discussed in Chapter 3 and as a result this is affecting the experimental output. These results though are far too inconclusive to make any kind of conclusions from.

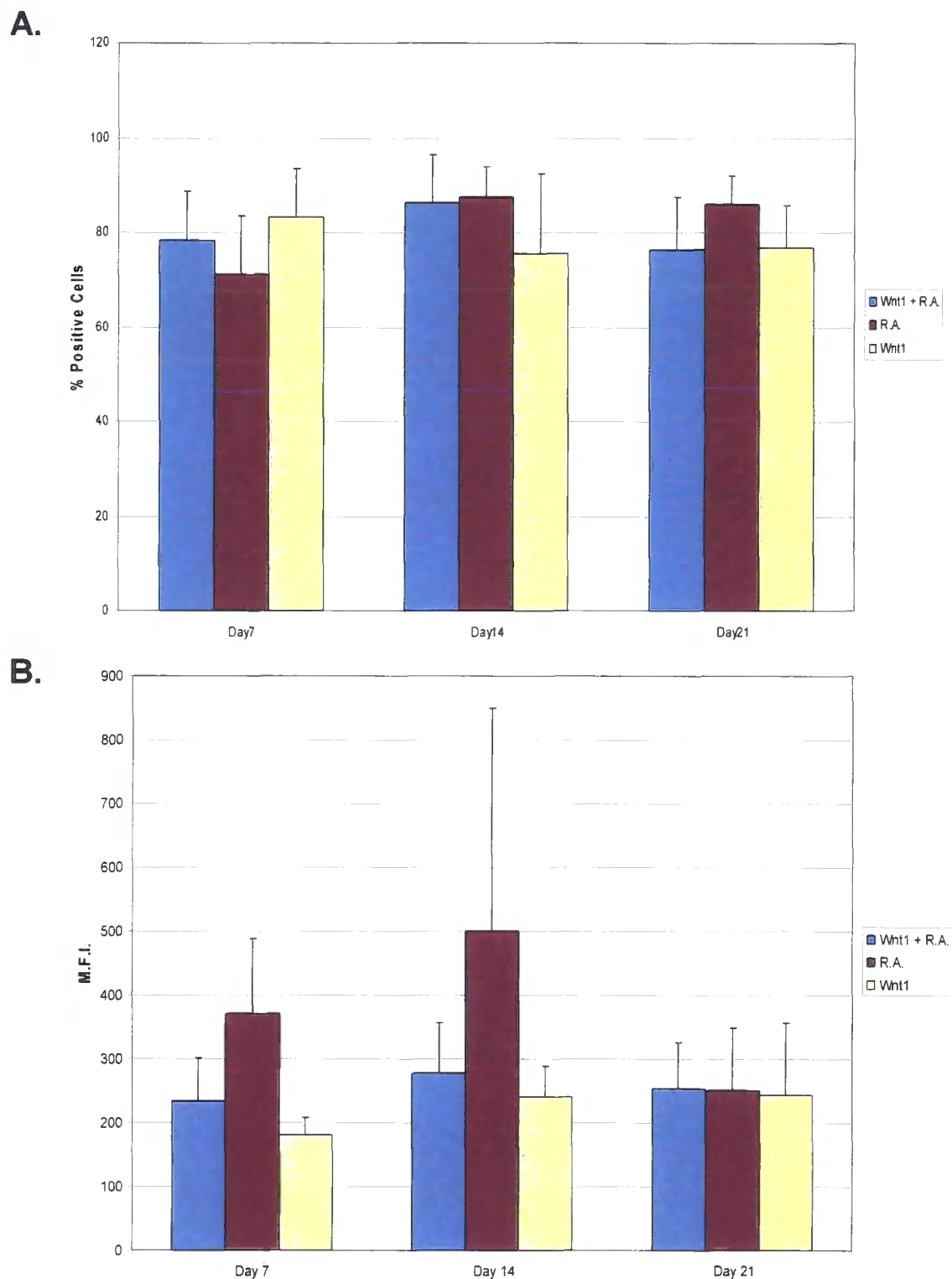
Figure 4.27: Nestin protein expression in EC cells treated with Wnt1 and R.A.

Figure 4.27A: The number of nestin antigen positive cells in Tera2.cl.SP12 cultures treated with 10µM retinoic acid only, 2ng/ml Wnt1 only, or a combination of both reagents after 7, 14 and 21 days. Error bars represent the S.D. of the mean. **B:** The intensity of nestin expression in the same culture conditions outlined above. Error bars represent the standard error of the mean. In all experiments n=3.

In the light of the variation seen in the flow cytometry data and the obvious disparity between retinoic acid only and retinoic acid plus Wnt1 treated cultures at 3 weeks in terms of their expression of Beta III tubulin, manual counts of cell number and Beta III tubulin positive cell number were performed. 5 fields of view for 3 biologically independent samples were counted using Image J. Hoescht stained nuclei were used as a measure to obtain total cell number, corresponding Beta III tubulin stained cells were counted to give a number of cells positive for this marker. Appendix D shows merged images of Beta III tubulin positive cells on a background of Hoescht stained nuclei to exemplify visually the nature of the counts. Figure 4.28 displays clearly that more cells were present in the Wnt1 only cultures, but that there was no significant difference between retinoic acid only and retinoic acid plus Wnt1 samples. Both of these conditions though differed to a statistically significant level from their Wnt1 only counterparts. The Wnt1 only condition was excluded from further statistical analysis as this condition by all the techniques used appeared unsuitable for use. Figure 4.29 shows both in terms of the average number of Beta III tubulin positive cells and percentage of Beta III tubulin positive cells per field of view that there is a statistically significant difference between the retinoic acid only and retinoic acid plus Wnt1 samples. The retinoic acid plus Wnt1 condition displays approximately double the number and percentage of Beta III tubulin positive cells in relation to its retinoic acid only counterpart. This is a promising result as it offers the possibility of producing more potentially neuronal (Beta III tubulin positive), less phenotypically mixed cultures from the Tera2.cl.SP12 cell line.

Figure 4.28: Cell counts in Tera2.cl.SP12 cultures treated with R.A., Wnt1 or both reagents for 3 weeks

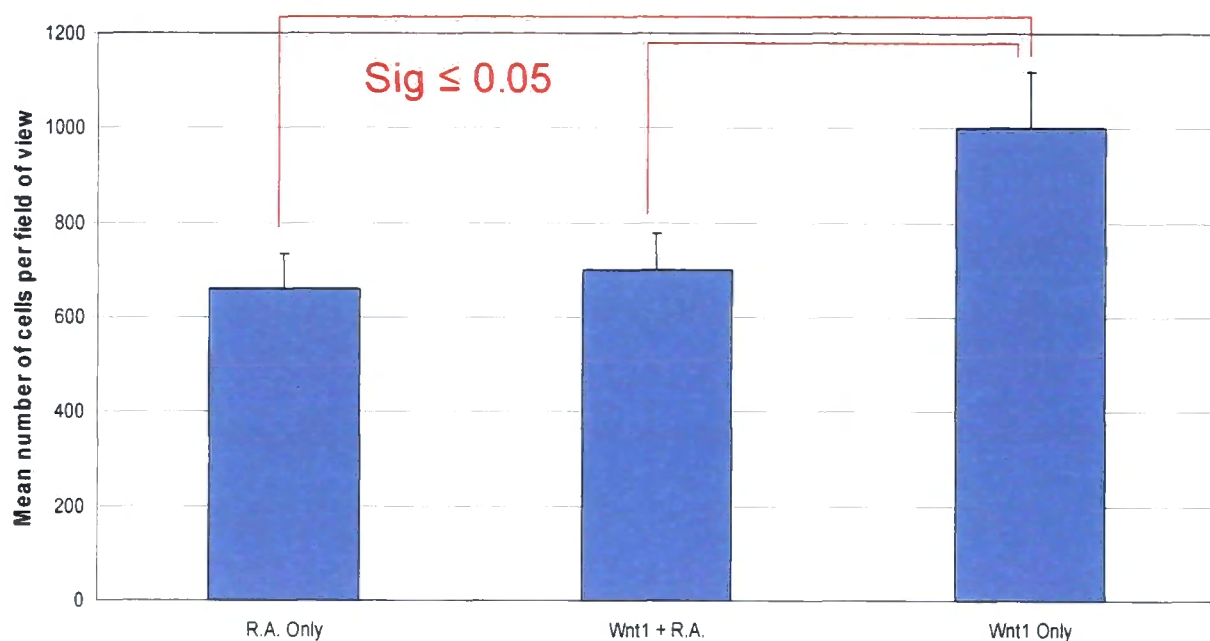


Figure 4.28: The mean number of cells per field of view at x20 magnification quantified by counting Hoescht stained nuclei in samples of Tera2.cl.SP12 cells treated with retinoic acid, Wnt1 or a combination of both reagents. 5 fields of view for 3 biological repeats were counted to give a mean value. The one way anova output indicates there is no statistically significant difference in cell numbers between retinoic acid only and Wnt1 plus retinoic acid cultures. However both of these two conditions display a clear statistically significant difference from their Wnt1 only counterparts.

Figure 4.29: The number and % of Beta III tubulin positive cells in Tera2.cl.SP12 cultures treated with R.A., Wnt1 or both reagents for 3 weeks

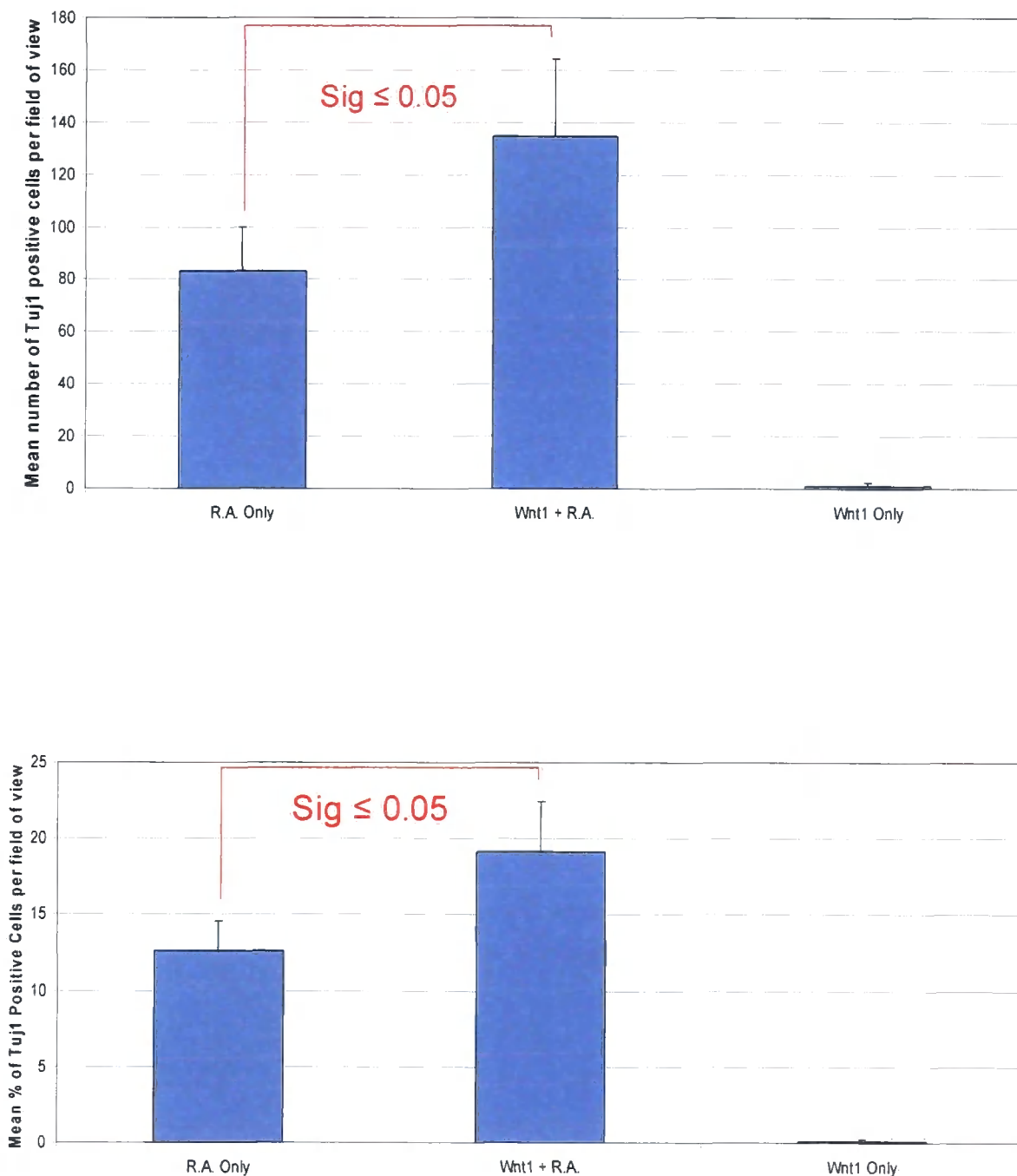


Figure 4.29: The mean number of Beta III tubulin positive cells (upper graph) and mean percentage of Beta III tubulin positive cells (lower graph) per field of view at x20 magnification, quantified by counting Beta III tubulin positive stained cells to get a mean number of cells positive for this marker and by counting Hoescht stained nuclei (a percentage was then taken by dividing the number of antigen positive cells by the number of cells) in Tera2.cl.SP12 cultures treated with retinoic acid, Wnt1 or a combination of both reagents. 5 fields of view for 3 biological repeats were counted to give a mean value. Student's T-test was used to assess if there was a statistically significant difference between the viable for use retinoic acid only and Wnt1 plus retinoic acid conditions. In terms of both number and percentage of Beta III tubulin positive cells there was a statistically significant difference (Sig. ≤ 0.05) found, indicating that the presence of Wnt1 causes a statistically relevant approximately 1.5 - 2 fold increase in the number and percentage of Beta III tubulin positive cells.

4.4 Discussion

The initial objective in this study was to elucidate if the exogenously applied commercially available human Wnt1 was able to influence the dopaminergic differentiation of the Tera2.cl.SP12 cell line, either alone or in concert with retinoic acid. Early indications were that retinoic acid was required and the entire range of techniques were all consistent in supporting this idea as the Wnt1 only condition appeared to be totally unsuitable for use at the concentration tested. Further work could be carried out to test the effects of varying the dose of this molecule to see if it could alone exert an effect on this cell line under different conditions. This though is possibly unlikely as retinoic acid is used in the vast majority of work using embryonal carcinoma cells. Perhaps therefore it would be more worthwhile to study the potential of different concentrations of Wnt1 in the presence of this molecule or after a pre-treatment with it.

To try and build on the findings in Chapter 3 where retinoic acid caused a peak in Nurr1 mRNA expression after 1 day in an analogous manner to that observed for Nurr1 protein in the Ntera2 embryonal carcinoma cell line (Misiuta et al., 2006) and given that Castelo Branco et al., 2003 had demonstrated Wnt1 could have a positive effect on Nurr1 positive precursors in rat mesencephalic cultures, this key dopaminergic transcription factor was investigated. Wnt1 appeared to have no significant effect on Nurr1 expression in Tera2.cl.SP12 cells treated with Wnt1 and retinoic acid and there was no augmentation of the peak in expression at 1 day when both reagents were used. Therefore it appears that Wnt1 does not directly impact on Nurr1 expression in Tera2.cl.SP12 cultures. However Prakash et al., 2006 were able to demonstrate that Wnt1 can maintain Otx2 expression and in turn repress Nkx2-2 expression, helping to promote acquisition of a dopaminergic phenotype. If Wnt1 could rescue the down regulation of Otx2 caused by retinoic acid seen in Chapter 3 then potentially via a route independent of Nurr1, up regulation of dopaminergic markers could be achieved. Otx2 caused a minimal amount of rescue but it appears in the embryonal carcinoma cell system that retinoic acid tends to exert the greatest influence over Otx2 mRNA expression. In collaboration Dr. V. Lakics tested primers for Nkx2-2 that worked on cDNA samples from human brain reference RNA

(Ambion) in real time PCR analyses, contrastingly in the Wnt1, retinoic acid and combined Wnt1 plus retinoic acid treated samples it was not possible to detect expression of it. Similar analyses were carried out independently by myself and yielded no result. Therefore either Nkx2-2 mRNA is not expressed in Tera2.cl.SP12 cells and their derivatives, or it is not detectable, this may be due to the cancerous aberrant nature of this cell line which may mean that there is a disruption or absence of the Nkx2-2 gene. Whatever the scenario the observation that Nkx2-2 is hard or not possible to detect may mean that the minimal level of rescue of Otx2 is of lesser importance as this system may not favour acquisition of other non dopaminergic fates if it doesn't possess the molecular machinery to produce them. As a result although the Otx2 and Nurr1 observations may not display strongly positive effects as might be hypothesised, they do not necessarily mean the Tera2.cl.SP12 cell line is an unsuitable model as there may be other routes to achieve a dopaminergic cell fate from cultures of it.

In addition Ono et al., 2007 had shown that Otx2 may have an inductive effect on Lmx1a. It is highly debateable if the small increases in Otx2 mRNA expression in Wnt1 plus retinoic acid treated cultures are able to impart such an effect on Lmx1a levels in the Tera2.cl.SP12 system; although further analysis of Otx2 protein levels would be beneficial in revealing more about this possible process. However Lmx1a mRNA expression is approximately double in the Wnt1 plus retinoic acid samples relative to their retinoic acid only counterparts at 7 days. So perhaps the minimal difference in Otx2 levels observed in the presence of Wnt1 and retinoic acid at 1 and 3 days is enough to influence the downstream pathway. Even if this is not the case a range of dopaminergic markers display an almost identical trend to Lmx1a in the regulation of their mRNA expression as does the neuronal marker Beta III tubulin. At a conceptual level this is in keeping with the *in vivo* work of Prakash et al., 2006, where Wnt1 possesses two main effects, the first of which is an early one where it is implicated in the formation of the mesencephalic dopaminergic progenitor domain. It is also congruous with the *in vitro* work of Castelo Branco et al., 2003, where Wnt1 again plays two possible roles, but the one of greater magnitude is an early ability to promote neurogenesis by a mechanism involving proliferation of Nurr1 positive precursors. Although none of the differences are statistically significant the trend is so strongly apparent and reproducible across a panel of markers that there does appear

to be via a mechanism that may or may not involve Otx2 an early dopaminergic differentiation enhancing effect at 7 days attributable to the presence of Wnt1 in cultures of Tera2.cl.SP12 cells treated with this glycoprotein and retinoic acid.

This early action of Wnt1 in the Tera2.cl.SP12 system is highly desirable not only in the context that it may aid in the design of a temporally efficient assay system using this cell line but also in the experimental rationale for further work that may include the use of Sonic hedgehog and Fibroblast growth factor 8. It is known from the work of Yan et al., 2005 that in human embryonic stem cells prior to any or before a threshold of Sox1 expression that Sonic hedgehog and Fibroblast growth factor 8 application is capable of promoting the formation of dopaminergic neurons. It has been suggested that Wnt1 may create a permissive environment for these inductive cues to act (Burbach & Smidt, 2006). After 7 days of culture Sox1 mRNA levels are still relatively low in the Tera2.cl.SP12 cell line following treatment with retinoic acid alone or in concert with Wnt1 (Figure 4.30). Therefore the positive effects of Wnt1 after 7 days may be able to be combined with the potentially optimal use of Sonic hedgehog and Fibroblast growth factor 8, with a view to ideally producing an enriched population of dopaminergic neurons, and in turn giving a coherent progression to this work.

Figure 4.30: The effects of Wnt1 and R.A. on Sox1 mRNA expression

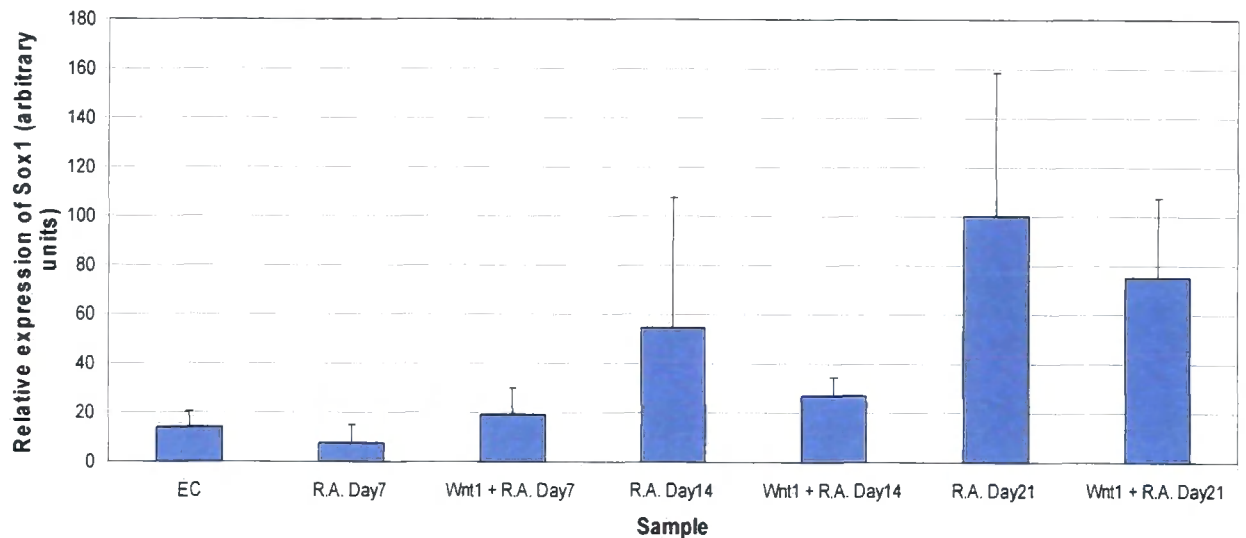


Figure 4.30: The relative expression of Sox1 mRNA in Tera2.cl.SP12 cells and their derivatives treated with 10 μ M retinoic acid only, or 10 μ M retinoic acid plus 2ng/ml Wnt1 for 7, 14 and 21 days. The one way anova output indicates there is no statistically significant difference between the two test conditions at 7 days and that these samples do not differ to a statistically significant level from the embryonal carcinoma stem cell starting material. In all experiments n=3 and error bars represent the standard deviation of the mean. Due to the relatively low levels of expression of Sox1 in the samples at 7 days, it may be an optimal temporal window/opportunity to integrate the use of Sonic hedgehog and Fibroblast growth factor 8 into the experimental design to try and facilitate the production of an enhanced population of dopaminergic neurons.

Before contemplating areas for additional study further, there are a couple of hypotheses outlined in the introductory section 4.1 to consider. The first of these is can Wnt1 enhance the yield of Beta III tubulin positive cells from Tera2.cl.SP12 cultures. In the presence of retinoic acid at 7 days Wnt1 appears to be able to up regulate the mRNA levels of the neuronal marker Beta III tubulin approximately 2 fold. Likewise at this time immunostaining for the same marker is much stronger in cultures treated with retinoic acid and Wnt1 in comparison to those that have been induced with retinoic acid alone. The difference between the two conditions is less apparent at 2 weeks both in terms of the immunostaining results and mRNA levels observed. It appears from this and earlier flow cytometry based work in Chapter 3 that at this developmental stage there is an interlude in the expression of this neuronal marker, possibly whilst other cell types predominate in the phenotypically mixed cell cultures. At 3 weeks at the mRNA level there again appears to be little difference in Beta III tubulin expression. However the immunostaining and cell counts reveal a different story as there is clearly a more intense expression of Beta III tubulin and almost double the number of cells express this neuronal marker in the retinoic acid plus Wnt1 samples relative to their retinoic acid only counterparts. This approximately 1.5 - 2 fold increase is in line with that observed in rat mesencephalic precursor cells (Castelo Branco et al., 2003) which may suggest Wnt1 has a conserved effect between species. In the work of Castelo Branco et al., 2003 there was no difference in tyrosine hydroxylase expression within the neuronal population, the flow cytometry data may possibly support the notion that the same is true in the Tera2.cl.SP12 cell line, but the results are too variable to make any firm conclusion. The immunostaining for tyrosine hydroxylase in both conditions appears to the naked eye to be comparable but is too faint to accurately quantify. Therefore the same effect may be present in the Tera2.cl.SP12 cells but this would require further repeats to validate this possible finding.

One interesting question raised by the immunocytochemistry data is; does Wnt1 in the presence of retinoic acid have two separate effects on the dopaminergic differentiation of Tera2.cl.SP12 cells? An early one at 7 days and a later activity at 3 weeks, in a fashion resembling the dual effects observed in Prakash et al., 2006. Alternatively does it have a single main early effect with a minor role later on that is harder to detect in a similar way to Castelo Branco et al., 2003, or does it exert an early effect

the features of which are only observed at later stages of the differentiation process? This final option is probably the most likely as the mRNA data indicate a difference only at 7 days. This is also seen via the more intense immunostaining. However at 7 days the Beta III tubulin positive staining is not seen in such clear neuronal body like and neuritic process type structures as the cultures earlier in the course of differentiation are not as morphologically developed. By 3 weeks the cultures have matured in morphology neuron like cells are able to be observed so the effect seen at 7 days is more apparent and quantifiable. It is possible there may be a lag time between the recognition of differences at the mRNA level being seen at the morphological and protein levels. However it may be that there is actually a difference at the protein level demonstrated by the more intense staining in the Wnt1 plus retinoic acid samples at 7 days, with a lag period to the morphological differences becoming more clearly observable at 21 days when the cultures are further developed. In the light of the trend at 7 days in the Wnt1 plus retinoic acid condition relative to retinoic acid only treated samples and the plateau effect observed for other markers, on balance it seems more likely that Wnt1 exerts its influence early but that morphologically this difference is apparent at later stages.

The final question to try and address given that Wnt1 appears able to direct the dopaminergic differentiation of Tera2.cl.SP12 cells is does Wnt1 act via a canonical pathway as expected? It appears possible that it does so, as the receptor for it is present and up regulated at the point when it exerts its maximum effect indicating Wnt1 in the presence of retinoic acid in this model system may be capable of self regulation of its action, mediated via an up regulation of its receptor. It would be of interest to try and confirm this at the protein level. Also beta-catenin mRNA is more prevalent at 7 days in the combined Wnt1 plus retinoic acid condition in comparison to the retinoic acid only samples. This may correlate with more stable beta-catenin protein being present, in turn facilitating the effects of Wnt1, although this too would need to be confirmed by protein based analysis. However it appears that Wnt1 is acting via a canonical mechanism, given that a part of this pathway glycogen synthase kinase - 3β could be a key junction at which signals from different pathways that could be activated by Wnts or Sonic hedgehog intersect (Castelo Branco & Arenas, 2006). This finding provides a further basis for study into the effects of Sonic hedgehog and Fibroblast growth factor 8 along with Wnt1 in the Tera2.cl.SP12

model. Therefore along with the other possible reasons discussed earlier there is a strong rationale for an experimental strategy devised to facilitate investigations into this area. Chapter 5 deals with the construction of this approach to try and elucidate a greater understanding of the potential for dopaminergic differentiation of the Tera2.cl.SP12 cell line.

Chapter 5

The effects of suspension culture, Sonic hedgehog, Fibroblast growth factor 8 and ascorbic acid, on the acquisition of a dopaminergic phenotype by human embryonal carcinoma stem cells

5.1 Introduction

In the previous Chapter Wnt1 in combination with retinoic acid was shown to have positive effects on the ability of Tera2.cl.SP12 embryonal carcinoma stem cells to acquire a neuronal and possibly more specifically a dopaminergic fate. Given the concept that Wnt1 may create a permissive environment for inductive factors such as Sonic hedgehog and Fibroblast growth factor 8 to act (Burbach & Smidt, 2006) the primary purpose of the experiments reported in this Chapter was to try and utilise these molecules in an optimal fashion to potentially yield an enriched population of dopaminergic neurons.

Sonic hedgehog and Fibroblast growth factor 8 have been used widely in attempts to try and produce dopaminergic neurons from a variety of stem cells. In the human Ntera2 embryonal carcinoma cell line there is conflicting evidence as to whether these molecules can exert an effect on the dopaminergic differentiation potential of this model system. Stull & Iacovitti, 2001, were only able to detect around 2% of cells treated with Sonic hedgehog/Fibroblast growth factor 8 as being tyrosine hydroxylase immunopositive. However these Ntera2 cells had already received a period of retinoic acid pre treatment and were possibly not susceptible to the effects of Sonic hedgehog/Fibroblast growth factor 8 application as they may have already become too differentiated. In work using human embryonic stem cells Yan et al., 2005 demonstrated that the early application of these two inductive molecules prior to or at a threshold of Sox1 expression was necessary to obtain a population of neurons displaying characteristics of a midbrain dopaminergic phenotype. Therefore it may be that the Ntera2 cells were not incapable of responding to Sonic hedgehog and Fibroblast growth factor 8 but that they were unable to do so due to the period of retinoic acid treatment they had previously received. The concept of there being an optimal early temporal window for use of these molecules presented by Yan et al., 2005 is in support of this idea. Further work in the Ntera2 cell system carried out in the absence of retinoic acid indicated that this system was able to show a positive dopaminergic differentiation response when treated with these two molecules in concert (Ravindran & Rao, 2006). This work was based on the sequential protocol of Lee et al., 2000, who used mouse embryonic stem cells. Both of these studies made

use of the formation of aggregates in suspension, termed embryoid bodies in the embryonic stem cell work, as part of the differentiation protocol.

In a more recent paper using human embryonic stem cells the concept of growing cells as aggregates in suspension was extended to produce structures that resemble neurospheres termed spherical neural masses (Cho et al., 2008). When these were then treated with Sonic hedgehog and Fibroblast growth factor 8 for 10 days, with ascorbic acid being added as well for the final 6 days of this 10 day period, a high percentage of tyrosine hydroxylase positive cells were formed. Indeed 86% of the total number of neurons were tyrosine hydroxylase positive in test cultures; given that Wnt1 was able to enhance the number of Beta III tubulin positive cells produced from Tera2.cl.SP12 cells, if its use could be linked to this differentiation protocol, there was a possible opportunity not only to enhance the yield of potential neurons but also the number of them displaying markers of a dopaminergic phenotype. In addition Tera2.cl.SP12 embryonal carcinoma stem cells have previously been shown to be able to produce neurosphere type structures rapidly when cultured in suspension in the presence of retinoic acid (Horrocks et al., 2003). Therefore this posed an interesting question, does growth of Tera2.cl.SP12 cells in suspension in the presence of retinoic acid alone or in combination with Wnt1 offer the possibility of enhanced dopaminergic differentiation in comparison to adherent culture? In the light of previous findings that Wnt1 exerts its greatest effect at 7 days and given that neurosphere type structures can be formed in this time frame, this would appear to be a logical point at which to test this hypothesis.

Although the direct approach of culturing retinoic acid induced Tera2.cl.SP12 cells in different oxygen environments seemed to exert no effect on the dopaminergic differentiation capacity of such cultures at the oxygen tensions tested in Chapter 3. The use of suspension culture may facilitate some effect if any is possible due to oxygen, as cells at the periphery of an aggregate are likely to experience a different set of conditions to those in the core, with the strong possibility that cells in the centre will receive a lower oxygen pressure (Gassmann et al., 1996). Whether or not there is an oxygen effect, the growth of the Tera2.cl.SP12 cells as aggregates essentially creates a more complex 3D microenvironment. Where not only may cell – cell interactions play a role but also there may be a different regulation of the extracellular

matrix, which itself may influence cell fate decisions, for example laminin in conjunction with basic Fibroblast growth factor and heparin may help induce the formation of dopaminergic neurons from rat neural stem cells (Yu et al., 2007). In addition the possibly shorter distances between cells growing in an aggregate may facilitate the effects of any soluble factors the cells under investigation may release, which could help drive the process of differentiation. Therefore there are a number of potentially favourable effects that could arise from growth of the Tera2.cl.SP12 cells in suspension. There is even the prospect that Wnt1 may exert a greater effect than it does when used in conjunction with retinoic acid on adherent cultures.

Once an assessment has been carried out to address the possibility of there being some differences between suspension and adherent culture of Tera2.cl.SP12 cells in the presence of retinoic acid alone or in combination with Wnt1, the premier (most favourable in relation to potential for dopaminergic differentiation) condition if there is one will be taken forward to extend the investigations. At this point either the cells will be grown in suspension or adherent cultures for 7 days with retinoic acid or retinoic acid plus Wnt1 being applied and then subsequently treated with Sonic hedgehog/Fibroblast growth factor 8 and retinoic acid for 4 days with ascorbic acid being added to this mix for the final 6 days (Figure 5.1) in a similar fashion to Cho et al., 2008 who omitted the retinoic acid. The choice to include retinoic acid is based on the previous findings in Chapter 4 where its use appeared necessary to drive the differentiation of and give control over the highly proliferative Tera2.cl.SP12 cells. However given the work of Stull & Iacovitti, 2001 where retinoic acid treatment of Ntera2 cultures prior to application of Sonic hedgehog and Fibroblast growth factor 8 possibly curtailed the effects of these two molecules, an additional set of experiments is to be included. In these investigations the most optimal (highly neuronal) condition will be selected from the samples treated as per figure 5.1. This set of samples will be compared to a set of cultures given an identical treatment for the first 7 days but after this time point the retinoic acid will be omitted and the 10 day scheme as per Cho et al., 2008 will be used to try and drive the dopaminergic differentiation process. The plus and minus retinoic acid conditions defined in Figure 5.2 will then be analysed to determine if this molecule has an inhibitory effect on dopaminergic differentiation in the context of this experimental strategy.

In total the duration of differentiation is no more than 17 days, sampling will also occur at 11 days to try and build up some impression of the changes in dopaminergic marker expression over time in the presence of the different inductive factors. This relatively short 17 day protocol though is designed with a view to downstream applications. If the Tera2.cl.SP12 cell line is a viable tool that can be utilised to carry out basic pharmacological screens then the procedures by which dopaminergic neurons are produced from it need to be temporally efficient. The effects of Wnt1 at 7 days, the ability of the embryonal carcinoma stem cells to rapidly form neurospheres and the propensity of them for neural differentiation may all combine to provide the required efficiency, especially if they can be linked neatly into the 10 day differentiation protocol of Cho et al., 2008. This work will further address the question not only of whether Tera2.cl.SP12 embryonal carcinoma stem cells possess the capacity for dopaminergic differentiation but also are they likely to be viable for any applied usage, or are they more suited to a role as a basic research tool? Other hypotheses may also be tested such as does Sonic hedgehog affect the signalling pathway outlined in Figure 4.2 as may be expected and does the initial presence of Wnt1 have any favourable effects in creating a permissive environment in which the inductive cues Sonic hedgehog and Fibroblast growth factor 8 can act to influence the dopaminergic differentiation of the embryonal carcinoma stem cells?

5.2 Cell Culture Methods

Suspension culture of EC cells with Retinoic acid and Wnt1

Cultures of Tera2.cl.SP12 EC cells were grown to confluency, the media aspirated from them, before being washed with 1x PBS, prior to treatment for 5 minutes with 0.25% (w/v) Trypsin/0.1% (w/v) EDTA (in HBSS) (Cambrex), to release the cells from the surface of the culture flask. The Trypsin was then neutralized by addition of 4ml of DMEMFG and any cells remaining loosely bound washed off the surface of the flask by pipetting the DMEMFG/Trypsin-EDTA mix against the side of the flask where cell attachment occurs. A further wash with 1x PBS was carried out to optimize the retrieval of EC cells for use. The cell suspension was then transferred to a 15ml Falcon tube and spun in a centrifuge (Eppendorf 5810R (swing bucket rotor A-4-62)) at 800rpm, 4°C, for 2 minutes. The excess fluid was removed to leave a cell pellet. This was resuspended in DMEMFG and cell number was determined using a haemocytometer. Once the cell number was known the EC cells were seeded out at a density of 2×10^6 cells per 90mm bacterial culture dish. They were then exposed to either a final concentration of 10 μ M all trans retinoic acid (Sigma), or a final concentration of 10 μ M all trans retinoic acid (Sigma) along with Wnt1 (Peprotech) at a final concentration of 2ng/ml for 7 days. Media was changed every 1-2 days to prevent EC cells adhering to the surface of the culture dish. A new culture dish was used for each individual culture every time the media was changed. To perform a media change the floating spheres of EC cells and the media they were differentiating in were collected using a serological pipette; transferred to a 15 or 50ml Falcon tube prior to being spun down in a centrifuge for 2 minutes at 800 rpm at 4°C. The media was then aspirated and the cell pellet resuspended in 10ml of fresh media. This was then pipetted into a fresh 90mm bacterial culture dish and a further 10ml of DMEMFG added. The culture was then treated with either retinoic acid alone or in combination with Wnt1 (as appropriate) to give exposure to the final concentrations outlined above.

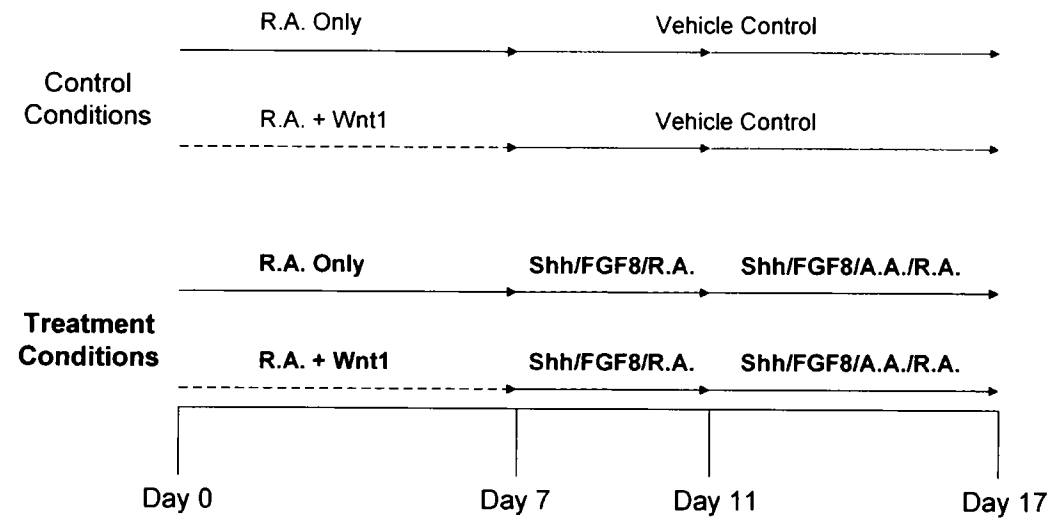
Treatment of suspension cultures with Shh/FGF8/A.A./R.A.

After 7 days in suspension culture with retinoic acid or Wnt1 plus retinoic acid samples not harvested for analysis at this point were plated out in total into 6 well plates or at a 1 in 5 dilution (to account for relative well area) into 24 well plates. This was done by collecting the floating spheres of EC cells and the media they were differentiating in using a serological pipette, transferring this mix to a 15 or 50ml Falcon tube prior to spinning it down in a centrifuge for 2 minutes at 800 rpm at 4°C. The media was then aspirated and the cell pellet resuspended with vigorous mechanical agitation (using a pipette to break up the clusters of treated EC cells) in an appropriate volume for the vessel the cells were about to be seeded into. In practice 3ml of media were used for samples that were to be plated out in 6 well plates (with all 3ml being transferred to the recipient well). Whilst for a 24 well plate the cell pellet was resuspended in 5ml of media, and after agitation 1ml of the solution containing approximately one fifth of the cells was added to the recipient culture vessel.

In the initial experiments immediately after plating out cells were exposed to final concentrations of 200ng/ml Shh (R & D Systems) (made up in 1x PBS), 100ng/ml FGF8 (Peprotech) (made up in 10mM Tris pH 8.0), and 10µM retinoic acid (Sigma) for 4 days, this treatment was continued for a further 6 days but 200µM ascorbic acid (Sigma) (made up in sterile distilled water) was also included. Vehicle controls exposed to only 1x PBS and 10mM Tris pH 8.0 for the first four days as well as sterile distilled water alongside these 2 components for the final 6 days were also run. Figure 5.1 gives a summary of the conditions used for the entire protocol including the initial 7 day suspension culture step. At all times cells were maintained at 37°C in a standard 5% CO₂ incubator.

Figure 5.1 - Shh/FGF8/A.A./R.A. Differentiation Protocol (based on Cho et al., 2008)

Shh/FGF8/A.A./R.A. Differentiation Protocol

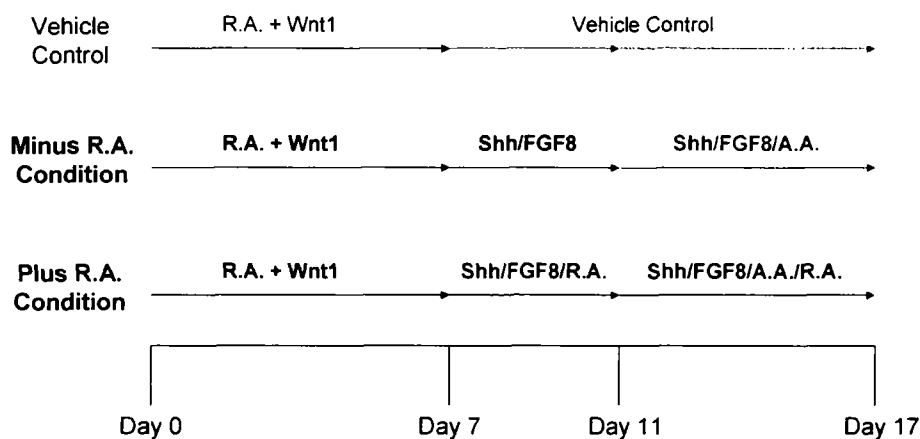


Note: Vehicle control consisted of treatment with 1x PBS and 10mM Tris pH 8.0 for 4 days (green arrow), this was continued for a further 6 days but with the addition of autoclaved sterile water to the regime as well (orange arrow).

Subsequent experiments investigated whether the inclusion of retinoic acid has a negative effect on the dopaminergic differentiation potential of the Tera2.cl.SP12 model. Samples were prepared as previously outlined for the plus R.A. samples; however for the minus R.A. samples as would be expected retinoic acid was omitted. This is summarised in Figure 5.2.

Figure 5.2 - Shh/FGF8/A.A. +/- R.A. Differentiation Protocol (based on Cho et al., 2008)

Shh/FGF8/A.A. +/- R.A. Differentiation Protocol



Note: Vehicle control consisted of treatment with 1x PBS and 10mM Tris pH 8.0 for 4 days (green arrow), this was continued for a further 6 days but with the addition of autoclaved sterile water to the regime as well (orange arrow).

5.3 Results

Prior to the assessment of whether or not Sonic hedgehog, Fibroblast growth factor 8 and ascorbic acid in the presence or absence of retinoic acid can drive the dopaminergic differentiation of the Tera2.cl.SP12 cell line. The first step in this study was to compare the effects of retinoic acid alone and in combination with Wnt1 under adherent and suspension culture conditions. If the suspension based method was comparable or favourable the rationale was then to integrate this approach into the protocol of Cho et al., 2008 with the embryonal carcinoma cell derived neurospheres replacing the spherical neural masses formed from the embryonic stem cells in that work. The spherical neural masses in Cho et al., 2008 were shown to express nestin, Sox1 and Beta III tubulin. Therefore the first step in these investigations was to use real time PCR analysis to facilitate a quantitative comparison of the levels of these three markers in Tera2.cl.SP12 cells grown in suspension or adherent conditions in the presence of retinoic acid or retinoic acid and Wnt1. Figure 5.3A demonstrates that there is a clear expression of nestin as would be expected when using embryonal carcinoma cells, and the levels at which this marker is present vary little between the conditions. Figure 5.3B indicates that Sox1 is present at higher levels in the adherent cultures, the lower levels present in the suspension cultures though may be favourable in the context of there being an advantage in applying Sonic hedgehog and Fibroblast growth factor 8 prior to or before a threshold of Sox1 expression as described by Yan et al., 2005. This was therefore an interesting result as it offered the prospect that suspension culture may be advantageous if pursuing the acquisition of a dopaminergic phenotype using a method involving Sonic hedgehog and Fibroblast growth factor 8 administration. There appeared however to be little difference in relation to the use of Wnt1 in conjunction with retinoic acid. In contrast Figure 5.3C shows that the combined use of suspension culture and Wnt1 plus retinoic acid may give more neuronal cultures. Interestingly Wnt1 in concert with retinoic acid does not have the same effect as observed previously in Chapter 4 on adherent cultures. Why Wnt1 may have had an effect in one set of experiments in Chapter 4 but seemingly does not in an identical set here is unknown, as it appears to exert an effect in the suspension cultures and this is of a similar scale to that previously observed. It may be that the aberrant nature of the cell culture model has caused this difference as if one passage

of cells used was more abnormal or flawed than is usual, then they could have been subsequently passaged (a number of times) and used for setting up each of the 3 repeats for which samples for analysis were collected. It could also be the Wnt1 used lost its activity, although this may be less likely given that the same batch was used for the suspension culture samples where it appears to have had a positive effect.

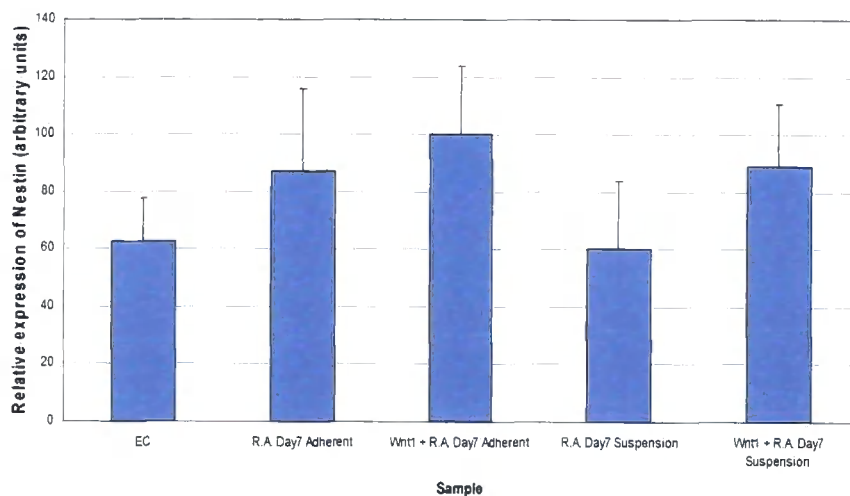
However the effect observed may not be due to the application of exogenous compounds at all. It may be the Wnt1 did lose its activity or was unable to exert any action on this occasion in either adherent or suspension cultures. The differences observed in these initial investigations may actually arise from differences in gene expression profiles that come about from the growth of the embryonal carcinoma cells in suspension rather than adherent conditions in a similar manner to that found with embryonic stem cells. Indeed the specific method of suspension culture of mouse embryoid bodies (hanging drop versus static suspension culture) can itself affect the gene expression profile observed (Mogi et al, 2009). This too may be the case in the embryonal carcinoma cell system and may account for some of the differences recorded.

Figures 5.4A, B and C show a similar pattern to that for Beta III tubulin for the dopaminergic markers, tyrosine hydroxylase and dopamine receptor 2, as well as the ventral midbrain marker Nkx6-1. The trend though is promising in that dopaminergic marker expression is highest in the Wnt1 plus retinoic acid suspension cultures. Figure 5.5A and C show that for Lmx1a and neurogenin2 the same trend is apparent; however there is little difference between the retinoic acid and retinoic acid plus Wnt1 conditions in relation to Msx1 expression. This possible component of the dopaminergic specification pathway is though up regulated in suspension cultures. Therefore given that both neuronal and dopaminergic phenotypic markers are up regulated in the Wnt1 plus retinoic acid suspension cultures, as are some of the factors (Lmx1a and neurogenin2) that may help specify a dopaminergic fate, it would appear this is the optimal condition. Although the inclusion of Wnt1 does not appear to affect Sox1 or Msx1 levels to any real extent, the use of suspension culture may give favourable levels of these two markers. The Wnt1 plus retinoic acid suspension culture condition or possibly even just its retinoic acid only suspension culture counterpart are as such likely to enhance the possibility of producing an enriched

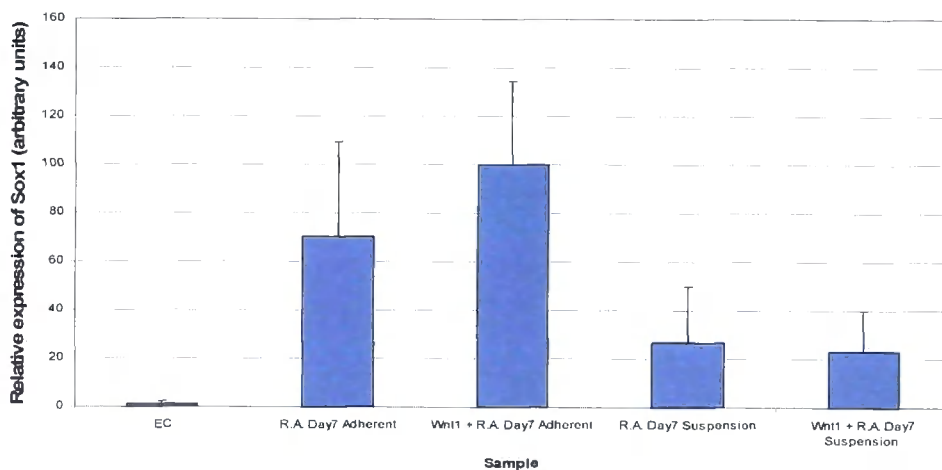
population of dopaminergic neurons. Figure 5.6A demonstrates that the mRNA for the transporter protein that allows ascorbic acid into cells is present in such cultures, as is the receptor for Sonic hedgehog (Figure 5.6B), as is the receptor for Fibroblast growth factor 8 (Figure 5.6C), although the mRNA levels for this have declined to a statistically significant level from those in the starting material. In an ideal scenario the expression of the Fibroblast growth factor 8 receptor would be studied at the protein level as falling quantities of mRNA may be present but the receptor may still be expressed and be functional at this stage. Likewise it would be more complete to confirm the presence of the other receptor and transporter protein at this level. However the indication is that all three molecules ascorbic acid, Sonic hedgehog and Fibroblast growth factor 8 may be able to exert an effect and thus hopefully promote the formation of potential neurons displaying dopaminergic characteristics. Indeed the system may be priming itself for a response to ascorbic acid and given the falling expression of the Fibroblast growth factor 8 receptor may be at a point where Fibroblast growth factor 8 application is necessary for it to have an effect, or its maximum effect on dopaminergic differentiation.

Figure 5.3: Nestin, Sox1 & Beta III tubulin expression in suspension & adherent cultures

A.



B.



C.

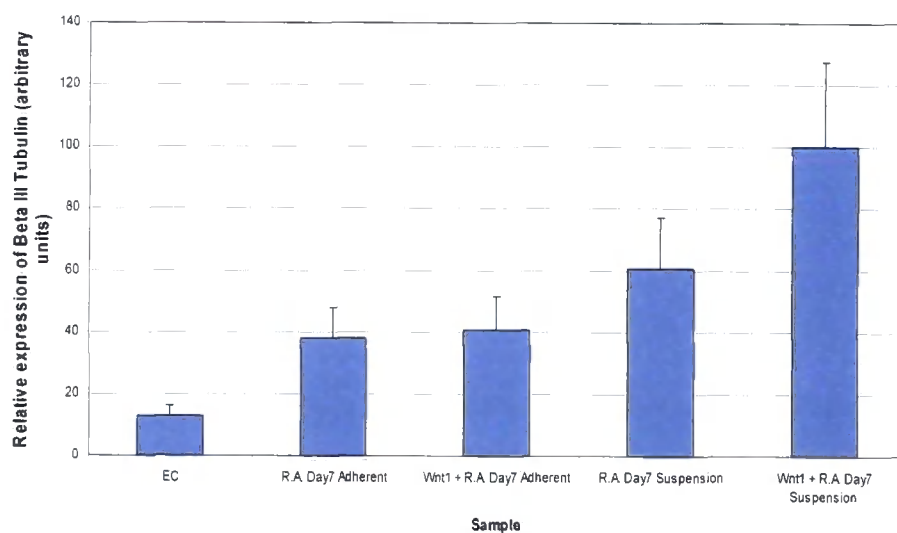
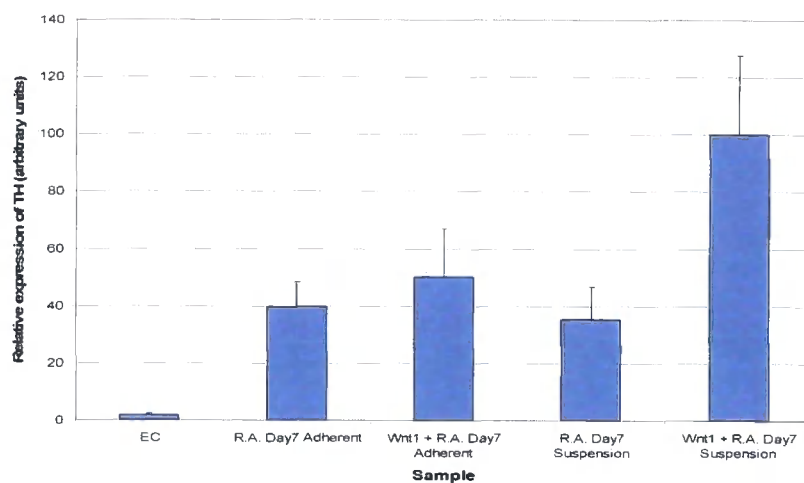


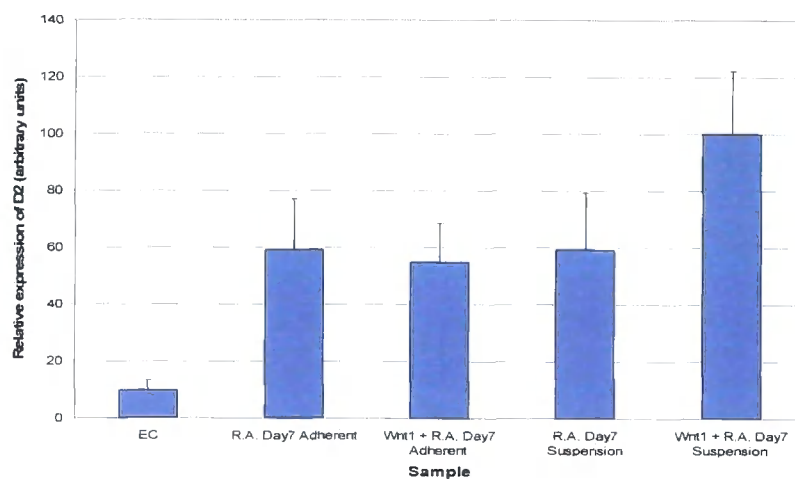
Figure 5.3A: Relative expression of Nestin mRNA in Tera2.cl.SP12 cultures and their derivatives grown in the presence of 10 μ M retinoic acid alone or in combination with 2ng/ml Wnt1 for 7 days as either adherent monolayers or free floating aggregates in suspension. **B:** Relative expression of Sox1 mRNA in the same conditions outlined above. **C:** Relative expression of Beta III tubulin mRNA again in the conditions outlined in part A. Error bars = S.D. of mean. In all experiments n=3.

Figure 5.4: TH, D2 & Nkx 6-1 expression in suspension & adherent cultures

A.



B.



C.

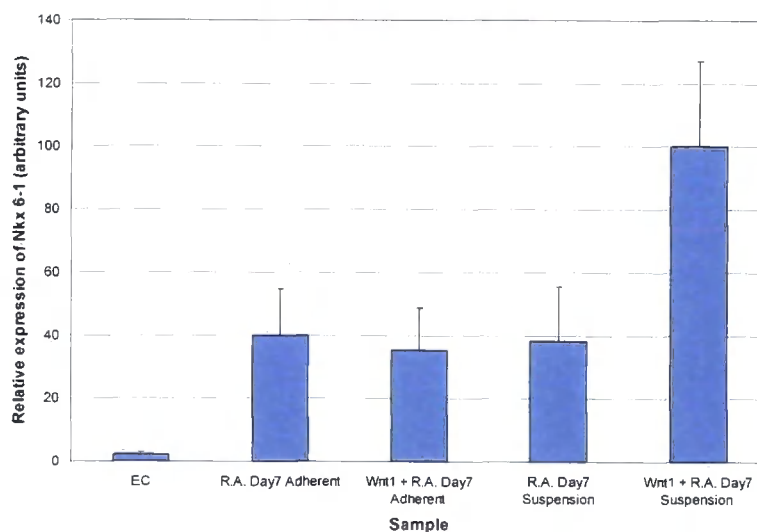


Figure 5.4A: Relative expression of TH mRNA in Tera2.cl.SP12 cultures and their derivatives grown in the presence of 10 μ M retinoic acid alone or in combination with 2ng/ml Wnt1 for 7 days as either adherent monolayers or free floating aggregates in suspension. **B:** Relative expression of D2 mRNA in the same conditions outlined above. **C:** Relative expression of Nkx 6-1 mRNA again in the conditions outlined in part A. Error bars = S.D. of mean. In all experiments n=3.

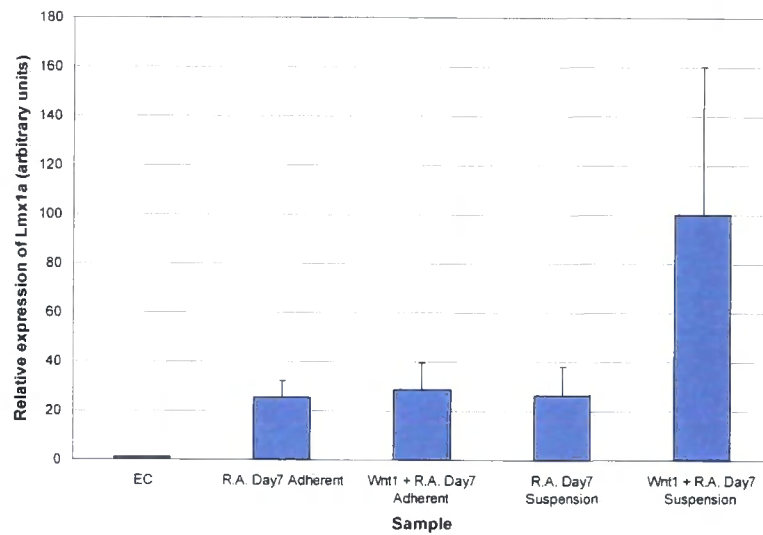
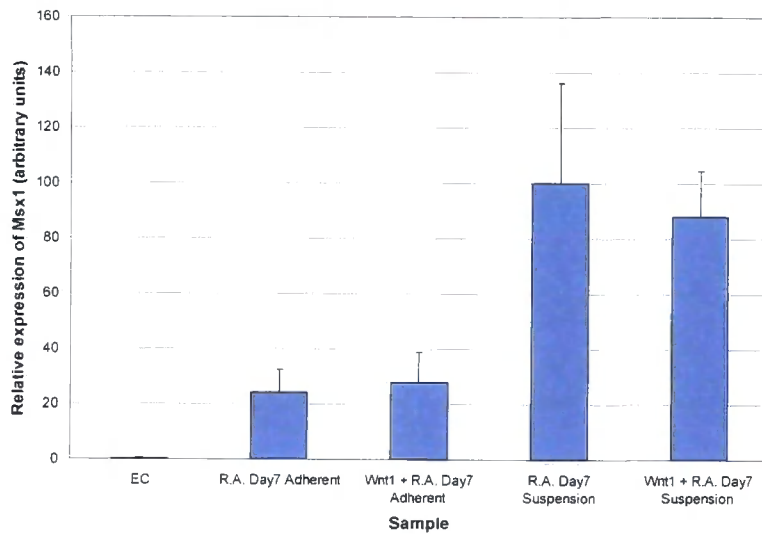
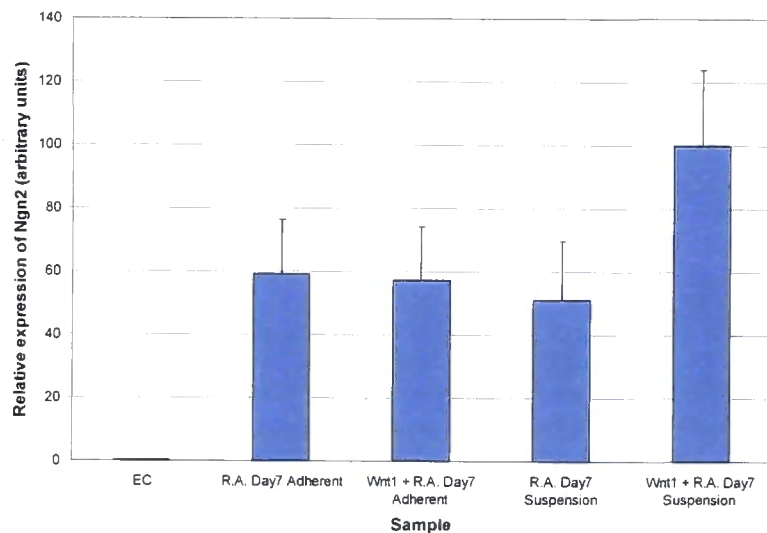
Figure 5.5: Lmx1a, Msx1 & Ngn2 expression in suspension & adherent cultures**A.****B.****C.**

Figure 5.5A: Relative expression of Lmx1a mRNA in Tera2.cl.SP12 cultures and their derivatives grown in the presence of 10 μ M retinoic acid alone or in combination with 2ng/ml Wnt1 for 7 days as either adherent monolayers or free floating aggregates in suspension. **B:** Relative expression of Msx1 mRNA in the same conditions outlined above. **C:** Relative expression of Ngn2 mRNA again in the conditions outlined in part A. Error bars = S.D. of mean. In all experiments n=3.

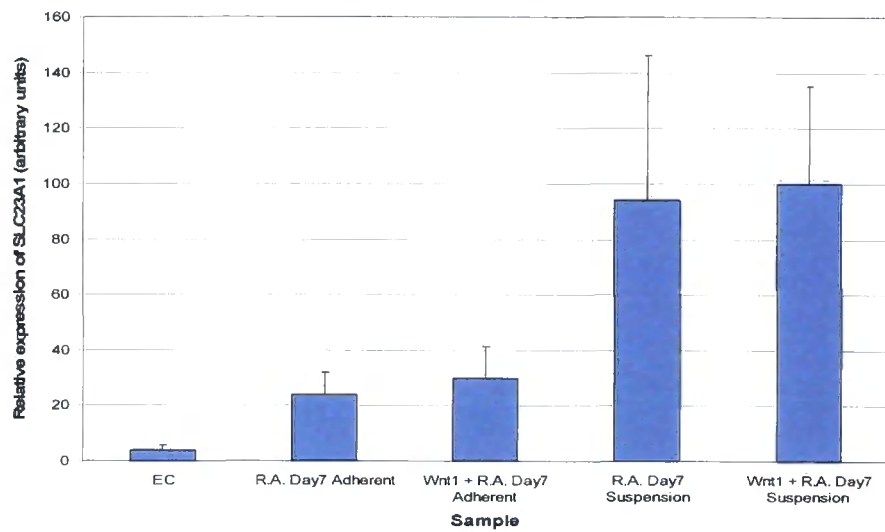
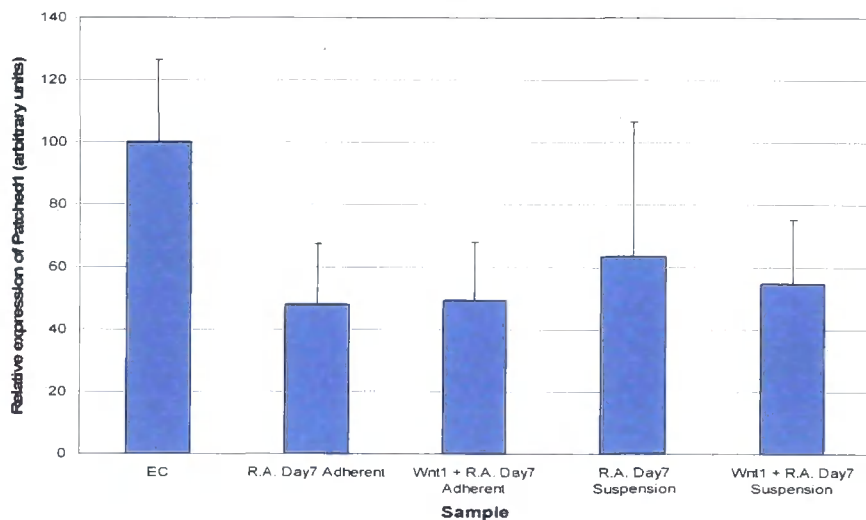
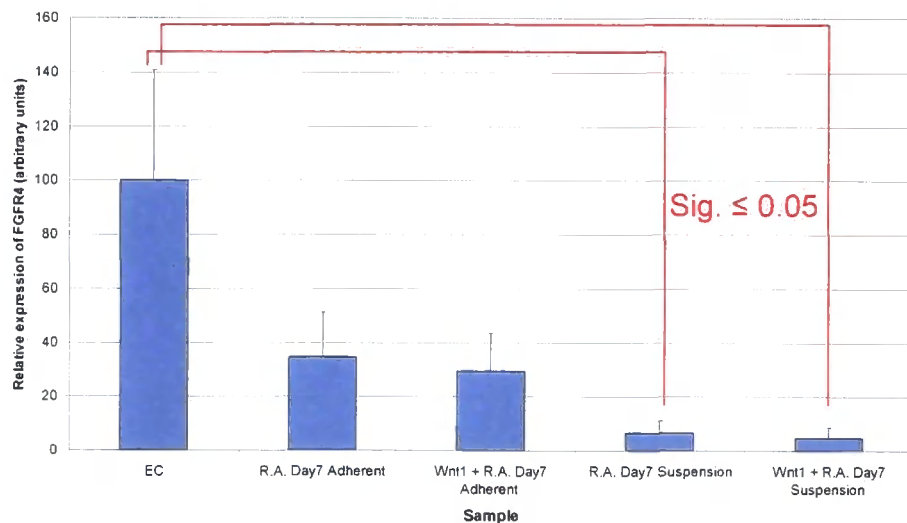
Figure 5.6: Transporter/receptor expression in suspension & adherent cultures**A.****B.****C.**

Figure 5.6A: Relative expression of SLC23A1 mRNA in Tera2.cl.SP12 cultures and their derivatives grown in the presence of 10 μ M retinoic acid alone or in combination with 2ng/ml Wnt1 for 7 days as either adherent monolayers or free floating aggregates in suspension. **B:** Relative expression of Patched 1 mRNA in the same conditions outlined above. **C:** Relative expression of FGFR4 mRNA again in the conditions outlined in part A. Error bars = S.D. of mean. In all experiments n=3. A one way anova with accompanying Tukey post hoc analysis was performed to elucidate the significant differences highlighted in red.

The 7 day retinoic acid and Wnt1 plus retinoic acid treated suspension cultures were taken forward as they appeared to offer the greatest chance of facilitating the formation of potential dopaminergic neurons. In addition the presence and absence of Wnt1 in the initial step gave rise to the opportunity to assess if Wnt1 application prior to Sonic hedgehog/Fibroblast growth factor 8 usage had any beneficial effects which could give support to the concept that it creates a permissive environment in which such inductive signals can act (Burbach & Smidt, 2006). When cells are grown in suspension and then plated out into adherent conditions as per Figure 5.1 they appear from the stage of entering adherent culture at 7 days more neuronal in appearance. Figure 5.7 shows morphological images of such cells after 1 day of Sonic hedgehog/Fibroblast growth factor 8/retinoic acid application and at the final 17 day time point. The cells appear to show more process like structures in the final 10 days of differentiation and differ considerably in morphology from the vehicle controls. Phase images though are not particularly conclusive so some immunocytochemistry was carried out to assess expression of the neuronal marker Beta III tubulin. Figure 5.8 shows both control and test cultures stain strongly for Beta III tubulin. Despite mechanical disruption of the neurospheres prior to seeding out into adherent culture plastic ware there are still some cell aggregates or sections of cells that remain in close proximity to each other. It is as such difficult to make a quantitative assessment of the number of neuronal cells.

Figure 5.7: Morphological Images of cells growing in the presence of a combination of Shh/FGF8/R.A. & A.A.

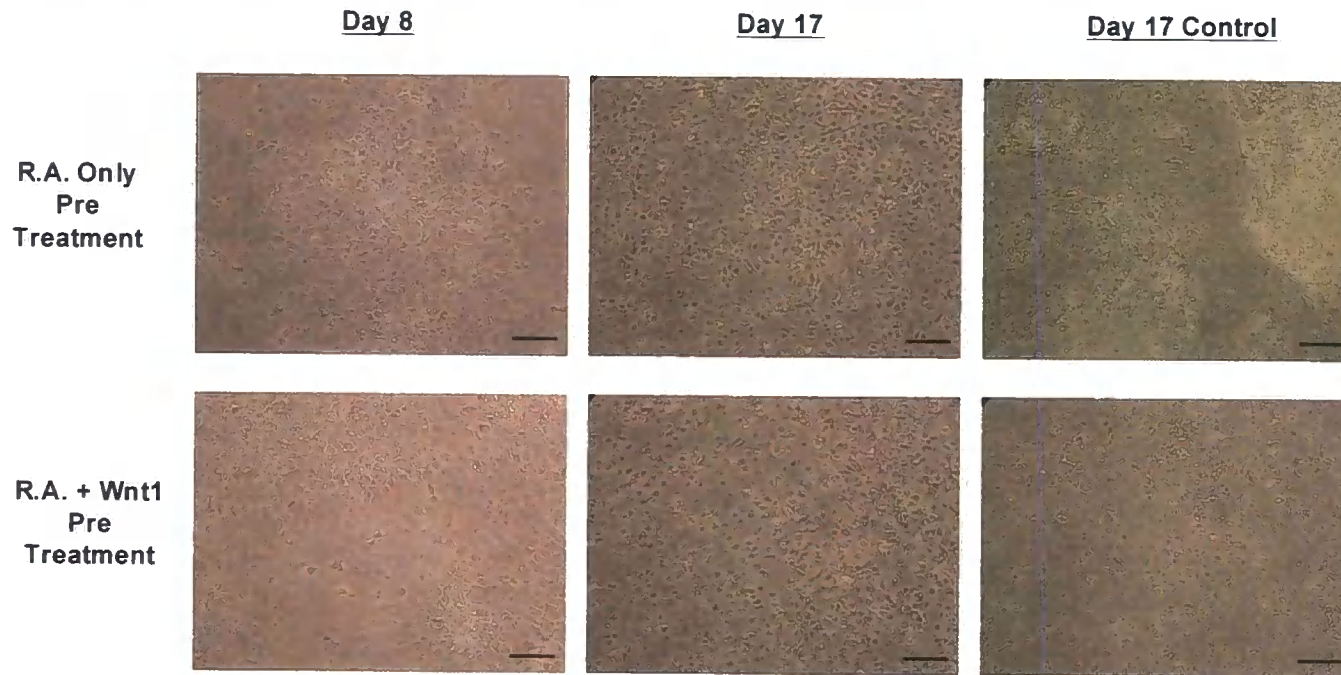


Figure 5.7: Phase images of cell morphology in cultures of Tera2.cl.SP12 cells pre treated for 7 days when grown in suspension with 10 μ M retinoic acid alone or in combination with 2ng/ml Wnt1 that were then seeded out into adherent cell culture plastic ware for the remaining 10 days of differentiation. Day 8 reflects the cultures following 1 day in adherent culture at this stage they were exposed to 200ng/ml Shh, 100ng/ml FGF8 and 10 μ M retinoic acid. Day 17 represents the cultures at the end of the differentiation protocol outlined in Figure 5.1. Day 17 control reflects the morphology of cultures that received the pre treatments outlined above for 7 days in suspension but that were then left in just media with the vehicles used to make the Shh, FGF8 and for the final 6 days A.A. up in prior to application. Scale bars represent 100 μ m.

Figure 5.8: Beta III tubulin immunostaining of Shh/FGF8/R.A. & A.A. treated Tera2.cl.SP12 cultures at 17 days

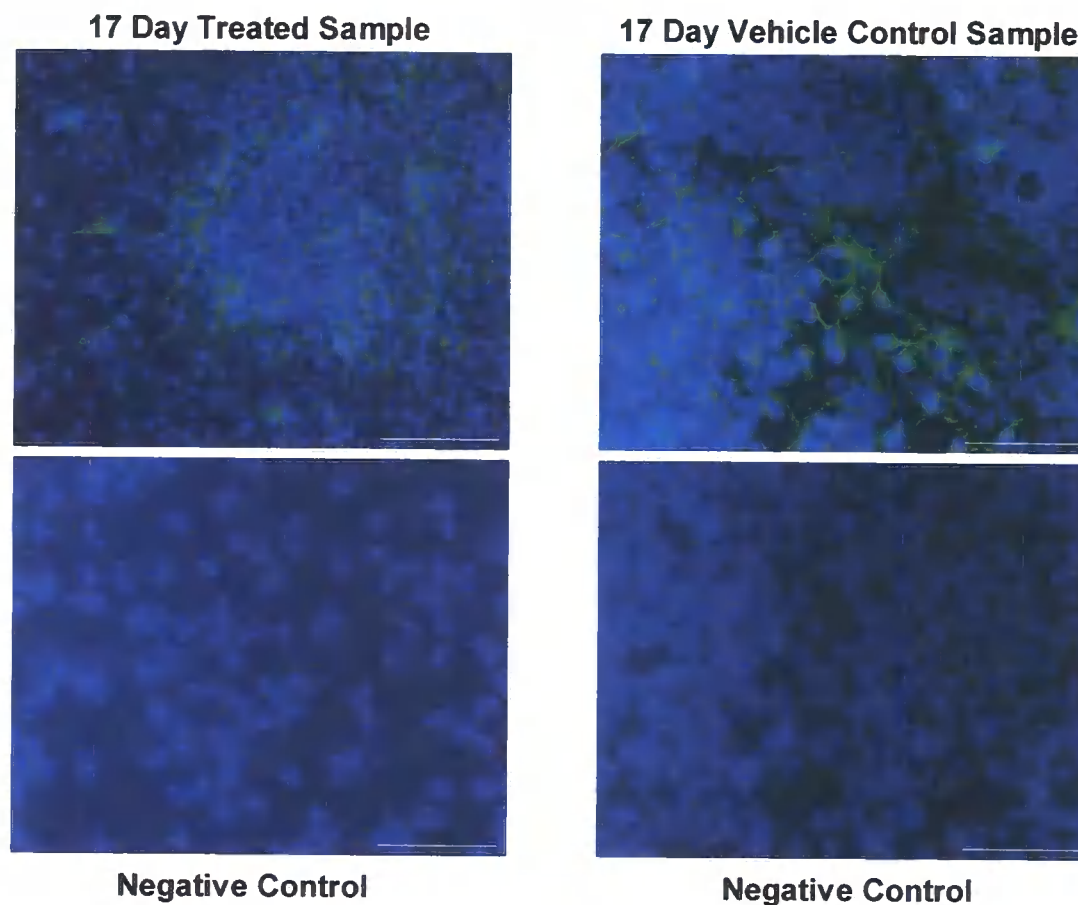


Figure 5.8: Beta III tubulin immunostaining of Tera2.cl.SP12 cells cultured for 7 days in suspension with 10 μ M retinoic acid and 2ng/ml Wnt1 prior to seeding out into 24 well plates where they were exposed to 200ng/ml Shh, 100ng/ml FGF8 & 10 μ M R.A. for 10 days with 200 μ M A.A. also being added to this cocktail for the final 6 days of differentiation. Vehicle controls were treated identically for the first 7 days but upon seeding out received only the vehicle solutions that the Shh/FGF8 and for the final 6 days A.A. were prepared in. Images were merged in Adobe Photoshop, Hoescht stained nuclei are blue, Beta III tubulin positive cells are represented by green (FITC) detection. Neuronal type processes and possible cell bodies are seen and as such this staining is viewed as a positive indicator that potentially neuronal cells have been produced. Due to the nature of the cultures it was very difficult to discern individual cells for cell counting but both cultures appeared clearly Beta III tubulin immunopositive, some of this clarity was lost upon merging the images due to the strong blue background. Scale bars represent 100 μ m. One representative image of n=3 biological repeats is shown.

Real time PCR was subsequently carried out to try and gain a relative estimate of how neuronal the cultures were based on the expression of the marker Beta III tubulin. Samples were taken at both 11 days and 17 days to attempt to gauge whether there was any pattern of neuronal induction. A 3 week retinoic acid treated adherent culture sample was also included as a comparative in an effort to relate this study to earlier work. First of all Figure 5.9 indicates that the treated samples vary from the vehicle controls suggesting the application of this cocktail of molecules has an effect. There is in addition a clear induction in Beta III tubulin expression from the untreated embryonal carcinoma cells to the 11 day and subsequently the 17 day samples. It is interesting that the Wnt1 plus retinoic acid pre treated samples at 11 days show on average almost double the expression of their retinoic acid only pre treated counterparts. This is in support of the concept that Wnt1 may create a permissive environment for Sonic hedgehog and Fibroblast growth factor 8 to act as inductive cues that drive the formation of dopaminergic neurons. The effect is still present but to a lesser degree after 17 days. The 3 week retinoic acid treated sample does not differ to a statistically significant level from the embryonal carcinoma cell sample, however the 17 day (retinoic acid plus Wnt1) sample does. This sample though does not differ to a statistically relevant level from the 3 week retinoic acid treated sample. The Sig. value of 0.062 is quite close to reaching a statistically significant value of ≤ 0.05 and given the variability which is undoubtedly seen when using embryonal carcinoma cell cultures, could be taken as a positive indicator, as often one passage of the three used is out of line with the other two, making it more difficult to observe a statistical effect even though the data are indicative of a strong trend, in this case towards a much more neuronal culture. This result is as such a promising start; a panel of dopaminergic markers were then screened to investigate if they displayed a similar expression pattern. Both phenotypic markers and factors that could be involved in specifying a dopaminergic fate were investigated.

In stark contrast to Beta III tubulin expression, Figure 5.10 demonstrates that tyrosine hydroxylase mRNA levels are down regulated by 17 days to a point below the basal expression in the starting material. The 11 and 17 day (retinoic acid) samples show slightly higher expression than their retinoic acid plus Wnt1 pre treated counterparts at the equivalent time points, with the effect being less apparent at 17 days. This is essentially almost the opposite of the trend seen for Beta III tubulin, posing the

question of how are these molecules that normally drive dopaminergic differentiation having a seemingly negative effect? One possible answer is that the presence of retinoic acid may be inhibitory to their function. This was tested subsequently. Another alternative is that the presence of retinoic acid is promoting acquisition of an alternative neurotransmitter phenotype. It may also be that the expected peak in tyrosine hydroxylase expression has passed and that a lower level of mRNA expression is not necessarily a negative indicator.

Figure 5.11 shows that for dopa decarboxylase there is an analogous expression pattern to that for tyrosine hydroxylase, although in both cases there is no statistically significant difference between the embryonal carcinoma cells starting material, 17 day ((retinoic acid plus Wnt1) most neuronal) sample and the 3 week retinoic acid treated comparative. However as is seen for dopamine receptor 1 in Figure 5.12 the embryonal carcinoma and 17 day (retinoic acid plus Wnt1) samples differ significantly from the 3 week comparative, supporting the trend that expression of a range of markers of a dopaminergic phenotype is relatively low in the 17 day (retinoic acid plus Wnt1) treated samples, despite them appearing highly neuronal in nature (assessed by the expression of Beta III tubulin). Figure 5.13 indicates that the trend is comparable for dopamine receptor 2, but that there is little difference between the mRNA levels observed at 11 and 17 days.

Figure 5.9: Beta III Tubulin mRNA expression in Tera2.cl.SP12 cultures treated with a combination of Shh, FGF8, R.A. & A.A.

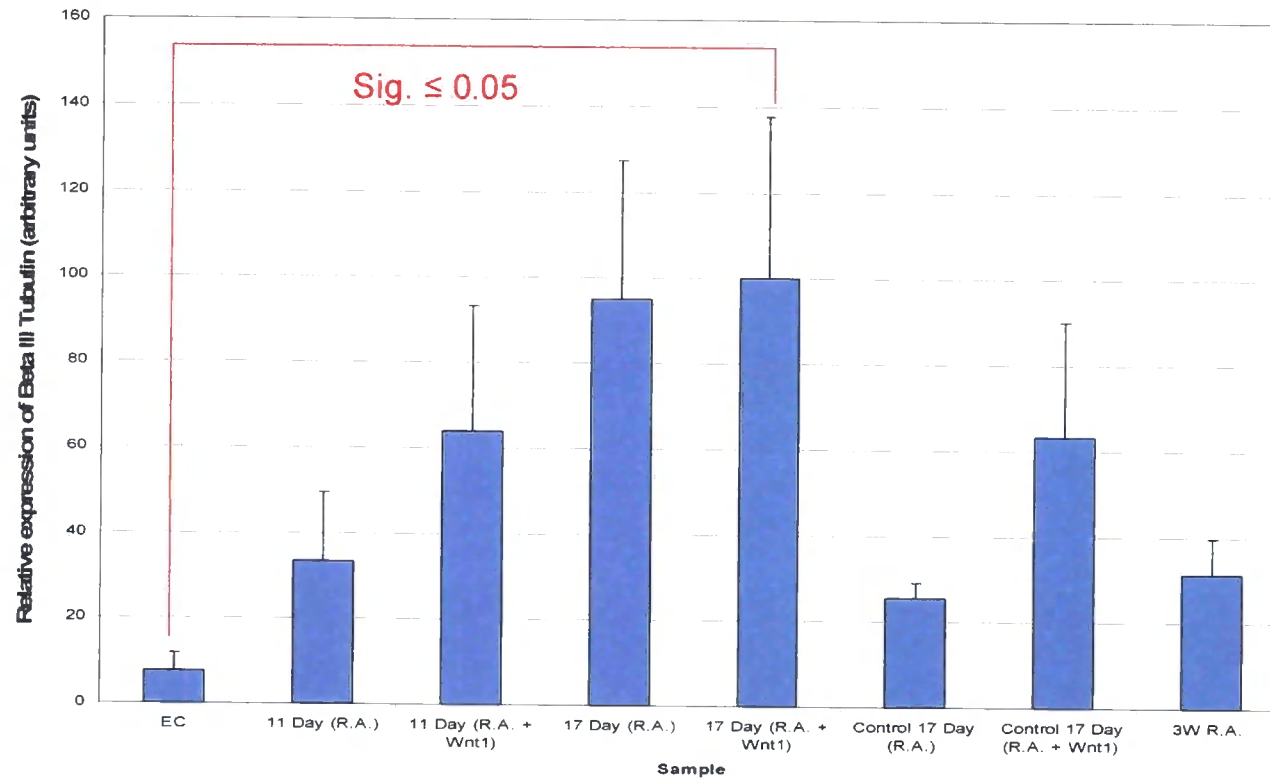


Figure 5.9: Relative expression of Beta III Tubulin mRNA in Tera2.cl.SP12 cells grown as in Figure 5.1; treated samples were collected at 11 and 17 days, controls at just the latter time point. The EC cell sample reflects the undifferentiated Tera2.cl.SP12 cells and the 3W R.A. sample reflects such cells following 3 weeks of retinoic acid induced differentiation in adherent conditions. This 3W R.A. set of samples was included for comparison purposes to try and help relate this work to previous findings. The one way anova output indicates that there is a significant difference between the EC and 17 Day (R.A. + Wnt1) samples. Error bars = S.D. of mean. In all experiments n=3.

Figure 5.10: TH mRNA expression in Tera2.cl.SP12 cultures treated with a combination of Shh, FGF8, R.A. & A.A.

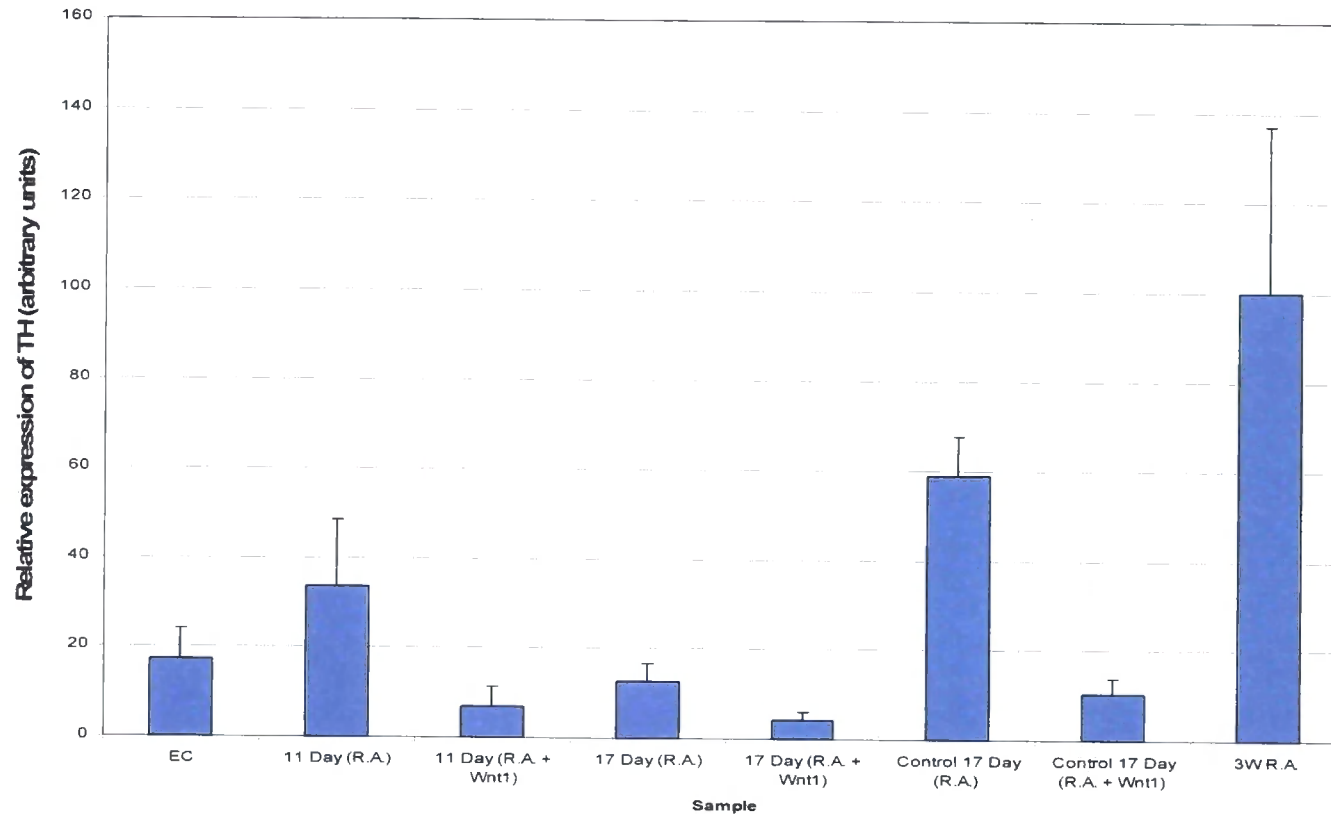


Figure 5.10: Relative expression of TH mRNA in Tera2.cl.SP12 cells grown as in Figure 5.1; treated samples were collected at 11 and 17 days, controls at just the latter time point. The EC cell sample reflects the undifferentiated Tera2.cl.SP12 cells and the 3W R.A. sample reflects such cells following 3 weeks of retinoic acid induced differentiation in adherent conditions. This 3W R.A. set of samples was included for comparison purposes to try and help relate this work to previous findings. The one way anova output indicates that there are no significant differences between the EC, 3W R.A. and 17 Day (R.A. + Wnt1) samples. Error bars = S.D. of mean. In all experiments n=3.

Figure 5.11: AADC mRNA expression in Tera2.cl.SP12 cultures treated with a combination of Shh, FGF8, R.A. & A.A.

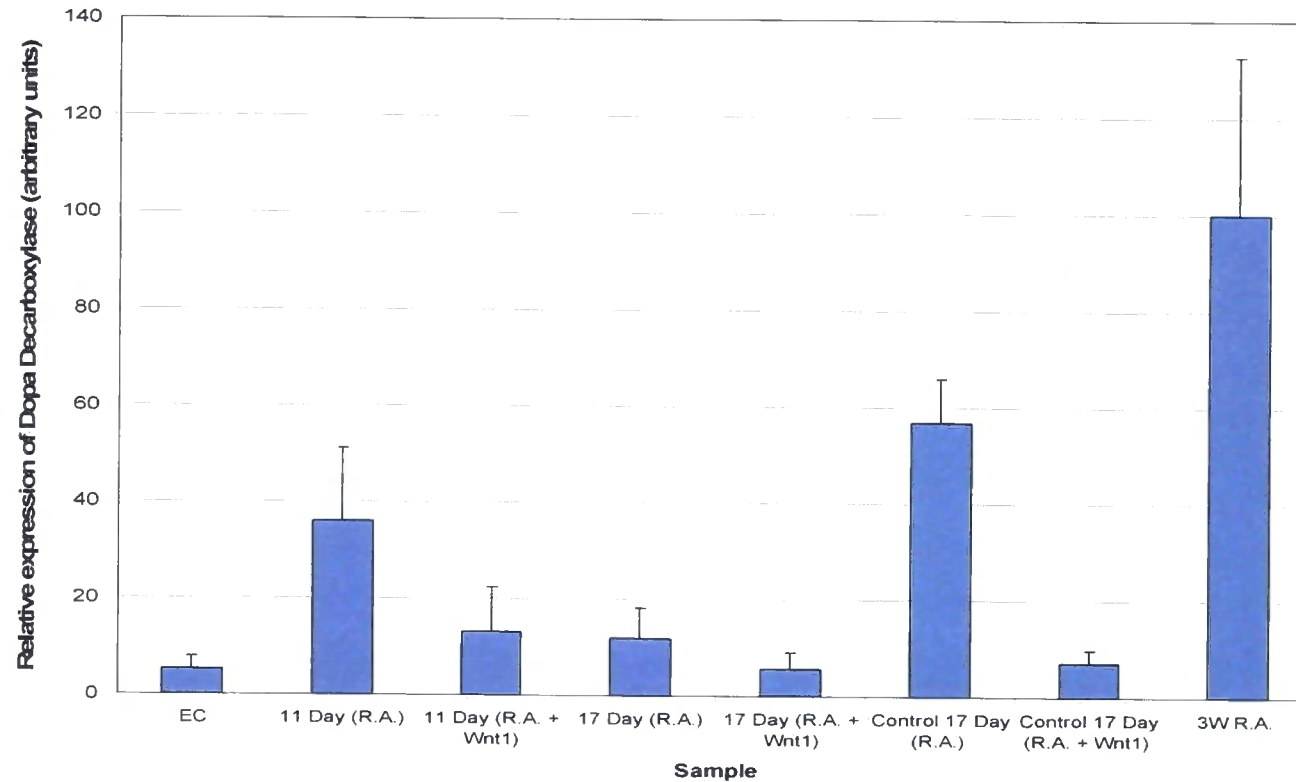


Figure 5.11: Relative expression of Dopa decarboxylase mRNA in Tera2.cl.SP12 cells grown as in Figure 5.1; treated samples were collected at 11 and 17 days, controls at just the latter time point. The EC cell sample reflects the undifferentiated Tera2.cl.SP12 cells and the 3W R.A. sample reflects such cells following 3 weeks of retinoic acid induced differentiation in adherent conditions. This 3W R.A. set of samples was included for comparison purposes to try and help relate this work to previous findings. The one way anova output indicates that there are no significant differences between the EC, 3W R.A. and 17 Day (R.A. + Wnt1) samples. Error bars = S.D. of mean. In all experiments $n=3$.

Figure 5.12: D1 mRNA expression in Tera2.cl.SP12 cultures treated with a combination of Shh, FGF8, R.A. & A.A.

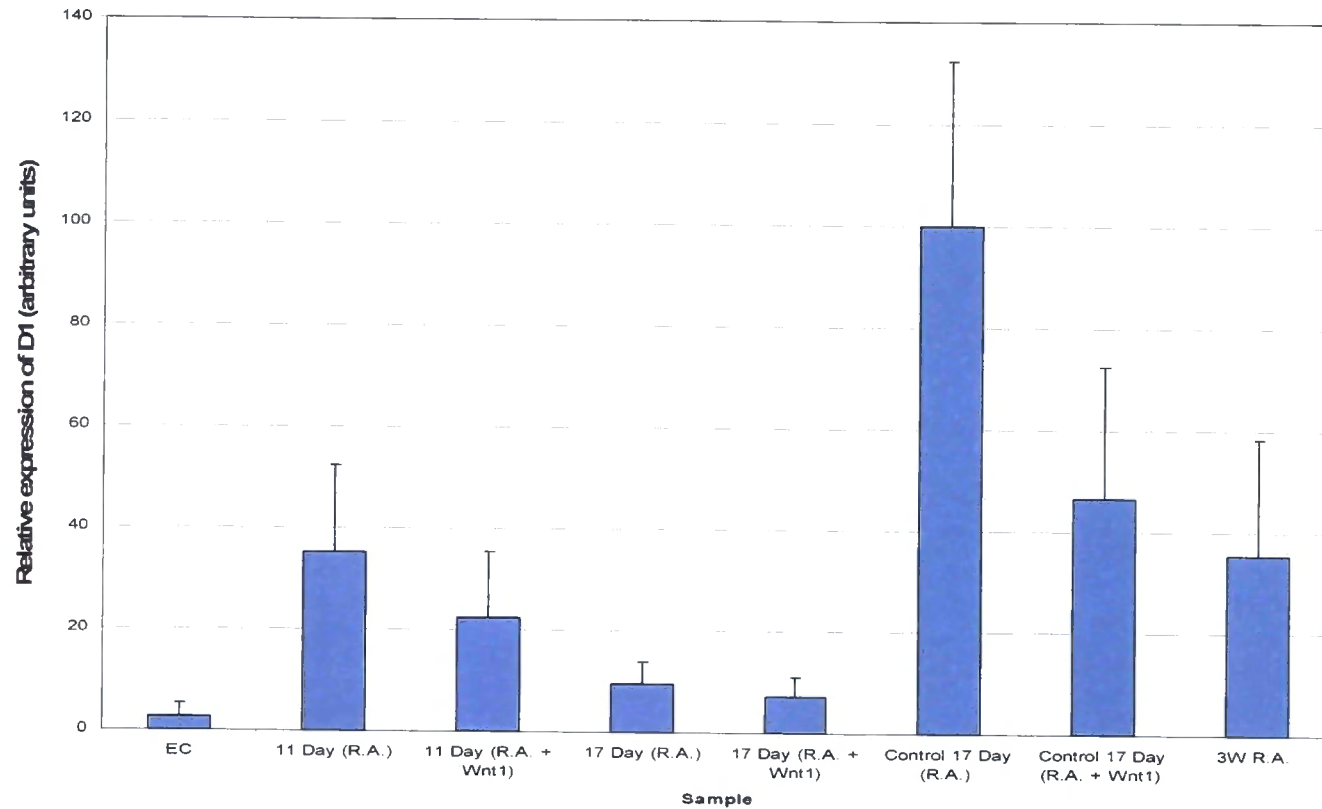


Figure 5.12: Relative expression of D1 mRNA in Tera2.cl.SP12 cells grown as in Figure 5.1; treated samples were collected at 11 and 17 days, controls at just the latter time point. The EC cell sample reflects the undifferentiated Tera2.cl.SP12 cells and the 3W R.A. sample reflects such cells following 3 weeks of retinoic acid induced differentiation in adherent conditions. This 3W R.A. set of samples was included for comparison purposes to try and help relate this work to previous findings. The one way anova output indicates that the 3W R.A. samples vary to a statistically significant level from both the EC and 17 Day (R.A. + Wnt1) samples (not shown on graph). Error bars = S.D. of mean. In all experiments n=3.

Figure 5.13: D2 mRNA expression in Tera2.cl.SP12 cultures treated with a combination of Shh, FGF8, R.A. & A.A.

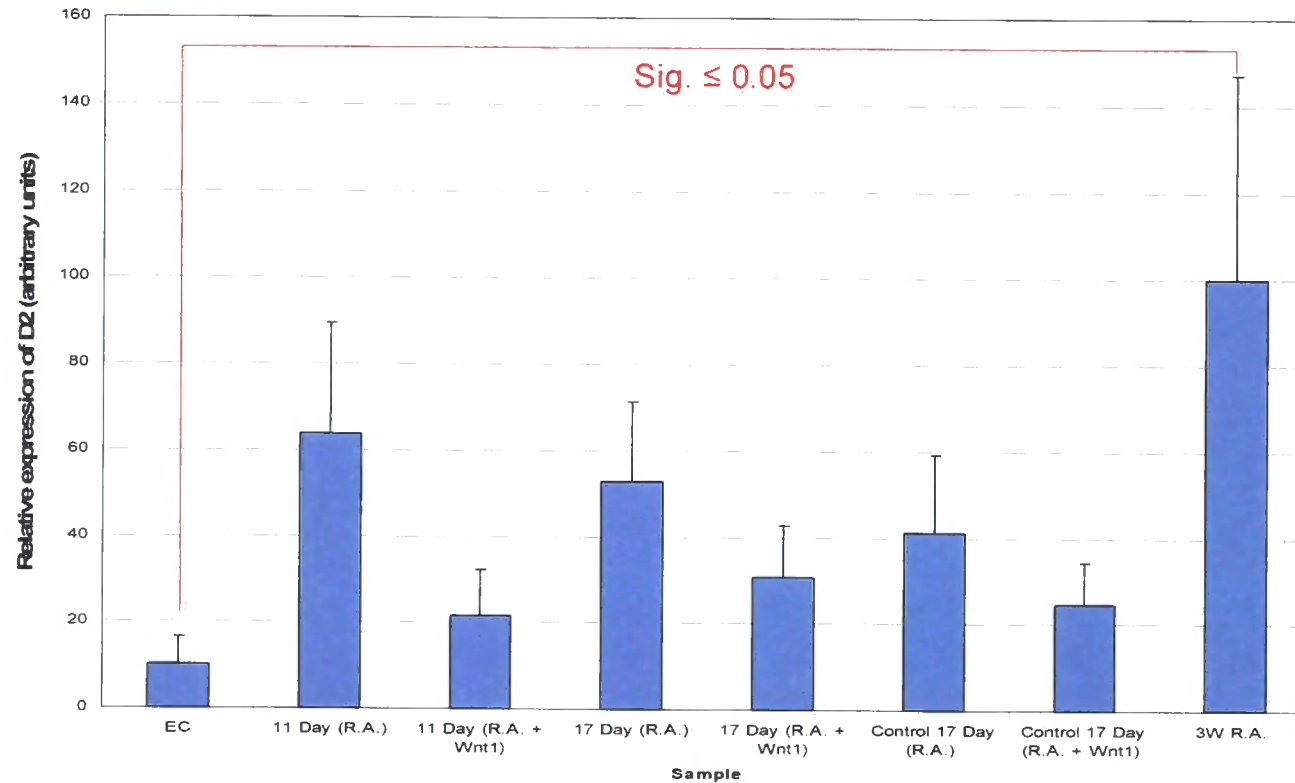


Figure 5.13: Relative expression of D2 mRNA in Tera2.cl.SP12 cells grown as in Figure 5.1; treated samples were collected at 11 and 17 days, controls at just the latter time point. The EC cell sample reflects the undifferentiated Tera2.cl.SP12 cells and the 3W R.A. sample reflects such cells following 3 weeks of retinoic acid induced differentiation in adherent conditions. This 3W R.A. set of samples was included for comparison purposes to try and help relate this work to previous findings. The one way anova output highlights the significant difference between the EC and 3W R.A. samples. Error bars = S.D. of mean. In all experiments n=3.

When factors that may be involved in specifying a dopaminergic fate as per Figure 4.2 were studied, at first as seen in Figure 5.14, *Lmx1a* within the context of the experimental error displayed a similar expression pattern to that observed for the markers of a dopaminergic phenotype. The 17 day (retinoic acid plus *Wnt1*) and embryonal carcinoma cell starting material differed to a statistically significant degree ($p \leq 0.05$) from the 3 week comparative supporting the notion that the expression of mRNA for the dopaminergic markers tested was low at this time point under the treatment conditions used.

However Figure 5.15 shows that for *Msx1* the downstream target of *Lmx1a* the expression profile is somewhat different. In this instance the 3 week retinoic acid treated comparative and 17 day (retinoic acid plus *Wnt1*) sample hardly differ at all, but both although not to a statistically significant level display a clear induction above the levels observed in the Tera2.cl.SP12 starting material. In fact *Msx1* expression is raised from 11 to 17 days particularly in the 17 day (retinoic acid plus *Wnt1*) samples which appear the most neuronal. It is interesting that the vehicle control samples display a particularly high level of *Msx1* expression in a similar fashion to some other markers in these conditions such as *Lmx1a* and Beta III tubulin. This may suggest that given a pre treatment with retinoic acid alone or in combination with *Wnt1*, that Tera2.cl.SP12 cultures are capable of controlled up regulation of dopaminergic and neuronal markers, or they may show a continued response to the earlier treatment with such factors. However even though they demonstrate this seemingly favourable ability to up regulate indicators of a dopaminergic or neuronal phenotype which is promising, it would need to be harnessed in a more controlled manner to potentially derive the greatest possible benefit from it. That is if such high levels of such markers are required at all, the process may be more subtle with lower levels being adequate to exert more delicate effects. There may even be an interaction between certain key dopaminergic inducing factors and metabolites such as dopamine itself; that make the process more intricate and mean that merely observing elevated levels of dopaminergic markers is not sufficient to produce the desired outcome of an enriched population of functional dopaminergic neurons.

There is though seemingly in the test conditions a difference in the regulation of *Msx1* mRNA levels. *Msx1* is postulated to act positively on neurogenin2 and to suppress

Nkx 6-1. Figure 5.16 shows that although neurogenin2 expression is not as great in 17 day (retinoic acid plus Wnt1) samples as it is in the 3 week retinoic acid treated comparatives, it does rise in its expression from 11 to 17 days when retinoic acid and Wnt1 are used in the initial 7 days. When just retinoic acid is used in this preliminary stage there is no real difference between the 11 and 17 day samples. This raises the possibility that Msx1 is at least in the Wnt1 plus retinoic acid pre treated samples having an effect on the up regulation of neurogenin2 from 11 to 17 days, although much more thorough investigation would be needed to make any firm conclusion as to the viability of this fledgling idea. Of interest though as can be seen in Figure 5.17 is that Nkx 6-1 levels drop off from 11 to 17 days, with the lowest expression being in the 17 day (retinoic acid plus Wnt1) samples, where Msx1 levels are relatively high. This raises the possibility that Msx1 may as expected be exerting an inhibitory effect on Nkx 6-1 expression. Nkx 6-1 is induced to a statistically significant level in the 3 week retinoic acid treated comparative samples in relation to the embryonal carcinoma stem cell starting material. Msx1 although not to a level of statistical relevance is induced in such samples in a comparable manner. Therefore the statistically significant difference in expression between 3 week retinoic acid treated and 17 day (retinoic acid plus Wnt1) samples is interesting not only in that the 17 day (retinoic acid plus Wnt1) samples express Nkx 6-1 more weakly than in the starting material, suggesting something within the system under investigation is having an effect, but this is seen when a known suppressor of Nkx 6-1, namely Msx1 is in relative terms showing an induction in its levels. Again this possible correlation would require further investigation but it does appear that components of the pathway described in Figure 4.2 thought to be influenced by Sonic hedgehog are seemingly regulated in a predictable fashion in its presence.

Figure 5.14: Lmx1a mRNA expression in Tera2.cl.SP12 cultures treated with a combination of Shh, FGF8, R.A. & A.A.

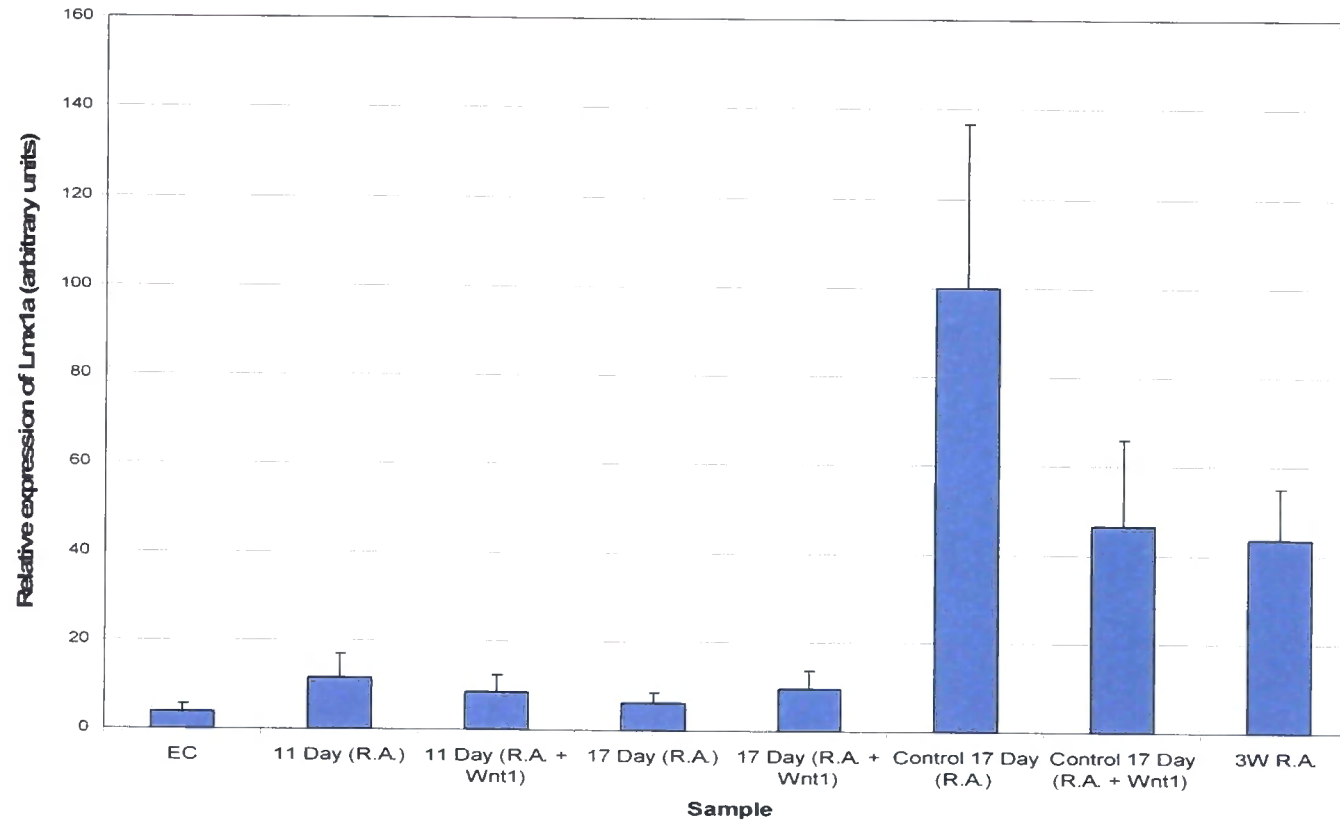


Figure 5.14: Relative expression of Lmx1a mRNA in Tera2.cl.SP12 cells grown as in Figure 5.1; treated samples were collected at 11 and 17 days, controls at just the latter time point. The EC cell sample reflects the undifferentiated Tera2.cl.SP12 cells and the 3W R.A. sample reflects such cells following 3 weeks of retinoic acid induced differentiation in adherent conditions. This 3W R.A. set of samples was included for comparison purposes to try and help relate this work to previous findings. The one way anova output indicates that the 3W R.A. samples vary to a statistically significant level from both the EC and 17 Day (R.A. + Wnt1) samples (not shown on graph). Error bars = S.D. of mean. In all experiments n=3.

Figure 5.15: Msx1 mRNA expression in Tera2.cl.SP12 cultures treated with a combination of Shh, FGF8, R.A. & A.A.

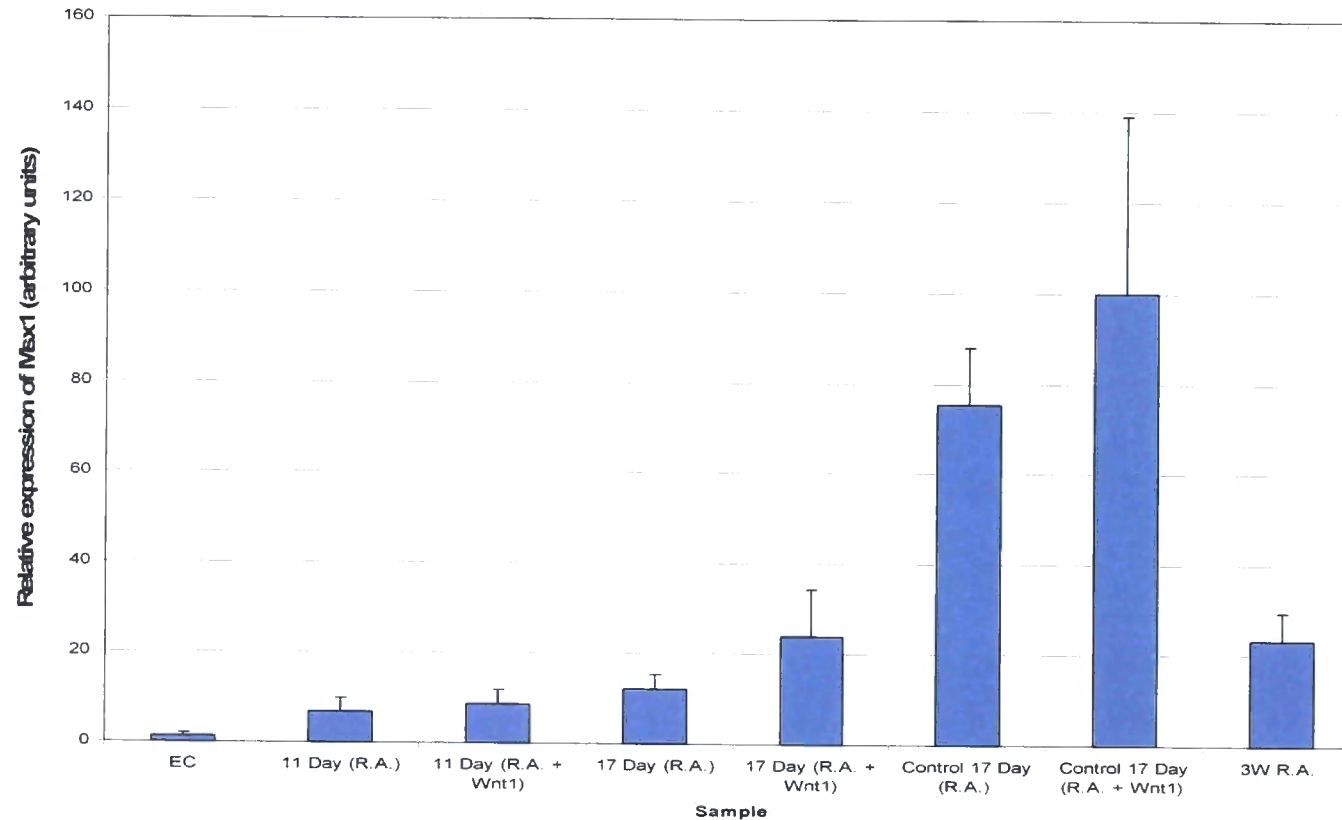


Figure 5.15: Relative expression of Msx1 mRNA in Tera2.cl.SP12 cells grown as in Figure 5.1; treated samples were collected at 11 and 17 days, controls at just the latter time point. The EC cell sample reflects the undifferentiated Tera2.cl.SP12 cells and the 3W R.A. sample reflects such cells following 3 weeks of retinoic acid induced differentiation in adherent conditions. This 3W R.A. set of samples was included for comparison purposes to try and help relate this work to previous findings. The one way anova output indicates that there are no significant differences between the EC, 3W R.A. and 17 Day (R.A. + Wnt1) samples. Error bars = S.D. of mean. In all experiments n=3.

Figure 5.16: Ngn2 mRNA expression in Tera2.cl.SP12 cultures treated with a combination of Shh, FGF8, R.A. & A.A.

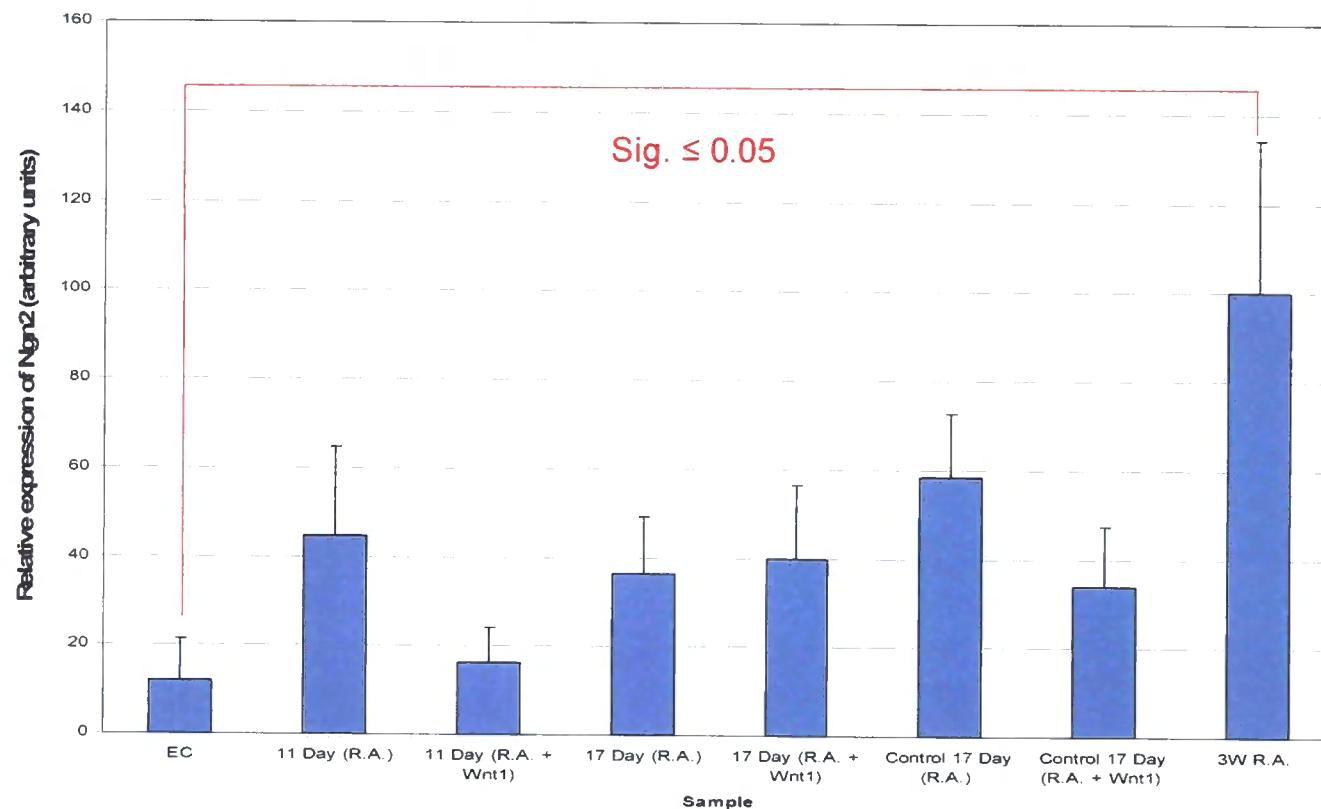


Figure 5.16: Relative expression of Ngn2 mRNA in Tera2.cl.SP12 cells grown as in Figure 5.1; treated samples were collected at 11 and 17 days, controls at just the latter time point. The EC cell sample reflects the undifferentiated Tera2.cl.SP12 cells and the 3W R.A. sample reflects such cells following 3 weeks of retinoic acid induced differentiation in adherent conditions. This 3W R.A. set of samples was included for comparison purposes to try and help relate this work to previous findings. The one way anova output highlights the significant difference between the EC and 3W R.A. samples. Error bars = S.D. of mean. In all experiments n=3.

Figure 5.17: Nkx 6-1 mRNA expression in Tera2.cl.SP12 cultures treated with a combination of Shh, FGF8, R.A. & A.A.

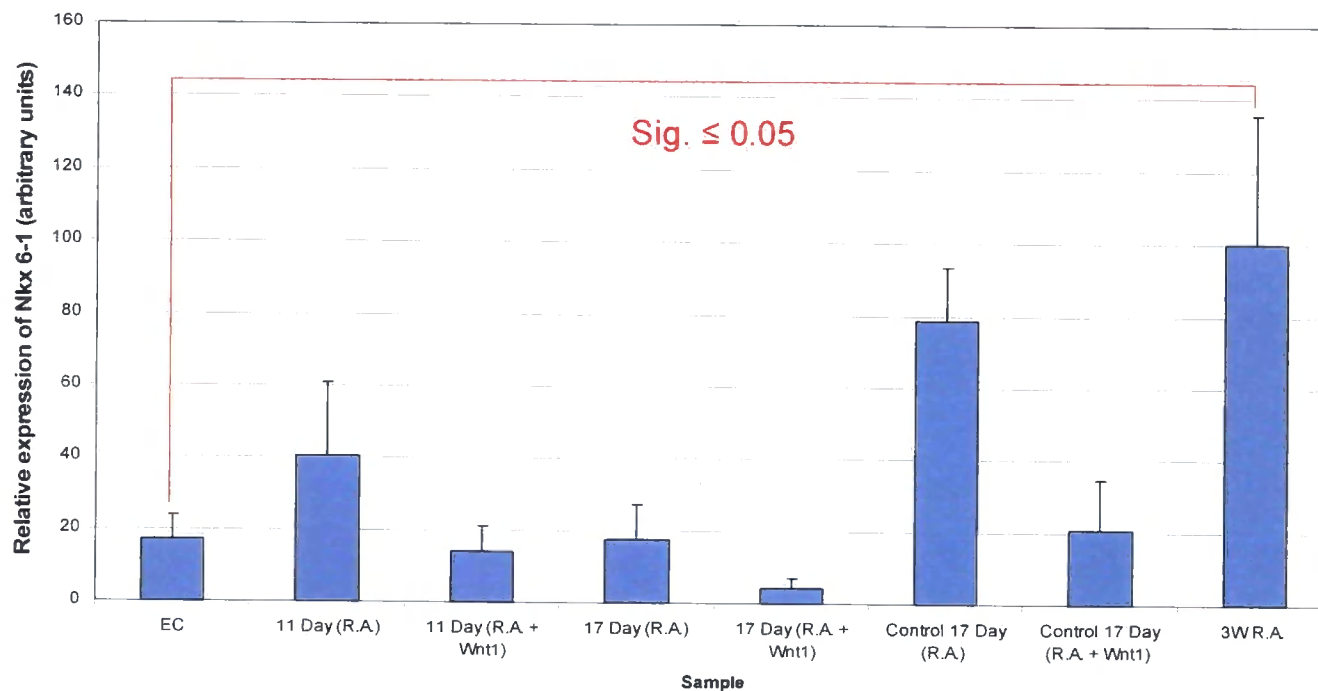


Figure 5.17: Relative expression of Nkx 6-1 mRNA in Tera2.cl.SP12 cells grown as in Figure 5.1; treated samples were collected at 11 and 17 days, controls at just the latter time point. The EC cell sample reflects the undifferentiated Tera2.cl.SP12 cells and the 3W R.A. sample reflects such cells following 3 weeks of retinoic acid induced differentiation in adherent conditions. This 3W R.A. set of samples was included for comparison purposes to try and help relate this work to previous findings. The one way anova output indicates that the 3W R.A. samples vary to a statistically significant level from both the EC and 17 Day (R.A. + Wnt1) samples (the EC to 3W R.A. difference is indicated on the graph, the other difference is not as it is probably of lesser relevance). Error bars = S.D. of mean. In all experiments n=3.

The question though still remains as to why the phenotypic markers appear to be relatively down regulated, especially given the suggestion that the pathway for dopaminergic specification thought to be influenced by Sonic hedgehog may well be active in the presence of this molecule within the context of this experimental strategy. One possible reason for this is that retinoic acid was included in the final 10 days of the differentiation protocol; feasibly this may have an inhibitory effect on dopaminergic differentiation or promote the acquisition of other neurotransmitter phenotypes. Therefore to address if this is likely to be the case retinoic acid was omitted for the final differentiation stages so as to replicate this part of the method of Cho et al., 2008. The cells were grown as per the scheme in Figure 5.2 with the most neuronal (as assessed by real time PCR) condition being selected for the initial stage. This was the 7 day retinoic acid plus Wnt1 suspension culture pre treatment followed by the 10 day protocol of Cho et al., 2008 termed the minus condition, which was then compared to the plus retinoic acid condition (that had previously been identified as the most neuronal) as defined in Figure 5.2. The comparison also included samples from the embryonal carcinoma cell starting material, the 3 week retinoic acid treated comparative and a 17 day vehicle control, as well as a MG63 sample that was not expected to show any dopaminergic or neuronal marker expression and as such this would act as a control to check in a non neural cell type that there was not a background expression of such markers.

Figure 5.18 shows that for dopa decarboxylase and dopamine receptor 2 there is hardly any difference between the plus and minus retinoic acid conditions. The trends are similar to those found previously for the other samples too, suggesting some consistency in the results obtained. A similar scenario is found for tyrosine hydroxylase and Beta III tubulin (Figure 5.19), there does appear to be a higher expression of tyrosine hydroxylase in the minus retinoic acid samples but this difference is not statistically significant. Figures 5.20 and 5.21 show the situation is analogous for the components of the pathway that Sonic hedgehog may affect to promote the acquisition of a dopaminergic phenotype.

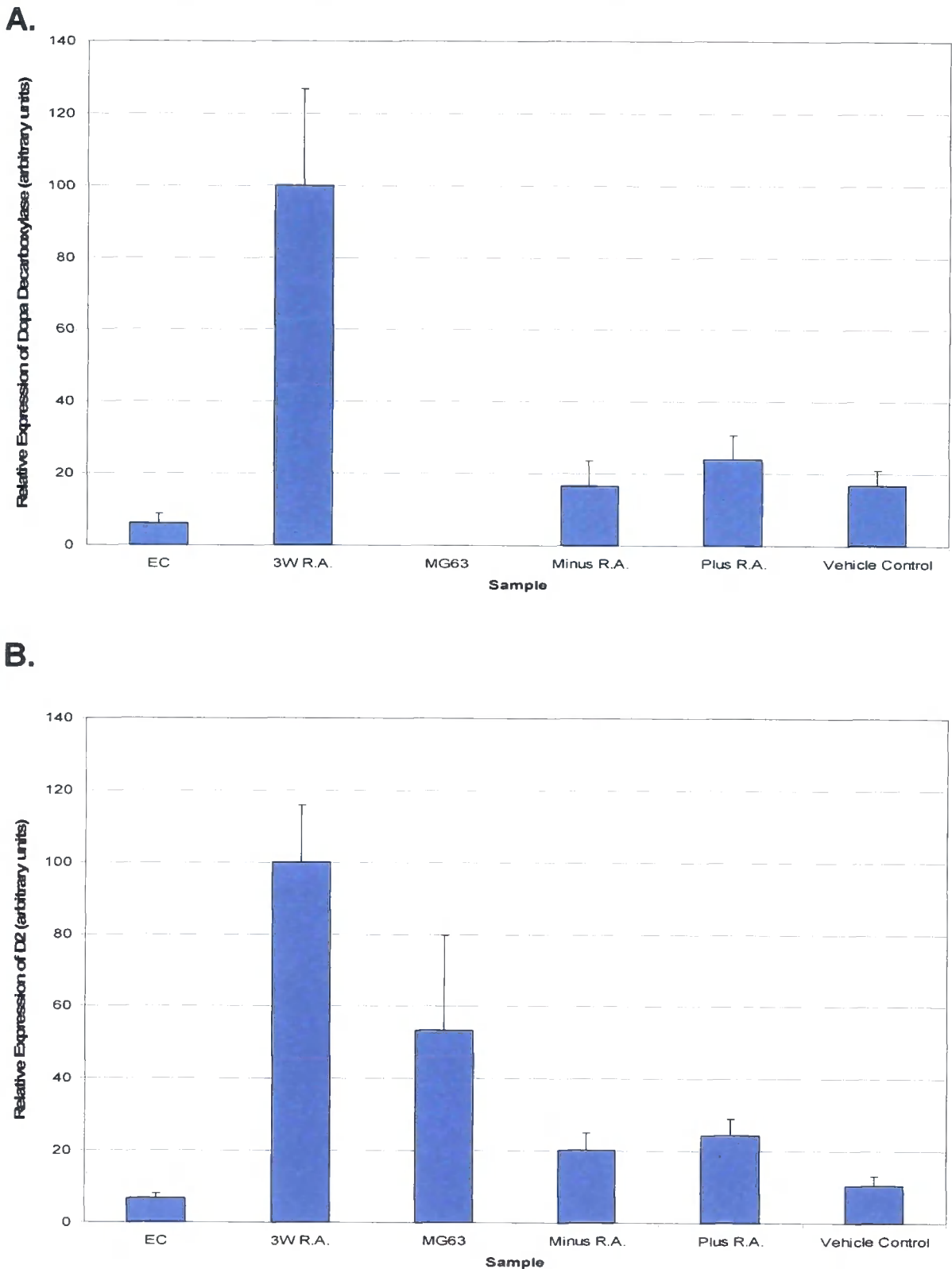
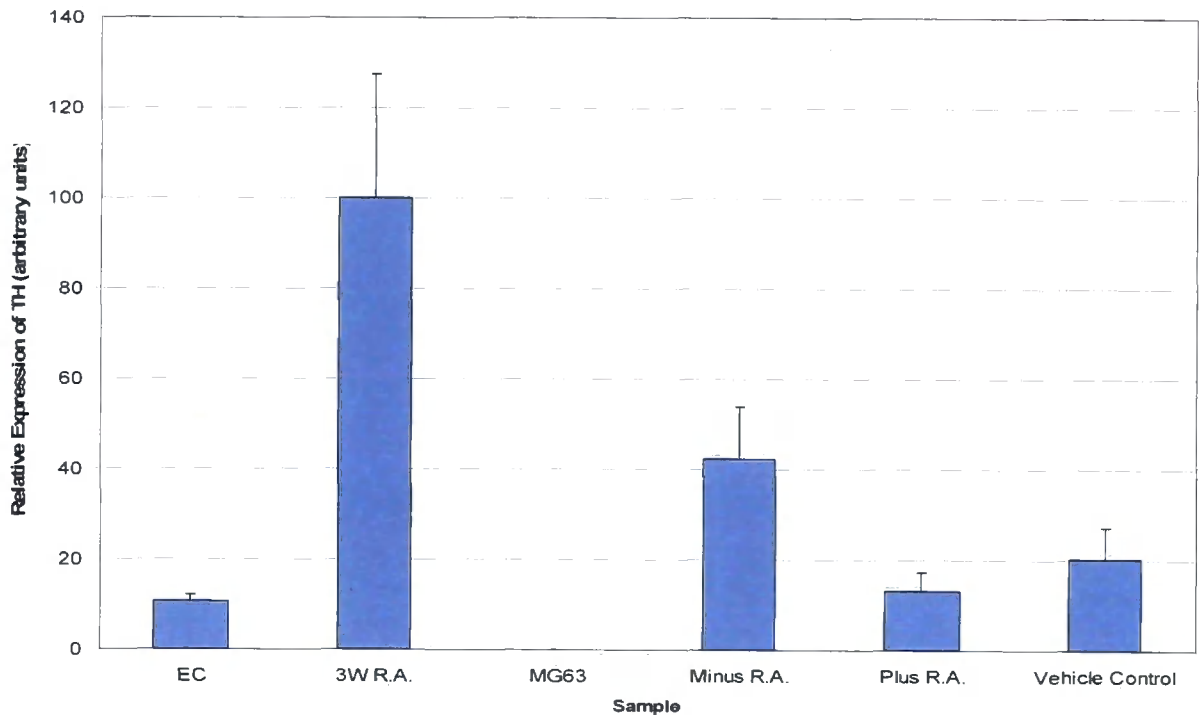
Figure 5.18: AADC & D2 expression in the plus and minus R.A. conditions

Figure 5.18A: Relative expression of Dopa decarboxylase mRNA in Tera2.cl.SP12 cultures treated as per the conditions defined in Figure 5.2, harvested at the terminus of the 17 day differentiation protocol. The EC cell sample reflects the undifferentiated Tera2.cl.SP12 cells and the 3W R.A. sample reflects such cells following 3 weeks of retinoic acid induced differentiation in adherent conditions. The MG63 condition was included as a control to assess if dopaminergic markers were expressed in a non neural cell line. **B:** Relative expression of D2 mRNA in identical conditions to those described in part A. Error bars = S.D. of mean. In all experiments n=3. Student's T-test was used to assess if there was a statistically significant difference between the plus and minus R.A. conditions, there was not in either instance.

Figure 5.19: TH & Beta III tubulin expression in the plus and minus R.A. conditions

A.



B.

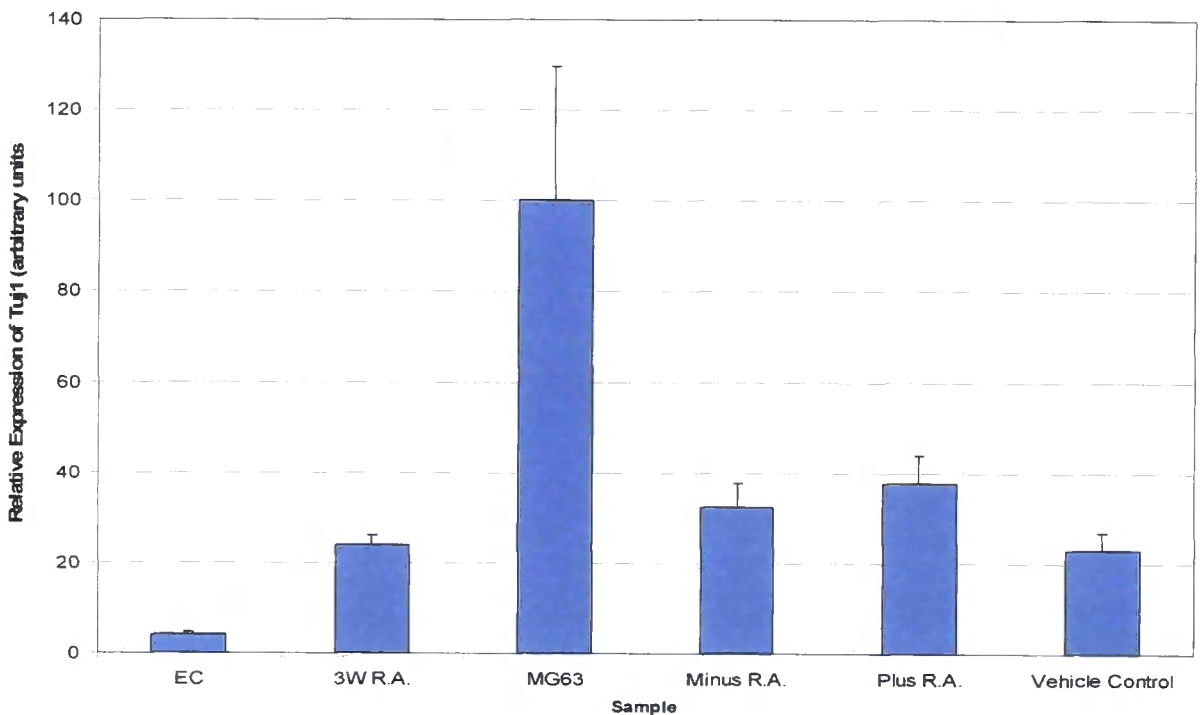


Figure 5.19A: Relative expression of TH mRNA in Tera2.cl.SP12 cultures treated as per the conditions defined in Figure 5.2, harvested at the terminus of the 17 day differentiation protocol. The EC cell sample reflects the undifferentiated Tera2.cl.SP12 cells and the 3W R.A. sample reflects such cells following 3 weeks of retinoic acid induced differentiation in adherent conditions. The MG63 condition was included as a control to assess if dopaminergic/neuronal markers were expressed in a non neural cell line. **B:** Relative expression of Beta III tubulin mRNA in identical conditions to those described in part A. Error bars = S.D. of mean. In all experiments n=3. Student's T-test was used to assess if there was a statistically significant difference between the plus and minus R.A. conditions, there was not in either instance.

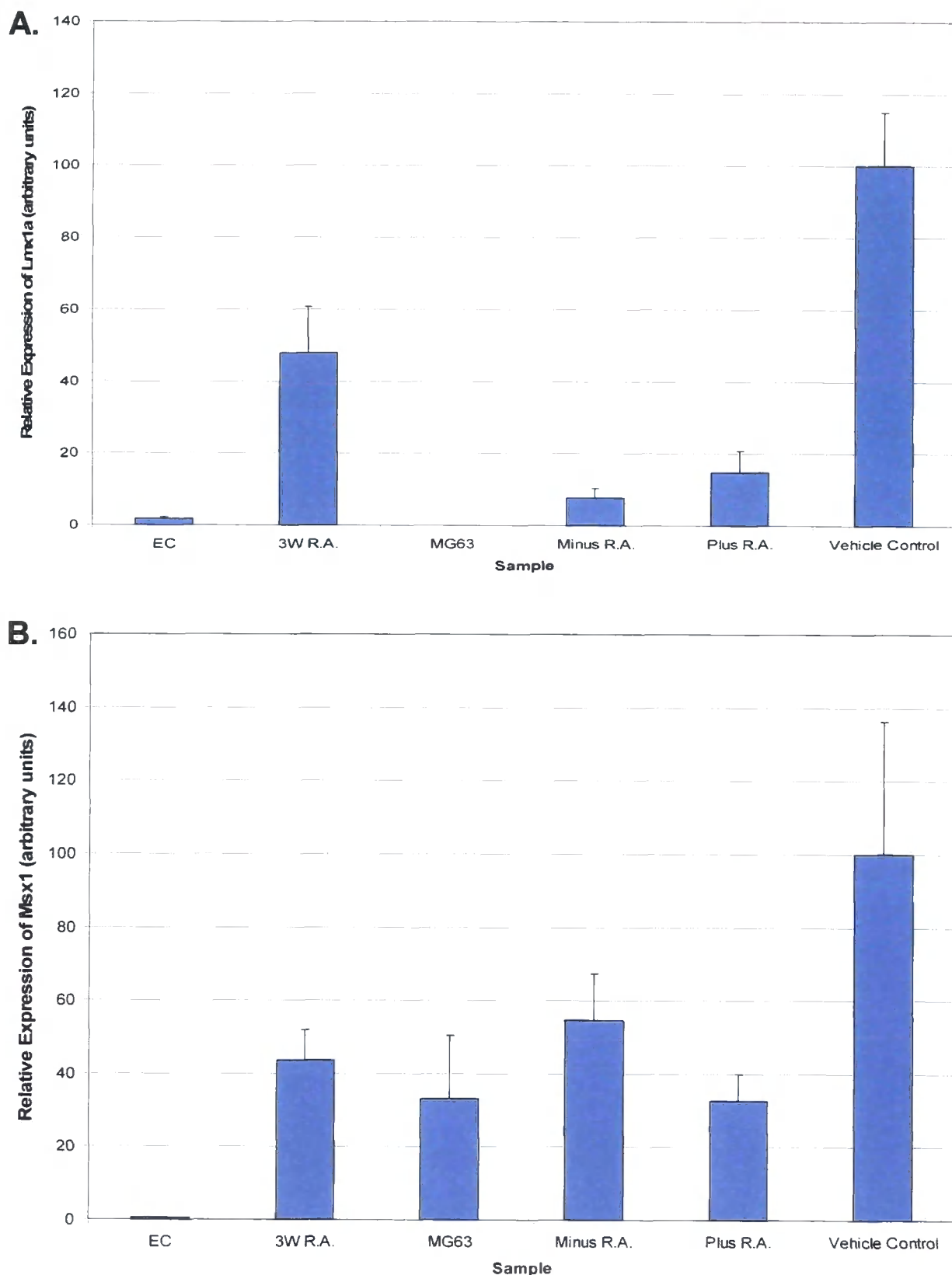
Figure 5.20: Lmx1a & Msx1 expression in the plus and minus R.A. conditions

Figure 5.20A: Relative expression of Lmx1a mRNA in Tera2.cl.SP12 cultures treated as per the conditions defined in Figure 5.2, harvested at the terminus of the 17 day differentiation protocol. The EC cell sample reflects the undifferentiated Tera2.cl.SP12 cells and the 3W R.A. sample reflects such cells following 3 weeks of retinoic acid induced differentiation in adherent conditions. The MG63 condition was included as a control to assess if dopaminergic markers were expressed in a non neural cell line. **B:** Relative expression of Msx1 mRNA in identical conditions to those described in part A. Error bars = S.D. of mean. In all experiments n=3. Student's T-test was used to assess if there was a statistically significant difference between the plus and minus R.A. conditions, there was not in either instance.

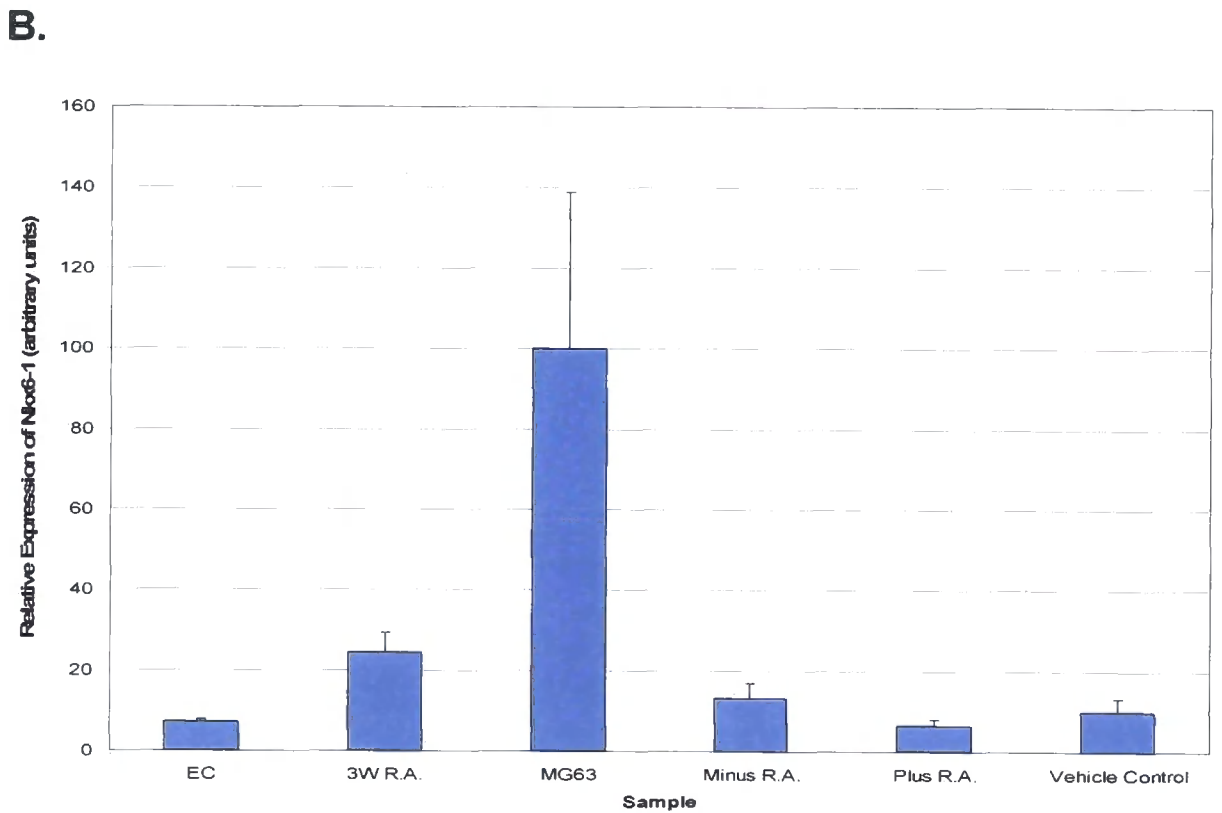
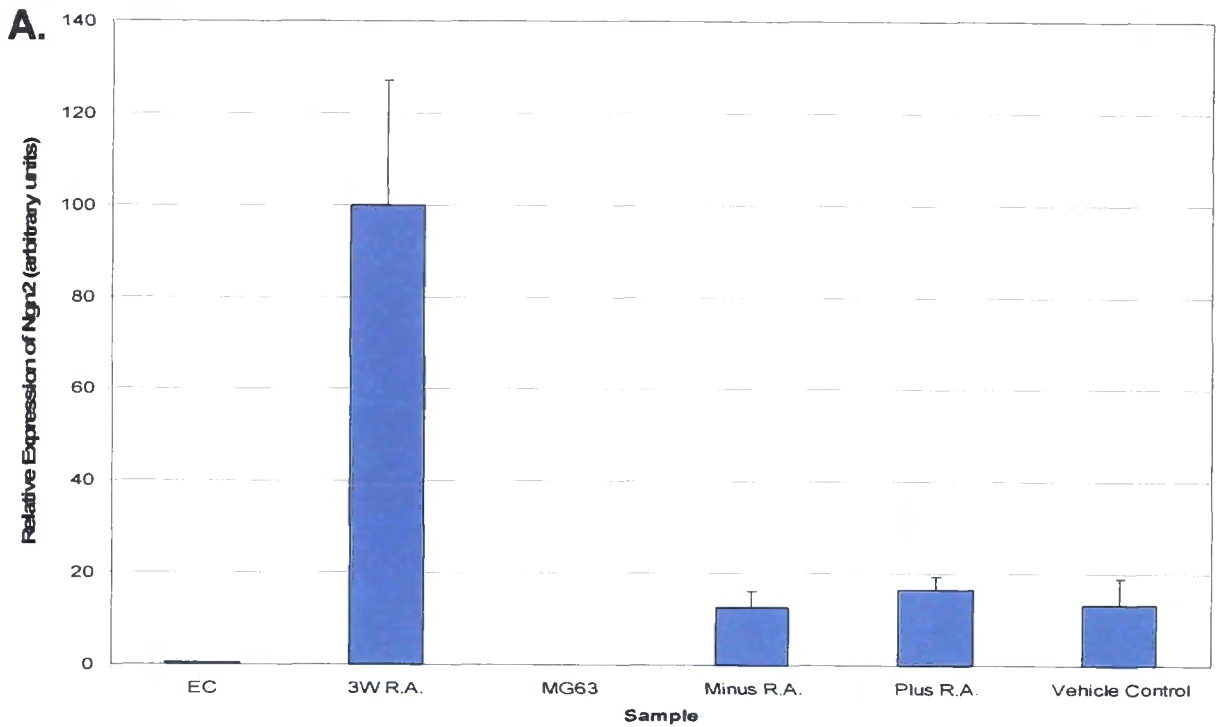
Figure 5.21: Ngn2 & Nkx 6-1 expression in the plus and minus R.A. conditions

Figure 5.21A: Relative expression of Ngn2 mRNA in Tera2.cl.SP12 cultures treated as per the conditions defined in Figure 5.2, harvested at the terminus of the 17 day differentiation protocol. The EC cell sample reflects the undifferentiated Tera2.cl.SP12 cells and the 3W R.A. sample reflects such cells following 3 weeks of retinoic acid induced differentiation in adherent conditions. The MG63 condition was included as a control to assess if dopaminergic markers were expressed in a non neural cell line. **B:** Relative expression of Nkx 6-1 mRNA in identical conditions to those described in part A. Error bars = S.D. of mean. In all experiments n=3. Student's T-test was used to assess if there was a statistically significant difference between the plus and minus R.A. conditions, there was not in either instance.

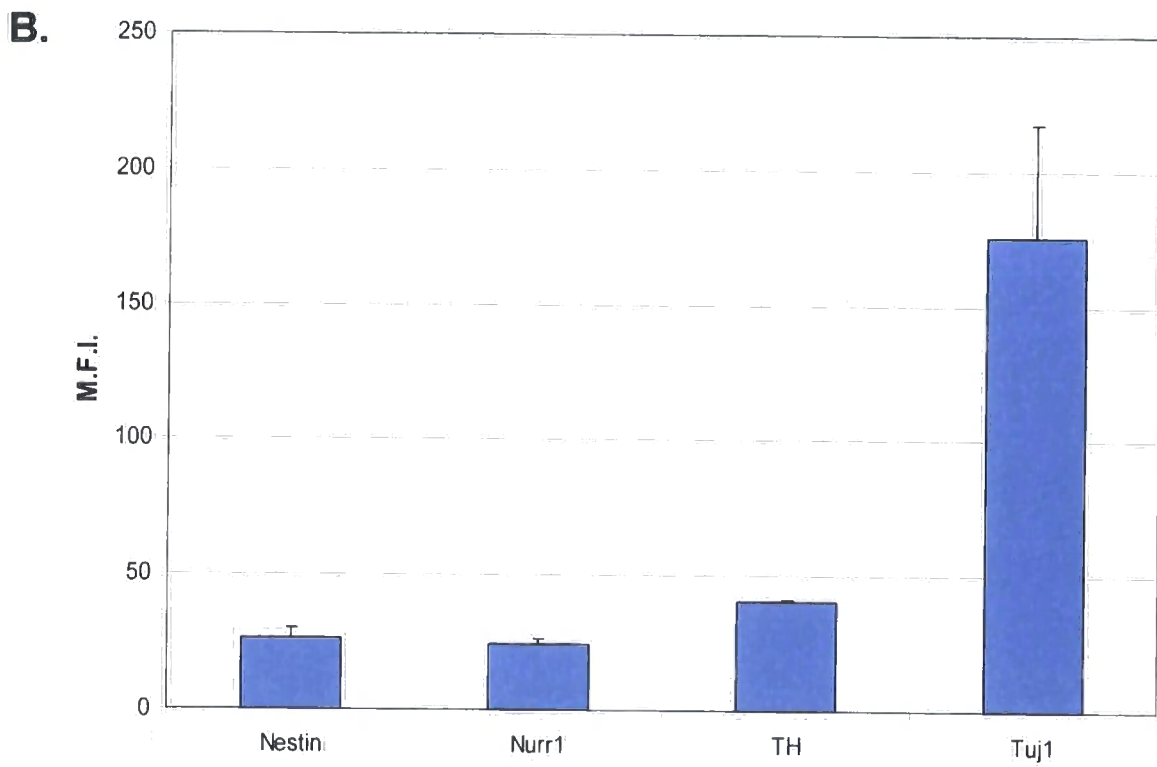
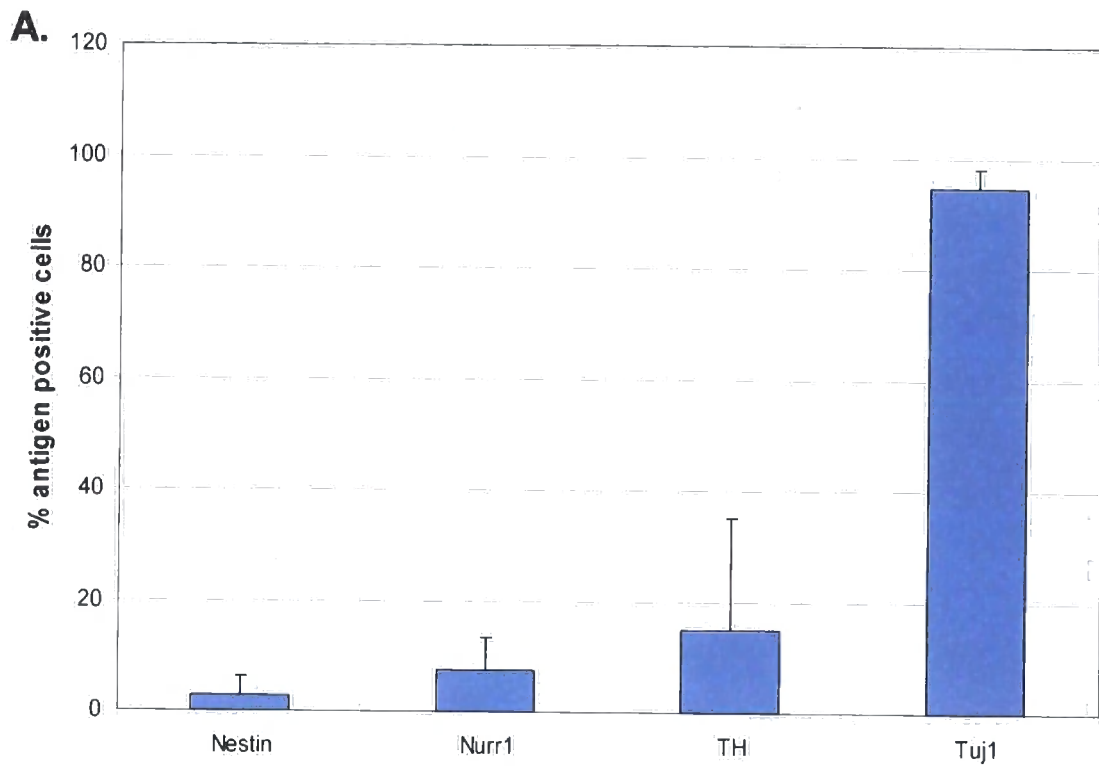
It is also of interest that dopa decarboxylase, tyrosine hydroxylase, Lmx1a and neurogenin2 are not detectable as would be expected in the non neural MG63 samples. However dopamine receptor 2, Beta III tubulin, Msx1 and Nkx 6-1 expression were detected in such samples. Although dopamine receptor 2 and Msx1 are relatively only moderately expressed and as is the case for Beta III tubulin and Nkx6-1 the variability in their expression is quite high. This may be due to the fact that the house keeping gene GAPDH used to normalise the expression data showed a highly different expression pattern in these MG63 samples in relation to their embryonal carcinoma cell derived counterparts. The raw cycle threshold data indicated it took usually around 25-26 cycles for detection of GAPDH in the MG63 cell samples, where as usually only 19-21 cycles were required for the embryonal carcinoma cell based samples. This is indicative of weaker expression of GAPDH in the MG63 cell samples which in turn could make any detectable expression in the test samples (for example Beta III tubulin) appear greater than it really is in relative terms to the expression in embryonal carcinoma cell derived material. Beta-actin the other house keeping gene used in this analysis was also expressed differently in MG63 samples in comparison to embryonal carcinoma cell based samples. The cycle threshold values for beta-actin were around 18-20 for embryonal carcinoma cell based specimens but ranged from 26-29 for MG63 cell samples. This highlights that even when using more than one house keeping gene disparities may arise due to variable expression of both the house keeping genes used, giving support to the concept that as wide a range as possible of house keeping genes should be utilised. It may however be that the data indicate false positives being recorded. For dopamine receptor 2 this is highly unlikely as detection occurs at between 36-39 cycles (out of 40). Such weak detection at so late a stage in the process is likely to only reflect the presence of a few transcripts or even a hint of contamination if the RNase treatment has not removed all the contaminating DNA that may be present. Given this in combination with the differences in house keeping gene expression it is possible but highly unlikely that false positives are being obtained that would have any effect on the data (this argument runs for Msx1 (detected at around 33-36 cycles) and Nkx6-1 (detected around 37-39 cycles)). There is also the possibility that as MG63 cells are cancerous that they may possess a low level aberrant expression of certain markers that would not be expected to be detected.

In the case of Beta III tubulin it would appear that although the house keeping gene issue may be of some relevance, that there is some detection. This may indicate the real time PCR results earlier in this Chapter and in Chapter 4 could be a result of false positives. This may be the case but this argument is not well supported by the immunostaining in this and the previous Chapter, that indicates cells are present that display the morphology expected of a potential neuron in culture. The variability in the level of expression seen also indicates that a broad false positive detection is less likely as more uniformity might be expected amongst the results if this was the case. The neurogenin2 real time PCR data in this Chapter also add support to the notion that there is an up regulation of neuronal marker expression and neurogenin2 was not detectable at all in the MG63 cell samples. It may be given their cancerous origins that MG63 cells express Beta III tubulin even though this may not be predicted. Alternatively the possibility of this seemingly false positive may arise more as a result of Beta III tubulin not being a solely neuronal marker, as a member of the tubulin family of proteins it may be that it is expressed more broadly and that it is not an ideal marker of a neuronal phenotype. In any future work it would be advisable to use a panel of neuronal markers such as microtubule associated protein 2, neurofilament 200 and Beta III tubulin in an analogous manner to the panel of dopaminergic markers tested. Alternatively another cell line expected to be negative for Beta III tubulin expression could be tested and if found to be negative could then be used as a negative control. If another cell line was tested and found to be negative in a predictable manner it would though raise the question of what is and isn't suitable for use as a negative control and also given the MG63 results just how specific a marker is Beta III tubulin? This would indicate the need for a panel of suspected neuronal markers to add validity to any further work.

Flow cytometry was subsequently carried out to try and assess further if the MG63 cell line was negative for the expression of the markers tested using immunological reagents. Figure 5.22 shows that for the neuroprogenitor/neuronal and dopaminergic markers tested in previous chapters, there is little to no expression in MG63 cells. The mean fluorescence intensity values for nestin, Nurr1 and tyrosine hydroxylase are equivalent to those for secondary only controls; there is a low level of Beta III tubulin expression but it is not as high as it would appear to be in the real time PCR analysis when related to mean fluorescence data for retinoic acid treated embryonal carcinoma

cells in Chapter 3. Also Beta III tubulin as a member of the tubulin family of cytoskeletal proteins may feasibly be present in other non neuronal cell types, so this is not a completely unsurprising result. On balance therefore it would appear that dopaminergic markers are not just expressed in any cell type but that they are actually able to be induced and regulated in a relatively controlled manner in the Tera2.cl.SP12 model under different conditions.

The finding that there is though some detection in a large number of cells of Beta III tubulin and a detection of tyrosine hydroxylase in particular and Nurr1 in a smaller but noticeable percentage of cells reinforces the concept discussed earlier that there may be some non specific binding of the antibodies used. The variability in expression and morphological features highlighted earlier are therefore critical features as they suggest that although there may be broad background detection, some specific clear predictable detection is still possible.

Figure 5.22: Flow cytometry analysis of MG63 cells

C.

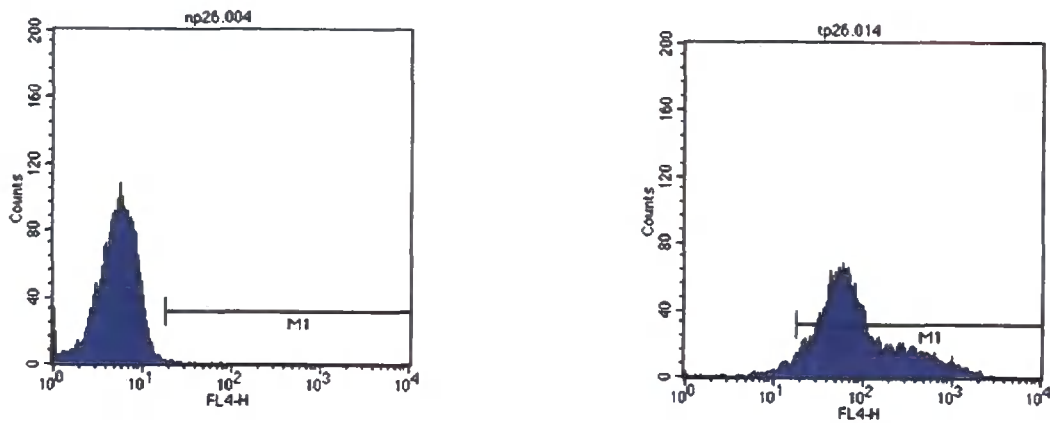


Figure 5.22A: The percentage of MG63 cells positive for expression of Nestin, Nurr1, TH and Beta III tubulin. The observed readings for Nestin, Nurr1 and TH are generally low in comparison to those for the retinoic acid treated derivatives of Tera2.cl.SP12 cells detailed in Chapters 3 & 4. **B:** The intensity of Nestin, Nurr1, TH and Beta III tubulin expression in MG63 cells. For the first 3 markers these values vary little from secondary only controls. For Beta III tubulin they reflect a relatively low but detectable level of expression akin to that observed for Tera2.cl.SP12 cells in Chapter 3. Error bars = S.D. of mean. In all experiments $n=3$. **C:** Flow cytometry traces showing how from the secondary only control sample where close to all cells were gated out as negative (left hand image) there is a shift to the right of the single peak for the test sample (right hand image). This detection of non neural MG63 cells positive for Beta III tubulin that is associated with a relatively weak (akin to that for EC cells recorded in Chapter 3 as mentioned above) but measurable intensity may reflect non specific binding of the antibody. However given that by other means detection in EC cells (which show comparable results to MG63 samples) of Beta III tubulin is weak it may be that this is not reflective of a strong expression of this marker but one that is detectable partly as a result of the sensitivity of the technique. It is though possible that Beta III tubulin is expressed in the non neural MG63 cells as it may be that it is not a totally neural specific marker, as neural markers have been found to be expressed by non neural cell types (stromal cells) such as in Deng et al, 2006.

The question though given that there appears to be little difference in expression at 17 days between the plus and minus retinoic acid conditions remains as to why there is a relatively low amount of mRNA present for a range of dopaminergic markers, when the embryonal carcinoma cells have been treated with a cocktail of molecules thought to drive dopaminergic differentiation. To probe deeper and change the point of comparison, Western blotting was used to try and gauge if the expression of neuronal and dopaminergic marker proteins reflected the trends seen in terms of the mRNA levels at the 17 day time point in the plus and minus retinoic acid conditions. Figure 5.23 shows that tyrosine hydroxylase protein is up regulated in both the plus and minus retinoic acid conditions, relative to the Tera2.cl.SP12 starting material. The test samples also display greater expression than in the 17 day vehicle control sample, supporting the concept that the more controlled approach is the superior method. As is the case for the mRNA data there appears to be a slightly greater expression in the minus retinoic acid condition. There is however very little correlation between the mRNA and protein data, as at the mRNA level tyrosine hydroxylase expression in the embryonal carcinoma cells is comparable to that in the vehicle control and plus retinoic acid conditions, with slightly more of it present in the minus retinoic acid samples. This is clearly not the case in terms of tyrosine hydroxylase protein expression. The minus retinoic acid condition may show a slightly greater induction than the vehicle control and plus retinoic acid samples, but in all three cases there is a marked induction of tyrosine hydroxylase protein relative to the Tera2.cl.SP12 starting material. Figure 5.23 also shows that for Beta III tubulin there is a slight but less tangible induction. This may be due to the fact that embryonal carcinoma stem cells show a propensity for neural differentiation and perhaps do express the slightly more generic marker Beta III tubulin at a basal detectable level. It may however be due to a general non specific binding of the antibody used. Although treatment using the protocols outlined in Figure 5.2 may enhance the neuronal nature of the cultures as observed in the real time PCR analyses it may also be the case that there is a remodelling of the Beta III tubulin present in such cultures, along with some induction. Further analysis using flow cytometry or the immunostaining shown previously (Figure 5.8) may give a better indication of the changing patterns of Beta III tubulin expression in this model system.

Figure 5.23: Beta III tubulin & TH protein expression in the plus & minus R.A. conditions

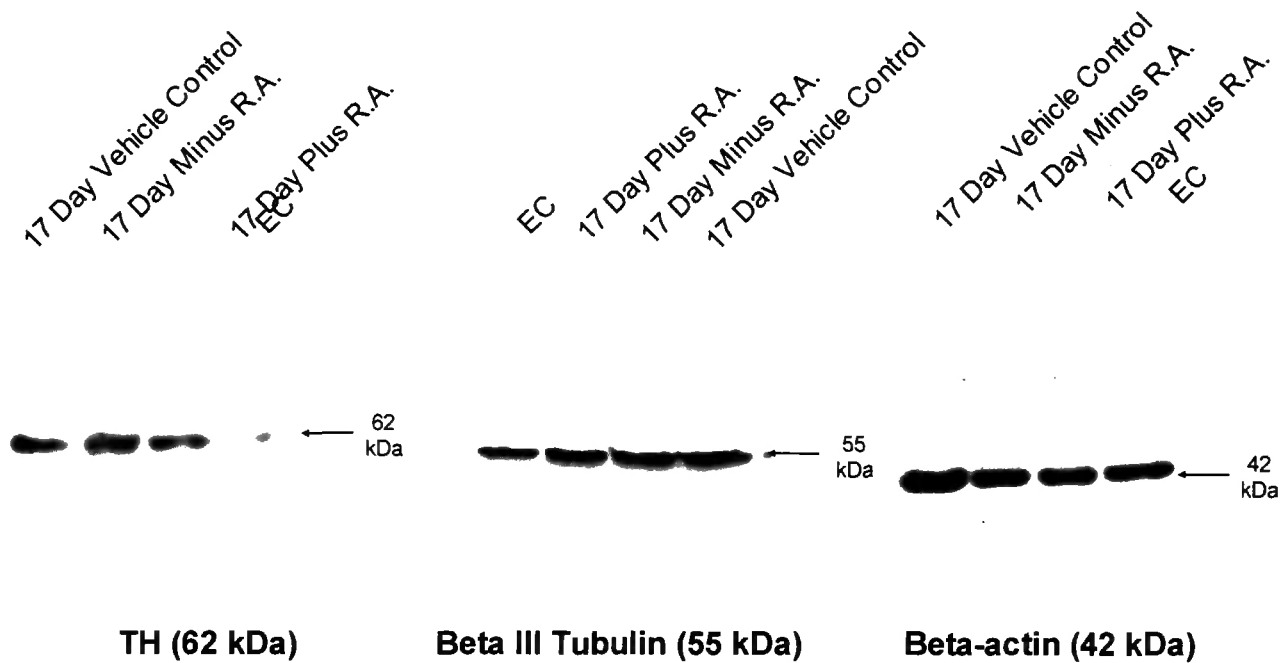


Figure 5.23: A representative Western blot image demonstrating TH and Beta III tubulin protein expression in samples harvested at the 17 day terminus of the differentiation protocol defined in Figure 5.2. The EC cell sample represents samples derived from cultures of undifferentiated Tera2.ci.SP12 embryonal carcinoma stem cells. 20 μ g of protein were loaded for each sample (n=3). Beta-actin was used as a loading control, as indicated the main bands for each antibody were of the predicted size. There is a faint additional band above the band of interest in the Beta III tubulin blot supporting the concept that there may be some non specific binding of this antibody. This is in keeping with the detection of this neural marker in the non neural MG63 cell line by flow cytometry, which may be due to non specific binding of the antibody or as a result of Beta III tubulin not being a totally specific marker of a neuronal phenotype.

It is clear though that mRNA and protein levels for markers of a dopaminergic phenotype are not regulated in a simple linear fashion. Figure 5.24 presents one possible model of how mRNA and protein expression for markers of a dopaminergic phenotype such as tyrosine hydroxylase may vary over time based on the data obtained. In an ideal scenario further protein based analysis using a panel of dopaminergic markers would be carried out as the presence of tyrosine hydroxylase may indicate the presence of adrenergic or noradrenergic neurons and as such this may account for the down regulation of other dopaminergic markers at the mRNA level, as indicators of other phenotypes may be up regulated. This though may not be that likely a scenario as tyrosine hydroxylase mRNA is down regulated in a comparable fashion to other dopaminergic markers, suggesting a conserved effect, if it was present as an indicator of another phenotype then it may feasibly be expected to be controlled in a different fashion. Further additional work could focus on assessing expression of alternative markers indicative of other neuronal phenotypes. Overall though there is some case to believe that under the conditions tested, in particular the minus retinoic acid condition that Tera2.cl.SP12 cells can respond to and be driven towards acquisition of a dopaminergic phenotype using this Sonic hedgehog/Fibroblast growth factor 8 based approach.

Figure 5.24: A summary of the trends in mRNA & protein expression in EC cells treated with a dopaminergic differentiation cocktail

A possible model of the trends in mRNA and protein expression for dopaminergic markers such as tyrosine hydroxylase in Tera2.ci.SP12 human embryonal carcinoma stem cells treated as per the scheme outlined

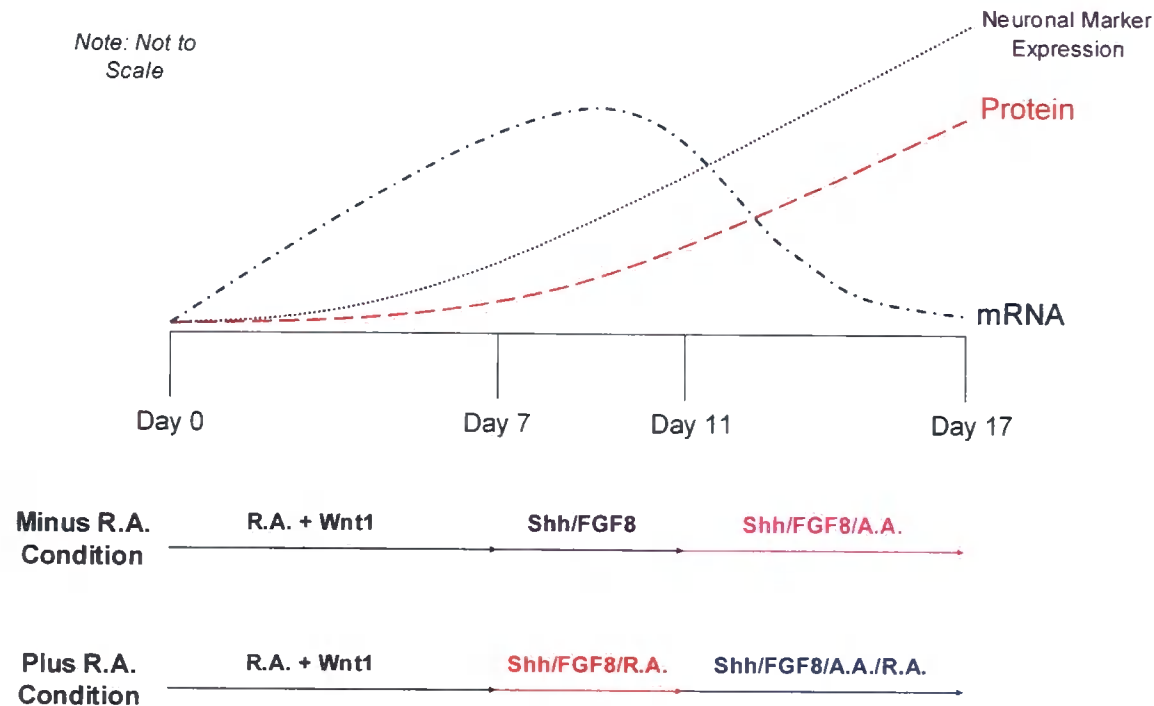


Figure 5.24: A possible model of the trends in mRNA and protein expression for dopaminergic markers such as tyrosine hydroxylase based upon the findings in this study. Neuronal marker expression is also shown as broadly increasing over the course of differentiation. There doesn't appear to be a direct correlation between mRNA and protein expression under the conditions tested, and there may well be a lag period where there is a rise in levels of protein as the mRNA levels start to decline. Both the plus and minus R.A. conditions are postulated to behave in a comparable manner throughout the entire process based upon the observation that there is little or no difference between the conditions at the end point of the differentiation protocol. This though would need to be verified experimentally if this work were to be extended.

5.4 Discussion

The first hypothesis to be addressed in this Chapter was whether suspension culture of the Tera2.cl.SP12 cell line could offer any advantages in the pursuit of the production of potential dopaminergic neurons relative to the use of standard adherent conditions. In the presence of retinoic acid alone and particularly in combination with Wnt1 suspension culture appears to offer some benefits. First of all neurospheres formed from Tera2.cl.SP12 cells treated with retinoic acid (Horrocks et al, 2003), or this molecule and Wnt1 for 7 days expressed nestin, Sox1 and Beta III tubulin in a comparable fashion to the spherical neural masses formed from human embryonic stem cells in the work of Cho et al., 2008. The spherical neural masses were then utilised to obtain a high yield of dopaminergic neurons, so given that the embryonal carcinoma cell derived neurospheres displayed a similar pattern of marker expression there was the prospect that they may be an adequate and representative substitute for the spherical neural masses when using the Tera2.cl.SP12 model. In addition suspension cultures tended to increase Beta III tubulin mRNA expression and lowered the levels of Sox1 mRNA present. In the light of the findings of Yan et al., 2005 that suggested when using human embryonic stem cells Sonic hedgehog and Fibroblast growth factor 8 should be applied early prior to or at a threshold of Sox1 expression, the lower levels of it in suspension cultures may be favourable in the context of integrating the effective 10 day Sonic hedgehog/Fibroblast growth factor 8 based differentiation protocol of Cho et al., 2008. It would be of interest in future studies to assess if Sox1 protein expression reflects the pattern seen for its mRNA. Suspension culture also appears to promote broadly neuronal differentiation as seen by elevated amounts of Beta III tubulin mRNA. However dopaminergic marker expression at 7 days appears to be affected less by suspension culture and more by the presence of Wnt1 as observed in Chapter 4. Some indicators such as Msx1 that is thought to be involved in the specification of a dopaminergic fate do though show an up regulation due to the use of suspension culture. A broader screen at different time points might help to extend this work to see if at other stages similar effects on factors associated with a dopaminergic phenotype are detectable. There is though seemingly promise in the use of suspension culture as part of the strategy to drive the dopaminergic differentiation of the Tera2.cl.SP12 cell line, as mentioned in the results this could be

via changes in gene expression profiles in a comparable manner to that found in mouse embryonic stem cells. This notion is also broadly in keeping with the work of Ravindran & Rao, 2006, who used an alternative embryonal carcinoma cell line, Ntera2 to produce dopaminergic neurons using a method involving embryoid body formation and the application of Sonic hedgehog/Fibroblast growth factor 8 in a similar fashion to the protocol pioneered by Lee et al., 2000 using mouse embryonic stem cells.

One anomaly that arose in these initial investigations though was the lack of an effect due to the presence of Wnt1 in combination with retinoic acid in adherent conditions. Wnt1 had previously been shown in Chapter 4 to up regulate dopaminergic marker mRNA expression at 7 days in relation to cultures treated with only retinoic acid. In suspension cultures at 7 days a comparable trend was seen, indicating that this molecule was able to exert an effect. However in the adherent samples tested in this chapter it emerges as having little or no influence on dopaminergic marker expression. In the light of the same stock of Wnt1 being used and the observable effects on suspension cultures this is unusual as the exogenous Wnt1 appears to only be inactive in the adherent set of cultures. This may be due to some flaw in the embryonal carcinoma cells used for these particular experiments as if one bad batch was split for use and passaged with a view to subsequent use any abnormality could be passed on to each set of cells utilised to provide the 3 biological repeats. The unpredictable, aberrant nature of the embryonal carcinoma cells makes this a possibility and could limit their use in the context of them not always providing reproducible results. There may be other reasons such as some mRNA may be degraded during storage, especially if for example the storage vessel temperature drops, although this may be expected to affect all samples it is possible it may have more impact on some than others, the duration of storage may more realistically have an effect. There are a myriad of other potential reasons but the unpredictable nature of the embryonal carcinoma cells is one of them and is an important consideration when contemplating their prospective use.

The next question was, can Sonic hedgehog and Fibroblast growth factor 8 alone and in conjunction with ascorbic acid influence the dopaminergic differentiation of Tera2.cl.SP12 embryonal carcinoma stem cells? In the Ntera2 cell line Stull &

Iacovitti, 2001 made the suggestion that Sonic hedgehog and Fibroblast growth factor 8 were not alone sufficient to drive the dopaminergic differentiation of such cultures (although here retinoic treated derivatives of the Ntera2 cells were used as previously discussed). However Ravindran & Rao, 2006 presented data indicating that in the absence of retinoic acid these molecules could be used to help form dopaminergic neurons from the Ntera2 cell line. Sonic hedgehog is thought to act via the mechanism outlined in Figure 4.2 and in the Tera2.cl.SP12 system it appears that Sonic hedgehog may up regulate the amount of Msx1 mRNA, whilst in turn Nkx 6-1 is repressed and neurogenin2 levels are slightly elevated. This would be expected if Sonic hedgehog was up regulating Msx1 and thus enabling it to act on its downstream signalling components. A greater effect may be expected to be seen on Lmx1a mRNA levels but as the first part of the signalling cascade perhaps if there is a peak in the expression of this factor it has already passed prior to the sampling points chosen. Further analysis starting with an assessment of the pattern of protein expression for these key transcription factors would be of benefit. Also if more time points could be included this would be of use to give a deeper understanding of the patterns of regulation of mRNA and protein for dopaminergic markers when applying Sonic hedgehog, Fibroblast growth factor 8 and ascorbic acid to the Tera2.cl.SP12 embryonal carcinoma stem cell system.

It is of interest that Msx1 is relatively up regulated and that it may influence neurogenin2 mRNA levels as this factor is thought to have broadly pro neural properties (Abeliovich & Hammond, 2007). This might be a part of the reason for the enhanced levels of the neuronal marker Beta III tubulin that are observed. The induction of the mRNA for Beta III tubulin from 11 to 17 days also indicates that the ascorbic acid in conjunction with the Sonic hedgehog and Fibroblast growth factor 8 may be having an effect on maturation of the cultures. It is also worthy of note that the Wnt1 plus retinoic acid pre treatment followed by 4 days of Sonic hedgehog and Fibroblast growth factor 8 application leads to almost twice the amount of Beta III tubulin mRNA expression relative to samples treated with only retinoic acid for the first 7 days. This may suggest that Wnt1 is creating a permissive environment for Sonic hedgehog and Fibroblast growth factor 8 to subsequently act in as inductive signals, to drive the dopaminergic differentiation process. Further work would be needed to address this in more detail, as it may be that the difference is in part or

wholly due to the fact that the Wnt1 plus retinoic acid combined cultures show higher levels of Beta III tubulin expression at 7 days and this may be carried forward to 11 days. It does though still indicate that there could be a positive synergy in the combined use of all three molecules.

Therefore given that the pathway thought to be induced by Sonic hedgehog is to some degree seemingly influenced by it at the mRNA level and the apparent ability of this molecule in concert with Fibroblast growth factor 8 and ascorbic acid to enhance the expression of the neuronal marker Beta III tubulin. It is a little surprising to find that a range of markers of a dopaminergic phenotype are relatively weakly expressed and in fact generally down regulated in terms of the level of their mRNA present at the terminus of the 17 day differentiation protocol. There was the possibility that this was due to the presence of retinoic acid, however the minus retinoic acid condition that replicates the method of Cho et al., 2008 shows no difference to its plus retinoic acid counterpart. This raises the question as to whether retinoic acid should be omitted if possible from the entire process as in Ravindran & Rao, 2006. It also offers the opportunity for further investigations into alternatives to retinoic acid, for example ascorbic acid which has been shown to enhance the yield of dopaminergic neurons from rat midbrain precursor cultures (Yan et al., 2001). There are also two other clear possibilities for this unexpected effect, the first is that false positives are arising when detecting Beta III tubulin expression giving a false impression of how neuronal the sample cultures are. This as discussed in the results is somewhat debateable especially given that neurogenin2 is up regulated and this marker is seen by some as broadly neuronal. However it is not impossible that cells reflective of a neuronal phenotype are not being formed at all or certainly to a lesser degree than might appear to be the case, in turn explaining the lack of dopaminergic marker expression. Alternatively it may be excess Sonic hedgehog is being applied possibly at a sub optimal temporal window in the differentiation process. Too much Sonic hedgehog may have an inhibitory effect and lead to fewer neurons of a potentially dopaminergic phenotype (Fasano & Studer, 2009; Joksimovic et al, 2009), giving an alternative explanation of the findings.

Another possible reason for the relatively negative effect on dopaminergic marker expression may be related to the drop in the levels of the mRNA for the receptor for

Fibroblast growth factor 8. Although the mRNA is present and the protein may be too perhaps a shorter period of neurosphere formation would be beneficial or earlier Fibroblast growth factor 8 application alongside Wnt1 and retinoic acid may be advantageous.

It may also be that low levels of mRNA do not correlate directly with the amount of protein present. This was found to be the case for tyrosine hydroxylase and it would be of use to expand this comparison to other markers in future investigations to try and lend support to the model whereby the peak in dopaminergic marker mRNA expression has passed prior to the sampling points. Markers of other phenotypes could be assessed for their expression to try and identify if an alternative neuronal population is being formed.

The MG63 cell control though highlights that such expression profiling approaches although valid need to be thorough, especially when considering more generic markers such as Beta III tubulin. On balance it appears that MG63 cells do not express dopaminergic markers in a regulated manner, but transcription factors such as *Msx1* can be detected in samples from these cells. It may be the case that certain elements associated with a particular phenotype in one cell or tissue play a totally different role in another cell or tissue type and this may account for the observation that some dopaminergic markers are detected in the non neural MG63 cell samples. This though underpins the absolute need if possible to assess a panel of markers for the phenotype of interest. If these can also be identified to be regulated in a predictable fashion this is an added bonus, as it helps put any findings into a specific context. In an ideal scenario both mRNA and protein data would be obtained because as the results in this chapter highlight there is seemingly not always a clear correlation between mRNA and protein expression.

The limitations of assessing marker expression also point to the need for functional testing. A clear development to this work would be to assess dopamine release using high performance liquid chromatography. If there was a clear evoked dopamine release in test conditions relative to controls upon treatment with potassium ions it would suggest that there were dopaminergic neurons present. This would if it were the case correlate with the protein data for tyrosine hydroxylase. Proteins are the

functional components of a cell and as such their expression may be viewed as more critical than that of the corresponding mRNA. If this work was investigated to a functional level it could be of great benefit. In addition if the Tera2.cl.SP12 cell line is to be used such testing is essential as any candidate neurons derived from it would need to show functional properties if they were to be utilised in pharmacological screening. The assessment of the electrophysiological properties and possibly even the transplantation of the cells derived in vitro into a Parkinsonian animal model such as a 6-hydroxydopamine treated mouse could also be of value, as they would give a further insight into the functional potential of embryonal carcinoma stem cell derived neurons.

Although it was hoped that some assessment could be made of the potential for applied usage of the Tera2.cl.SP12 model, the current data are not sufficient to base any conclusive decisions on, they do suggest that the 17 day approach could offer the prospect of an enhanced yield of dopaminergic neurons but further protein expression data are needed prior to at least some form of functional test such as a dopamine release assay. It appears that there may be idiosyncrasies in such cultures that dictate that the patterns of mRNA and protein expression vary differentially with time in the complex process of attempting to achieve dopaminergic differentiation but currently the data are still too open to interpretation to really make a judgement on the potential of the Tera2.cl.SP12 cell line as a model to study dopaminergic neurogenesis. Therefore to try and gain a better insight into the suitability of this model system for this purpose, it was decided to make a direct comparison of it relative to the Ntera2 cell line that has been utilised in the desired context in the available literature, using another well established method, that of co-culture with PA6 cells; this forms the basis of Chapter 6.

Chapter 6

The use of a mesodermal
cell co-culture method to
make a comparison of the
dopaminergic
differentiation potential of
the Human EC cell
systems Tera2.cl.SP12 and
Ntera2.D1

6.1 Introduction

The primary purpose of this final results Chapter is to compare the Tera2.cl.SP12 embryonal carcinoma cell line to a suitable counterpart that has been used in the published literature to study dopaminergic neurogenesis. The Ntera2 cell line was chosen to make the comparison against for a number of reasons. First of all, it is an embryonal carcinoma cell line so suffers from the same generic drawbacks of being of cancerous origin, secondly it is from the same species, this purports the use of the same primers, antibodies and other reagents used for the Tera2.cl.SP12 cell line is feasible; finally it has been used to produce neurons representative of a dopaminergic phenotype using a range of established methods. For example Ravindran & Rao, 2006, utilised a Sonic hedgehog based protocol and Schwartz et al., 2005, were able to demonstrate dopaminergic differentiation of this cell line using a PA6 based co-culture methodology. However Stull & Iacovitti, 2001, had questioned the ability of Sonic hedgehog and Fibroblast growth factor 8 to drive the dopaminergic differentiation of the Ntera2 cell line, this though may be due to the use of these molecules on Ntera2 cells that had already received a lengthy retinoic acid induction. Still this poses some unanswered questions as to the potential of this cell line if using a Sonic hedgehog based approach, especially when this earlier work is compared/contrasted to the findings of Ravindran & Rao, 2006. In addition in Chapter 5 the ability of Tera2.cl.SP12 cells to respond to these molecules is observable but the means by which they regulate differentiation of such cells is still open to interpretation. Therefore as a result the PA6 based co-culture method was chosen as it offered a technically straight forward, reasonably rapid means of comparing the Ntera2 system to the Tera2.cl.SP12 model; that had worked previously using the Ntera2 cell line (Schwartz et al., 2005). PA6 co-culture has also been shown to influence the dopaminergic differentiation of mouse (Kawasaki et al., 2000), primate (Kawasaki et al., 2002) and human (Zeng et al., 2004, Vazin et al., 2008) embryonic stem cells, so would appear to possess a stromal derived inducing activity that is sufficiently conserved to have an effect on a range of cells from different species and sources. Further support for choosing this particular method comes from the work of Hayashi et al., 2008 where Wnt5a was highlighted as a key part of the dopaminergic neuron inducing activity of PA6 cells. In the light of the positive

findings in Chapter 4 using Wnt1 to direct the differentiation of the Tera2.cl.SP12 cell line there is a strong possibility that another member of the Wnt family, namely Wnt5a may be able to exert an effect in this cell system.

There is the concept that the stromal derived inducing activity of PA6 cells may contain two components one related to the cell surface and the other a soluble element (Kawasaki et al., 2000). Vazin et al., 2008, extended this idea suggesting that the cell surface activity may account for the neural inducing effect of PA6 cells on human embryonic stem cells, whilst the soluble component is responsible for the specific dopaminergic neuron inducing activity (Vazin et al., 2008). Therefore although various strategies have been tried for example using PA6 conditioned medium or filter membranes to separate the PA6 cells from those under investigation (Kawasaki et al., 2000), the decision was made to utilise the method of directly co-culturing the PA6 cells with the embryonal carcinoma stem cells as it offered the greatest number of potential avenues to achieve dopaminergic differentiation.

To try and eliminate stress on the Ntera2 cells, Schwartz et al., 2005 tested the ability of mitomycin C treated, mitotically inactivated PA6 cells to drive the dopaminergic differentiation of this embryonal carcinoma cell line. Such treatment of the PA6 cells did not detract to any significant degree from the potency of them to promote acquisition of a dopaminergic phenotype, and helped prevent over crowding, in turn reducing the burden on the culture media (Schwartz et al., 2005). The embryonal carcinoma cells though are the more highly proliferative cell type, and as such steps to control their proliferation and differentiation may be an alternative means by which to prevent over grown co-cultures. In Chapter 3 after just 1 day retinoic acid was shown to cause a statistically significant increase in expression of the neuronal marker Beta III tubulin. Therefore although the main focus of this work is to compare the Tera2.cl.SP12 model to its Ntera2 counterpart, an additional hypothesis to investigate is whether a short pre treatment of the Tera2.cl.SP12 cells with retinoic acid has any benefits when such cells are subsequently co-cultured with PA6 cells.

In the light of work by Perrier et al., 2004, who demonstrated that other stromal cells such as the MS5 line could be used in strategies aimed at the production of dopaminergic neurons and given the availability of primary stromal cells in the

laboratory, one final hypothesis was put under scrutiny. This was can rat mesenchymal stem cells (otherwise known as bone marrow stromal cells) be used to drive the dopaminergic differentiation of embryonal carcinoma stem cells (or their retinoic acid induced derivatives)?

Mesenchymal stem cells have been reported as a potential source of material from which dopaminergic neurons can be derived directly *in vitro* (Trzaska et al., 2007). These cells displayed expression of dopaminergic markers and were able to secrete dopamine (Trzaska et al., 2007). This work was supported by that of Barzilay et al., 2008 who were able to demonstrate dopaminergic marker expression and dopamine secretion upon depolarisation. Further work where RE1 – silencing factor, a known suppressor of mature neuronal genes in neuronal progenitors, was artificially reduced in mesenchymal stem cells facilitated the production of more functionally mature neurons (Trzaska et al., 2008). This work though promising is based on the premise that mesenchymal stem cells can trans-differentiate across the germ layer boundary to form cells of different lineages (normally mesenchymal stem cells would form cells of mesodermal origin such as fat, cartilage and bone, not those of ectodermal origin such as neurons). As such this is an area surrounded by much controversy with a body of evidence being presented that suggests cytoskeletal collapse may be a mechanism by which mesenchymal stem cells acquire neuronal morphologies (Hardy et al., 2008). There is also work that indicates mesenchymal stem cells may express neural proteins in standard culture (Deng et al., 2006). In addition the time course of neuronal differentiation could be brought into question as it might not be expected that production of dopaminergic neurons is possible in 4-6 days as observed in Barzilay et al., 2008, especially given that cells have to cross the germ layer boundary and neurogenesis is usually not a particularly rapid process even in neural cell types or those cell lines with a propensity for this differentiation pathway. On balance though it is not inconceivable that 6 days may be adequate if the correct inductive cocktail is supplied, given that a cell *in vitro* is highly unlikely to mirror in a precise fashion events *in vivo*, as exemplified by the unexpectedly rapid nature of PA6 driven dopaminergic differentiation in primate embryonic stem cell cultures (Kawasaki et al., 2002). The functional ability of cells representative of a dopaminergic phenotype derived from mesenchymal stem cells clouds the picture further creating greater uncertainty.

However one aspect of the potential of mesenchymal stem cells for use that can more readily be agreed upon appears to be their capacity to provide trophic factors that may influence neural and more specifically neuronal development (Hardy et al., 2008). Bone marrow stromal cells from adult mice were used in conjunction with Sonic hedgehog and Fibroblast growth factor 8 to help generate neurons characteristic of a dopaminergic phenotype from embryonic stem cells (Shintani et al., 2008). Therefore to add another dimension to this work, the effects of primary rat mesenchymal stem cells in co-culture on the dopaminergic differentiation potential of embryonal carcinoma cells were decided upon as an area for investigation. The fact the cells are of rat origin also opens up the possibility of at least being able to make an inference as to whether highly conserved factors play a role in dopaminergic differentiation across a number of species. In addition if the mesenchymal stem cells in co-culture can exert an effect on the embryonal carcinoma cells they offer an additional means by which to compare the dopaminergic differentiation capability of the two cell lines Ntera2 and Tera2.cl.SP12 under investigation, which is the principle focus of this Chapter.

6.2 Cell Culture Methods

Maintenance of mouse PA6 Stromal Cells

PA6 cells from the Riken Bioresource Centre Japan (<http://www.brc.riken.go.jp/inf/en/index.shtml>) were maintained and grown in alpha-Minimal Essential Medium (α MEM; Invitrogen) supplemented with 10% (v/v) fetal calf serum (Sigma), and 100U/ml penicillin/streptomycin (Gibco) in T25 culture flasks (Nunc) at 37°C in a 5% CO₂ incubator (Sanyo) prior to use. 0.25% (w/v) Trypsin/ 0.1% (w/v) EDTA (in HBSS) (Cambrex) was used to passage cells 1:4 once they had grown to a confluent state, on a weekly basis as per the instructions provided upon purchase of the cell line. Passages U+8 to U+12 cells were used as passage U+8 cells were provided from Riken Bioresource centre and subsequent passaging lead to the choice of passages used.

Maintenance of Primary Rat Mesenchymal Stem Cells

Primary rat MSCs (all passage 1) were kindly provided by Steven Allan Hardy of the Przyborski Lab, Durham University. They were maintained in Dulbecco's modified Eagle's medium (DMEM; PAA) supplemented with 10% (v/v) fetal calf serum (Sigma), 2mM L-glutamine (Cambrex), 100U/ml penicillin/streptomycin (Gibco) and 1x non essential amino acids (Invitrogen) in T75 culture flasks (Nunc) at 37°C in a 5% CO₂ incubator (Sanyo) prior to use.

Co-culture Differentiation

PA6 cells or primary rat MSCs were grown to confluency under the conditions outlined above, the media aspirated from them, before being washed with 1x PBS, prior to treatment for 5 minutes with 0.25% (w/v) Trypsin/ 0.1% (w/v) EDTA (in HBSS) (Cambrex), to release the cells from the surface of the culture flask. The Trypsin was then neutralized by addition of 4ml of DMEMFG for MSC cultures or α MEM supplemented as described above for PA6 cultures and any cells remaining loosely bound washed off the surface of the flask by pipetting the DMEMFG or

α MEM/Trypsin-EDTA mix against the side of the flask where cell attachment occurs. A further wash with 1x PBS was carried out to optimize the retrieval of cells for use. The cell containing mix was then transferred to a 15ml Falcon tube and spun in a centrifuge (Eppendorf 5810R (swing bucket rotor A-4-62)) at 1000rpm, 4°C, for 3 minutes for PA6 cells or 250g, 4°C, for 5 minutes for the MSCs. The excess fluid was removed to leave a cell pellet. This was resuspended in Glasgow minimum essential medium (GMEM) (Invitrogen) supplemented with 10% (v/v) Knockout Serum Replacement (Invitrogen), 0.1x non essential amino acids (Invitrogen), 1mM sodium pyruvate (Invitrogen) and 0.1mM β -Mercaptoethanol (Invitrogen) as appropriate and cell number was obtained by using a haemocytometer.

Once cell number was known MSCs or PA6 cells were plated out at a density of 5000-10000 cells/cm² (Schwartz et al., 2005) in 6 or 12 well plates (Nunc) in Glasgow minimum essential medium (Invitrogen) supplemented with 10% (v/v) Knockout Serum Replacement (Invitrogen), 0.1x non essential amino acids (Invitrogen), 1mM sodium pyruvate (Invitrogen) and 0.1mM β -Mercaptoethanol (Invitrogen). The cells were allowed to settle whilst the EC cells were prepared for use as follows.

Cultures of Tera2.cl.SP12 or Ntera2.D1 EC cells were grown to confluency, the media aspirated from them, before being washed with 1x PBS, prior to treatment for 5 minutes with 0.25% (w/v) Trypsin/ 0.1% (w/v) EDTA (in HBSS) (Cambrex), to release the cells from the surface of the culture flask. The Trypsin was then neutralized by addition of 4ml of DMEMFG and any cells remaining loosely bound washed off the surface of the flask by pipetting the DMEMFG/Trypsin-EDTA mix against the side of the flask where cell attachment occurs. A further wash with 1x PBS was carried out to optimize the retrieval of EC cells for use. The cell containing mix was then transferred to a 15ml Falcon tube and spun in a centrifuge (Eppendorf 5810R (swing bucket rotor A-4-62)) at 800rpm, 4°C, for 2 minutes. The excess fluid was removed to leave an EC cell pellet. This was resuspended in GMEM supplemented as outlined above and cell number was determined using a haemocytometer.

Once cell number was known Tera2.cl.SP12 or Ntera2.D1 EC cells in GMEM supplemented as described above were added at a density of 2000 cells/cm² (Schwartz et al., 2005) into the wells containing MSCs or PA6 cells. The co-cultures were then topped up with media to a final volume of 3-5ml. Media was changed after the first 4 days and every day thereafter up to 17 days which had previously been shown to be sufficient time to achieve robust tyrosine hydroxylase expression (Schwartz et al., 2005). All cultures were maintained at 37°C in a 5% CO₂ incubator (Sanyo).

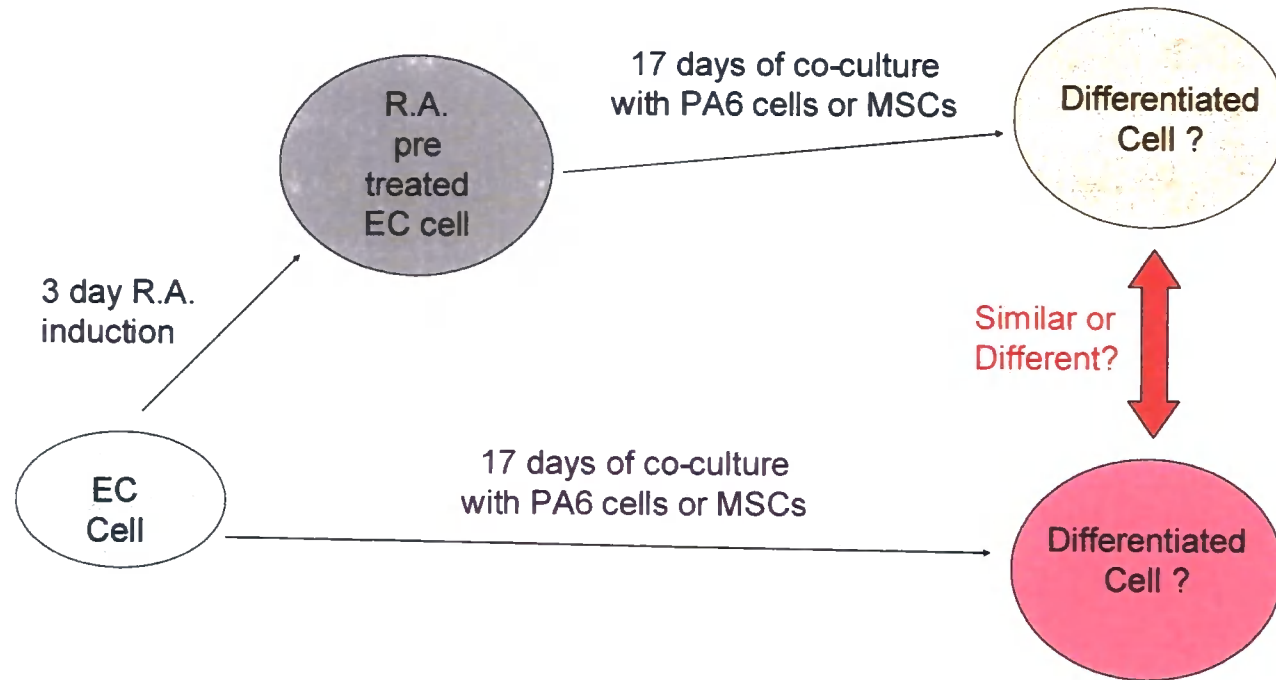
Retinoic acid pre treatment of Tera2.cl.SP12 cells

Cultures of Tera2.cl.SP12 EC cells were grown to confluency, the media aspirated from them, before being washed with 1x PBS, prior to treatment for 5 minutes with 0.25% (w/v) Trypsin/ 0.1% (w/v) EDTA (in HBSS) (Cambrex), to release the cells from the surface of the culture flask. The Trypsin was then neutralized by addition of 4ml of DMEMFG and any cells remaining loosely bound washed off the surface of the flask by pipetting the DMEMFG/Trypsin-EDTA mix against the side of the flask where cell attachment occurs. A further wash with 1x PBS was carried out to optimize the retrieval of EC cells for use. The cell containing mix was then transferred to a 15ml Falcon tube and spun in a centrifuge (Eppendorf 5810R (swing bucket rotor A-4-62)) at 800rpm, 4°C, for 2 minutes. The excess fluid was removed to leave an EC cell pellet. This was resuspended in DMEMFG and cell number was obtained by using a haemocytometer. Once the cell number was known the EC cells were seeded out in DMEMFG at 0.5x10⁶ cells per T25 flask (Nunc). After seeding out cells were induced to differentiate by exposure to a final concentration of 10µM all trans retinoic acid (Sigma) for 3 days. Cells were grown in a standard 37°C, 5% CO₂ incubator (Sanyo).

After 3 days cells were seeded out into co-cultures in an identical fashion to their untreated embryonal carcinoma cell counterparts (as described previously in the co-culture differentiation section). Figure 6.1 illustrates the two different approaches.

Figure 6.1: Schematic of co-culture studies

Summary of co-culture differentiation methods



Note: Not to Scale

6.3 Results

The initial comparison of the Tera2.cl.SP12 model to the Ntera2 system was made by using real time PCR to compare the relative levels of mRNA for neuronal and dopaminergic markers in samples from the two embryonal carcinoma cell lines grown in co-culture with PA6 cells or mesenchymal stem cells. Figure 6.2A shows that dopamine receptor 2 mRNA is most up regulated in its expression in Tera2.cl.SP12 samples co-cultured with PA6 cells. However the induction due to PA6 cell co-culture is not statistically significant as there is substantial variation in the results obtained. It is though regardless of this on average markedly up regulated relative to the other conditions. In contrast mesenchymal stem cell or PA6 co-culture of the Ntera2 line for 17 days causes a statistically significant increase in dopamine receptor 2 mRNA levels, reflecting a difference between the starting material and test samples, which show much less variation and as such facilitate the statistical effect. These samples though in raw terms are on average displaying an induction that is smaller in magnitude than that seen with the Tera2.cl.SP12 cell line co-cultured in the presence of PA6 cells. Figure 6.2B shows for dopa decarboxylase there is a large variation in its expression in most of the samples and as such little can be taken from this result. It may though suggest given that such co-cultures appear by eye to be very over grown that there is insufficient regulation of the differentiation of the embryonal carcinoma cells.

Tyrosine hydroxylase expression was also examined in such samples and as can be seen in Figure 6.3A the trend is comparable to that for dopamine receptor 2 in that PA6 cells in co-culture with the Tera2.cl.SP12 cell line show the greatest average induction. However once more the Ntera2 co-culture samples display relatively lower mean levels of mRNA for the marker of interest but the induction observed is statistically significant. Again this is most probably due to less variation between test samples. Figure 6.3B demonstrates that for the neuronal marker Beta III tubulin the trend is comparable to that for indicators of a dopaminergic phenotype with PA6/Tera2.cl.SP12 co-culture offering the greatest increase in mRNA expression. On this occasion there is a statistically significant induction from the Tera2.cl.SP12 starting material to the 17 day PA6/Tera2.cl.SP12 co-culture samples, which in

addition display a statistically significant increase in expression relative to the PA6/Ntera2 condition. This gives some support to the trend observed that suggests PA6/Tera2.cl.SP12 co-culture is producing the most neuronal and possibly more specifically dopaminergic cultures, if higher levels of marker expression are viewed as a positive gauge, which in this relatively simple system is probably the most appropriate measure.

When markers associated with the acquisition of a dopaminergic phenotype were assessed a comparable trend was discovered once again. Figure 6.4A shows that *Lmx1a* mRNA is most up regulated in PA6/Tera2.cl.SP12 co-cultures and this increase relative to the starting material is in this instance significant. Both Ntera2 co-culture samples show statistically relevant inductions but much lower mean levels relative to the PA6 /Tera2.cl.SP12 co-culture condition. Figure 6.4B demonstrates that *Msx1* is up regulated to a statistically significant degree in all co-culture samples relative to the appropriate starting material. The trend though once more points to PA6/Tera2.cl.SP12 co-culture offering the greatest opportunity to derive a substantial average increase in marker expression. Figure 6.5A indicates that for *neurogenin2* the same trend of a strong increase in PA6/Tera2.cl.SP12 co-culture samples holds true. Although there is a statistically significant increase in expression in MSC/Ntera2 co-cultures in relation to the Ntera2 starting material this is probably of little biological relevance in the context of the broader trends. Likewise Figure 6.5B shows that on average PA6/Tera2.cl.SP12 co-culture provides the greatest up regulation in marker expression (for *Nkx 6-1*), yet again it is the Ntera2 co-culture samples that differ significantly from their respective starting material. One question though to arise from this analysis of *Nkx 6-1* mRNA expression is if *Msx1* levels are up regulated in PA6/Tera2.cl.SP12 co-culture samples and this factor is a suppressor of *Nkx 6-1*, then why and also how is *Nkx 6-1* up regulated as well? One possible explanation is that it could be due to different cells within the cultures expressing the two different markers independently. This is feasible given that Tera2.cl.SP12 cells have been shown to be able to produce a mixture of cell types (Stewart et al, 2003).

One other factor to be considered when carrying out co-culture experiments is that the interaction between the cell types may operate in both directions. That is to say stromal cells may influence the differentiation of embryonal carcinoma cells but these

cells themselves may exert an effect on their stromal cell counterparts. Although the dopamine receptor 2 and tyrosine hydroxylase primers are to the best of our knowledge human specific, there is the possibility that for these and the other probes that detection of transcripts for the markers tested may arise from the expression of them in the PA6 cells or mesenchymal stem cells that have been potentially activated by the presence of the embryonal carcinoma stem cells in co-culture. In practice this may or may not be the case and both stromal cell types appear negative for the markers tested when grown in isolation but the prospect of this two way interaction is nonetheless worthy of consideration. Given that the tyrosine hydroxylase antibody used in Figure 6.6 that is able to detect both human and mouse tyrosine hydroxylase protein, does so in PA6 cells and it is generally accepted that mRNA needs to be present to form protein, then given that no tyrosine hydroxylase transcript is found in the stromal cells it is reasonable to think at least for this marker that any result is due to stromal cell induction of the embryonal carcinoma cells (and not the reverse scenario). The observation that the trends for tyrosine hydroxylase are seen for other markers of a dopaminergic phenotype lends support to the notion that in this system it is the stromal cells primarily exerting an effect on the embryonal carcinoma cells and not the reciprocal interaction taking place. The human specific nestin immunostaining in Figure 6.13 is also indicative that there are few if any stromal cells present at the point of harvesting the samples for analysis due to the highly proliferative embryonal carcinoma cells dominating the co-cultures. Therefore it would not be expected that induction of markers in the stromal cells due to embryonal carcinoma cell co-culture would be likely to occur, but it is still valid to think that this is theoretically possible. One way to alleviate this issue in future work would be to use conditioned media from stromal cell cultures to try and drive the differentiation of the embryonal carcinoma stem cells.

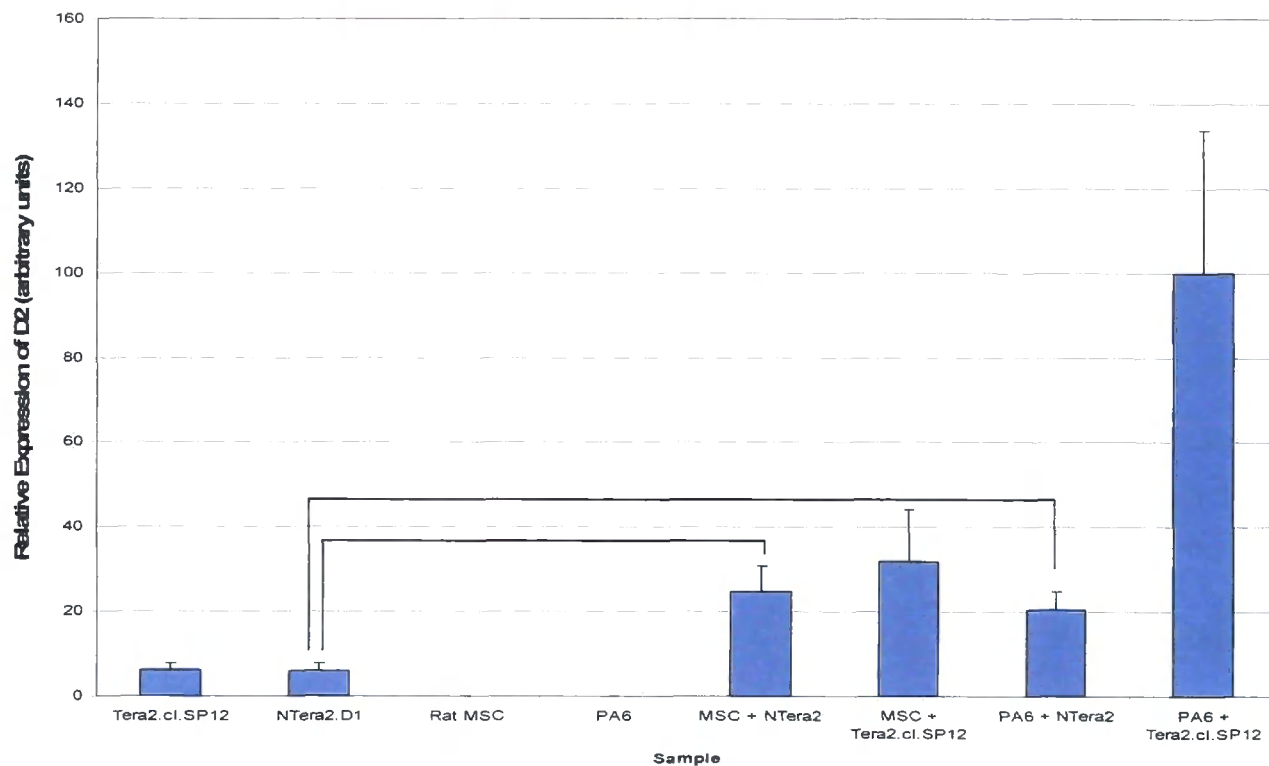
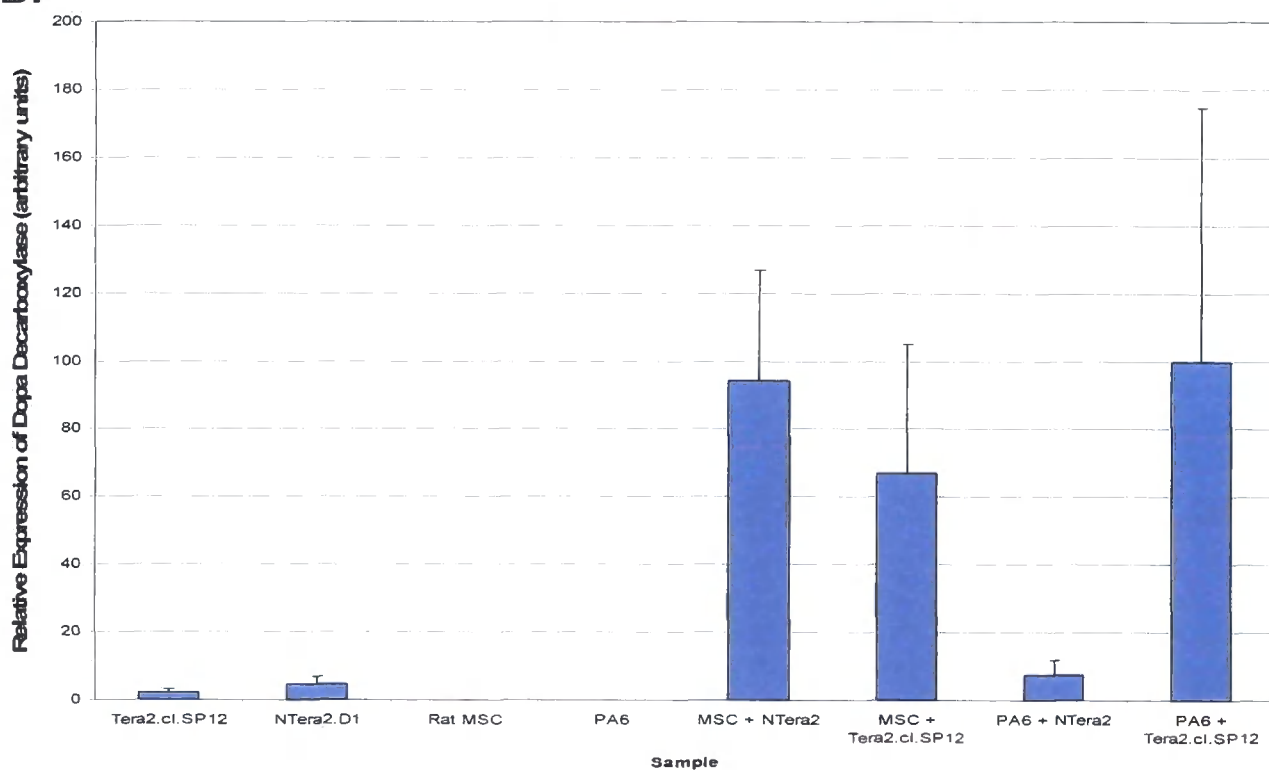
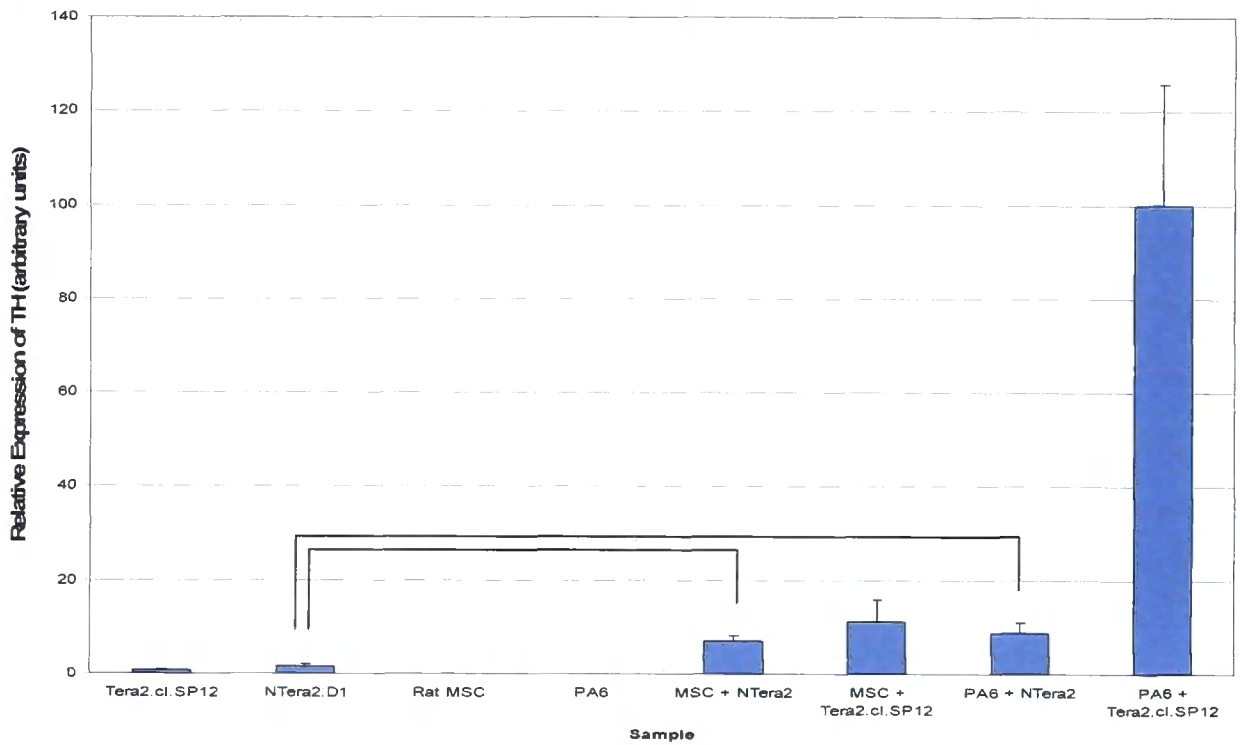
Figure 6.2: D2 & AADC mRNA expression in EC/Stromal cell co-cultures**A.****B.**

Figure 6.2A: Relative expression of human D2 mRNA in samples from two human EC cell lines (Tera2.ci.SP12 & Ntera2), rat MSCs & murine PA6 cells, as well as 17 day old co-cultures of the EC and stromal cell types. Statistically significant differences between samples calculated using a one way anova with accompanying Tukey post hoc analysis are indicated by black bars where appropriate and indicate a significance ≤ 0.05 . **B:** As above but for human dopa decarboxylase (and there are no statistically relevant differences). In all cases error bars represent the standard deviation of the mean; $n=3$.

Figure 6.3: TH & Beta III tubulin mRNA expression in EC/Stromal cell co-cultures

A.



B.

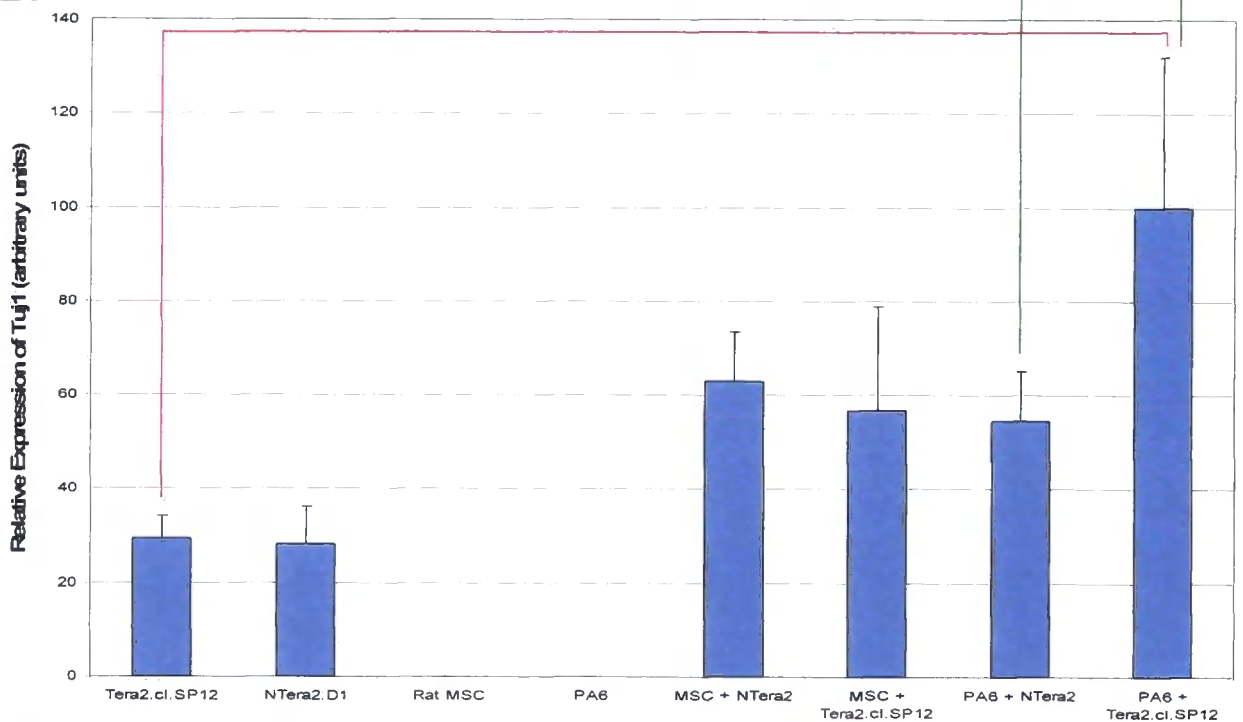


Figure 6.3A: Relative expression of human TH mRNA in samples from two human EC cell lines (Tera2.cl.SP12 & Ntera2), rat MSCs & murine PA6 cells, as well as 17 day old co-cultures of the EC and stromal cell types. Statistically significant differences between samples calculated using a one way anova with accompanying Tukey post hoc analysis are indicated by black bars where appropriate and indicate a significance ≤ 0.05 . **B:** As above but for human Beta III tubulin, the purple bars indicate a significant difference between the untreated Tera2.cl.SP12 cells and their 17 day PA6 co-cultured counterparts worked out using a one way anova with Tukey post hoc analysis (sig ≤ 0.05). Green bars indicate a statistically significant difference between Ntera2 and Tera2.cl.SP12 samples co-cultured with PA6 cells for 17 days, defined using Student's T-test (sig ≤ 0.05). In all cases error bars represent the standard deviation of the mean; $n=3$.

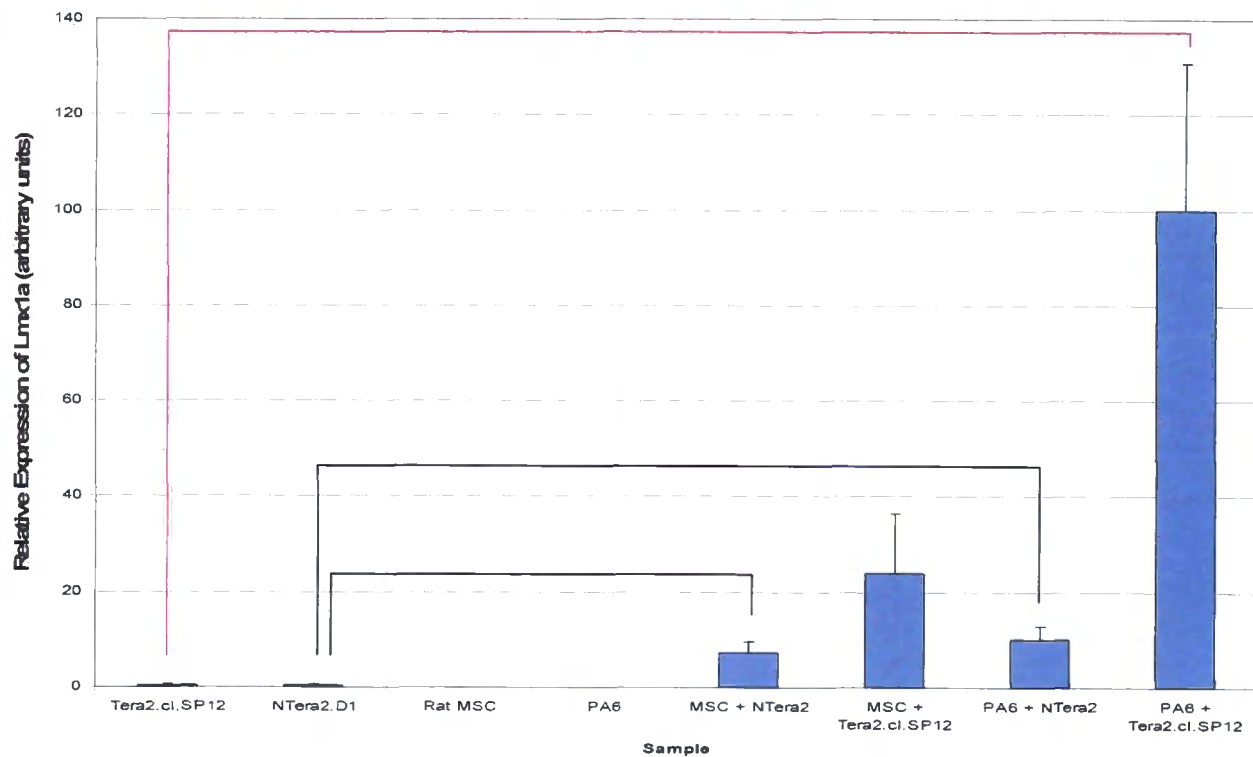
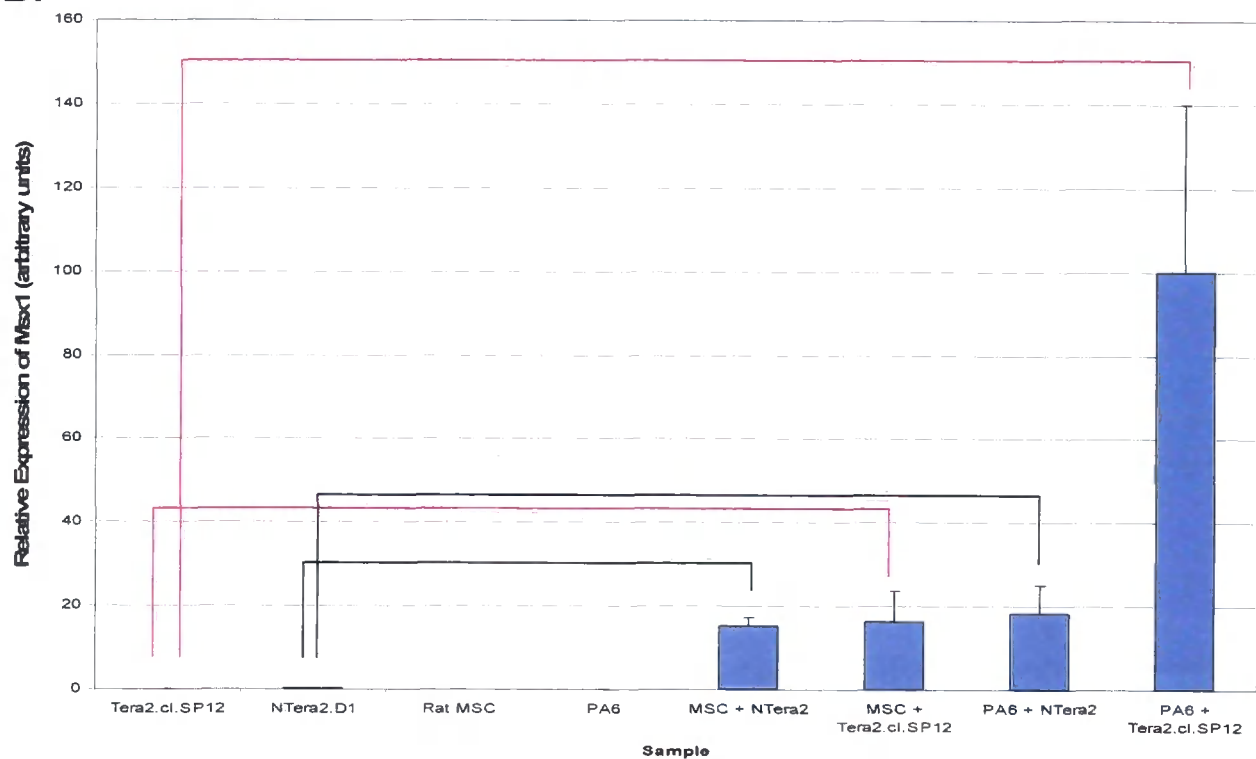
Figure 6.4: Lmx1a & Msx1 mRNA expression in EC/Stromal cell co-cultures**A.****B.**

Figure 6.4A: Relative expression of human Lmx1a mRNA in samples from two human EC cell lines (Tera2.cl.SP12 & Ntera2), rat MSCs & murine PA6 cells, as well as 17 day old co-cultures of the EC and stromal cell types. Statistically significant differences calculated using a one way anova with accompanying Tukey post hoc analysis are indicated by black bars for Ntera2 based samples and purple bars for Tera2.cl.SP12 based samples where appropriate and indicate a significance ≤ 0.05 . **B:** As above but for human Msx1. In all cases error bars represent the standard deviation of the mean; $n=3$.

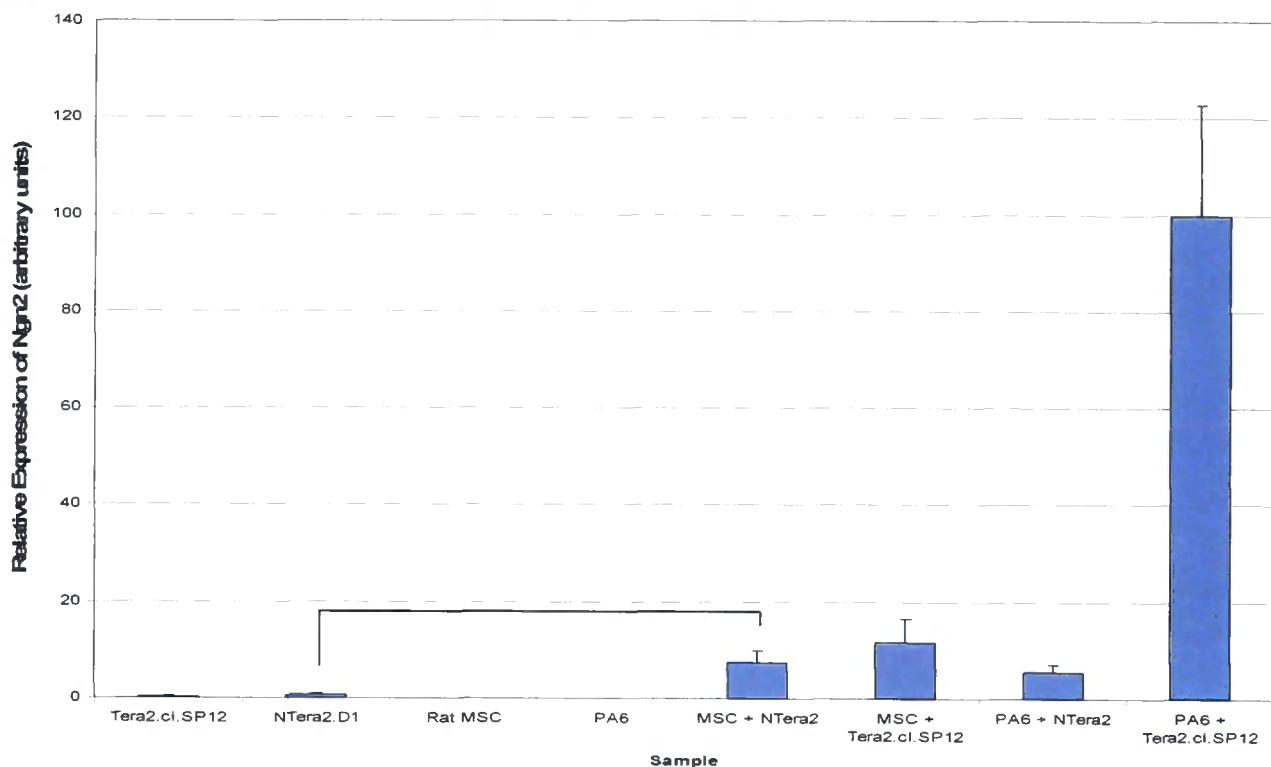
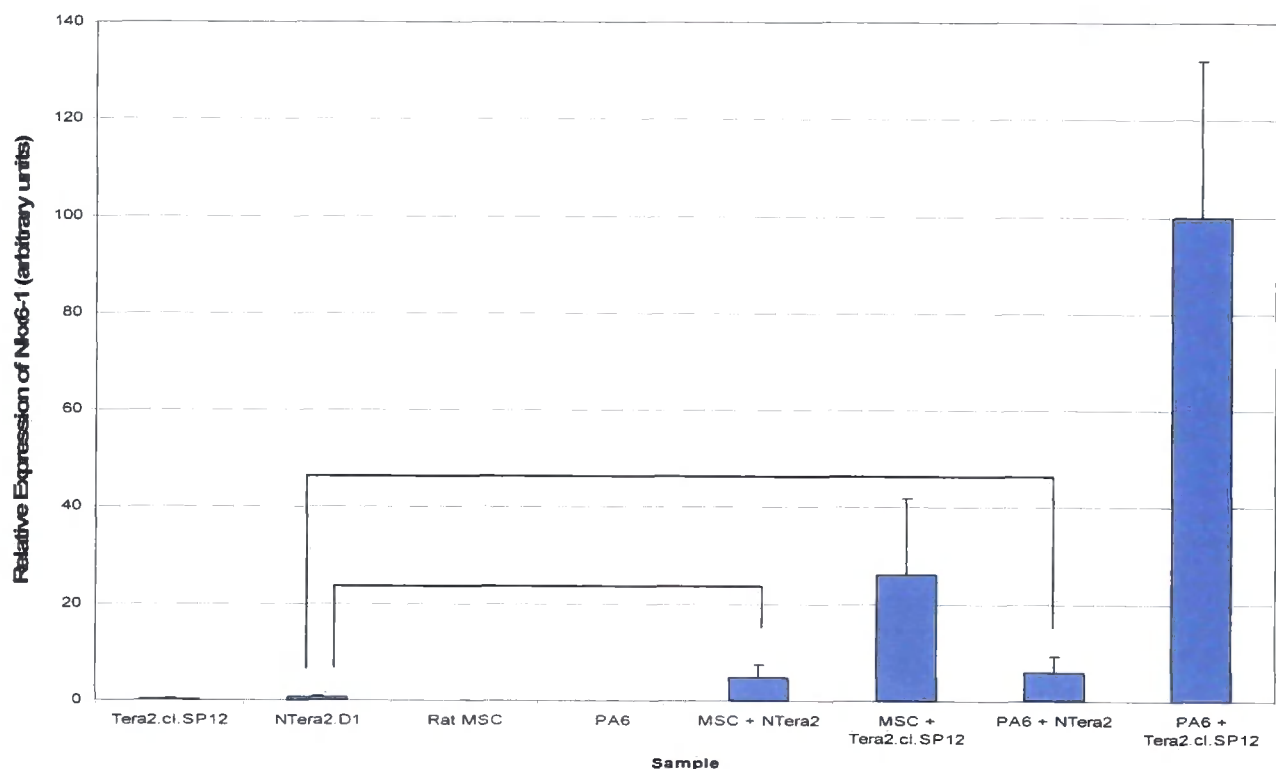
Figure 6.5: Ngn2 & Nkx 6-1 mRNA expression in EC/Stromal cell co-cultures**A.****B.**

Figure 6.5A: Relative expression of human Ngn2 mRNA in samples from two human EC cell lines (Tera2.ci.SP12 & Ntera2), rat MSCs & murine PA6 cells, as well as 17 day old co-cultures of the EC and stromal cell types. Statistically significant differences between samples calculated using a one way anova with accompanying Tukey post hoc analysis are indicated by black bars where appropriate and indicate a significance ≤ 0.05 . **B:** As above but for human Nkx 6-1. In all cases error bars represent the standard deviation of the mean: $n=3$.

Overall therefore it appears feasible these results may indicate that there is a lack of control in the co-culture system with the up regulation observed occurring in a hap hazard or less well ordered fashion than is desirable, possibly due to a lack of retinoic acid to drive the initial differentiation of the embryonal carcinoma cells. One other more basic observation that can be made from all the real time PCR analyses is that the primers used were human specific in their detection, as there was no amplification in any of the murine PA6 or rat mesenchymal stem cell samples for any of the markers investigated. Although this would need to be checked more thoroughly by testing the primers on rat or mouse sources known to be positive for the phenotypic indicators investigated. Also to check that a comparable quantity of cDNA was used, the amount of it in rat, mouse and a representative human sample was quantified. Table 6.1 shows a similar amount of cDNA was present for each species supporting the concept that the lack of detection was probably due to the primers only being suitable for amplification from human sources.

Table 6.1: Quantification of cDNA

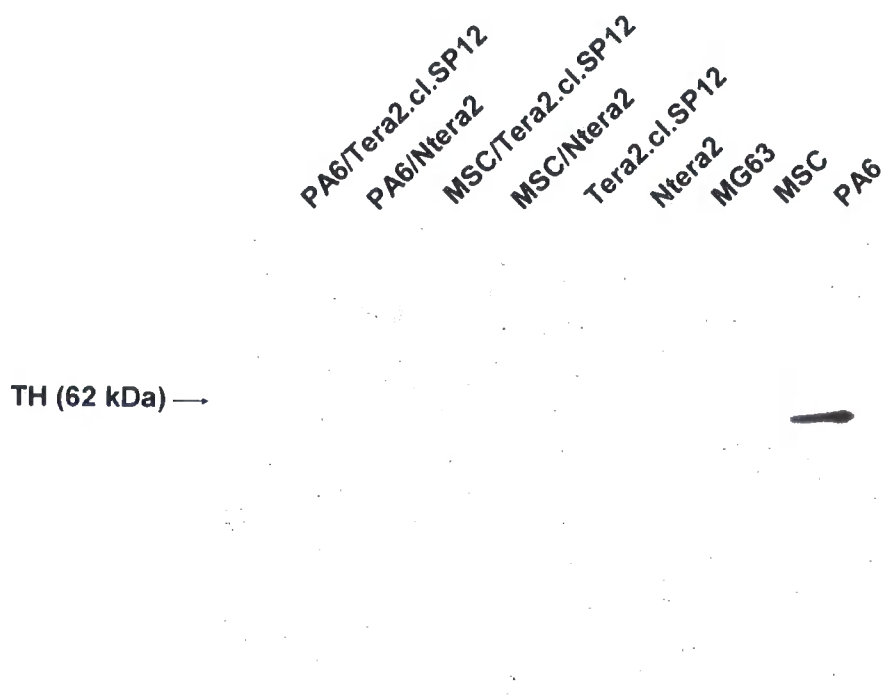
Sample (n=3)	Mean Quantity of cDNA (ng/ μ L)	Standard Deviation of Mean (ng/ μ L)
Human (Ntera2)	263.62	8.03
Mouse (PA6)	247.19	14.53
Rat (MSC)	247.14	9.63

To try and build on this initial real time PCR data that suggested Tera2.cl.SP12 cells could show a comparable or greater up regulation of dopaminergic marker mRNA relative to their Ntera2 counterparts when co-cultured with PA6 cells or mesenchymal stem cells, Western blotting was employed to extend the comparison to the level of protein expression. Figure 6.6 indicates that there is little difference in Beta III tubulin protein expression between any of the test co-culture conditions and the two types of embryonal carcinoma cell under investigation. There is only a weak to no real detection of this marker though in the PA6 or mesenchymal stem cell samples. On top of this tyrosine hydroxylase protein expression is not observed in either

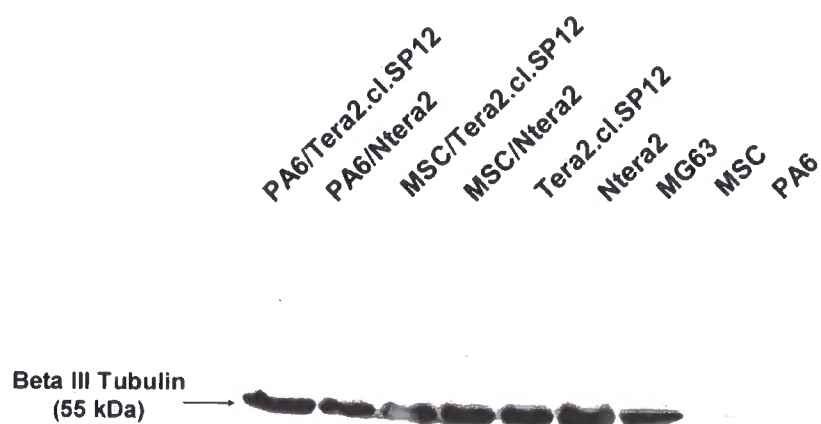
embryonal carcinoma cell line or the non neural MG63 cell samples as might be predicted. However it is only weakly detected in the PA6/Tera2.cl.SP12 co-culture samples and is absent in any of the other co-culture conditions. It does appear to be detectable in the PA6 cells and may be very weakly present in the mesenchymal stem cells. This observation is not totally unsurprising as stromal cell types have been shown previously to express neural markers (Deng et al, 2006). The disparity between detection of tyrosine hydroxylase protein in Figure 6.6 and the lack of detection of its mRNA in Figure 6.3 most probably arises from the antibody used being able to detect tyrosine hydroxylase in a range of species including both human and mouse, where as the primers supplied for tyrosine hydroxylase are thought on reliable grounds to be human specific. The observation that the detection of tyrosine hydroxylase is stronger in the PA6 cell only than in the PA6/Ntera2 and PA6/Tera2.cl.SP12 co-culture samples would suggest few if any PA6 cells are left at 17 days and that the embryonal carcinoma cells comprise close to or the entire culture. This is supported further by the human specific nestin staining in Figure 6.13 that indicates few if any non human cells remain at the point of harvesting the samples for analysis and is also consistent with the appearance of such cultures prior to harvesting. This protein data may also support the notion that there is a lack of control over the differentiation process as although mRNA levels are up regulated this increase is not observable at the protein level. The only trend that could be picked out is that tyrosine hydroxylase protein is detectable in the PA6/Tera2.cl.SP12 co-culture samples, which show the greatest mean induction of this and other markers in terms of mRNA expression. Thus it would appear that Tera2.cl.SP12 cells again compare favourably to their Ntera2 counterparts, although the conditions for investigation could be improved upon.

Figure 6.6: TH & Beta III tubulin protein expression in EC/Stromal cell co-cultures

A.



B.



C.

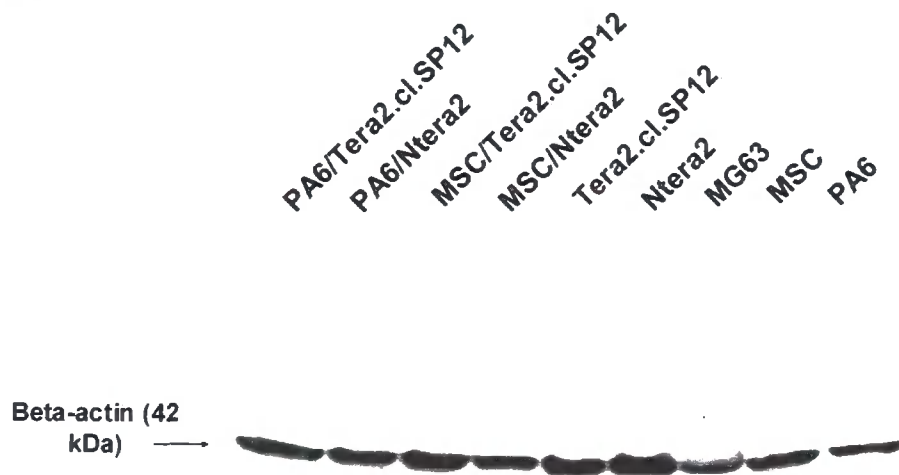


Figure 6.6A: TH protein expression assessed by Western blotting in samples from two human EC cell lines (Tera2.ci.SP12 & Ntera2), rat MSCs & murine PA6 cells, as well as 17 day old co-cultures of the EC and stromal cell types. Non neural MG63 samples were also included as a control. 20 μ L of protein were loaded for each sample. **B:** As for A. above but this blot indicates expression of Beta III tubulin not TH. **C:** A blot showing beta-actin expression in comparable samples to those in A. and B. above, beta-actin was used as a loading control. Representative images from n=3 repeats using biologically independent samples are shown. The detection of Beta III tubulin in the MG63 cell samples is consistent with the flow cytometry analysis in Chapter 5 and may reflect non specific binding of the antibody. An alternative theory is that it may be possible to detect neural markers (that are perhaps not totally specific for a particular phenotype) in non neural cell types in a similar manner to the detection of neural markers in stromal cells in Deng et al, 2006. This would also help explain the detection of TH in the stromal PA6 cells.

Prior to changing the experimental conditions one final assessment of the two embryonal carcinoma cell lines in co-culture with PA6 cells or mesenchymal stem cells was made using immunocytochemistry. This facilitated the possibility of observing neuronal cell morphology and also the detection of tyrosine hydroxylase in more than just one test condition as a small area of tyrosine hydroxylase positive cells could possibly be detected that may not be sufficient in number to provide a clear band on a Western blot. First of all though the Ntera2, PA6 and mesenchymal stem cells had to be screened for their expression of tyrosine hydroxylase and Beta III tubulin the two markers that were to be studied in test samples. Figure 6.7 demonstrates that Ntera2 cells give a diffuse faint stain for Beta III tubulin that is barely detectable and are essentially negative in their staining for tyrosine hydroxylase. Mesenchymal stem cells and PA6 cells showed no real detectable staining for tyrosine hydroxylase or Beta III tubulin (data not shown). Therefore any staining observed is likely to be due to the presence of these markers in embryonal carcinoma cell derivatives following their co-culture driven differentiation. One point the lack of tyrosine hydroxylase staining of stromal cell cultures highlights though is that the strength of expression and sensitivity of detection are key factors when considering expression data. Tyrosine hydroxylase is detectable given the amount of sample loaded in the Western blot analysis in Figure 6.6, however it is not detectable by immunostaining. This disparity may arise from the differences between the protein being detectable and situations where it is truly present in a manner that is meaningful. If tyrosine hydroxylase was found in process like structures indicative of a neuronal morphology it may be deemed an indicator of a dopaminergic phenotype, but in stromal cells this is unlikely to be the case and it may be more an artefact of culture and the sensitivity of detection offered by molecular expression analyses. In addition some immunological reagents may perform more effectively for one technique such as Western blotting than another such as immunostaining, as a cytosolic protein, tyrosine hydroxylase may by its very nature also not lend itself to detection by immunocytochemistry. Figure 6.8 shows that Beta III tubulin staining is detectable in all co-culture conditions. There are some neuronal type cells but there are not long clear processes as seen in Chapter 4 when Wnt1 was used in conjunction with retinoic acid for 3 weeks. In addition only fairly small areas of the entire culture show positive staining, although most areas are extremely confluent with cells as can be seen from the amount of Hoescht stained nuclei and phase images. Figure 6.9

indicates the same pattern is true for tyrosine hydroxylase staining. It is detectable in all co-culture samples but only in very rare patches and the positive cells are a tiny proportion of the entire cultures. It may be these small areas are stromal cells (as tyrosine hydroxylase was detected in PA6 cells by Western blot) but given the associated morphologies that are more characteristic of a potential neurite in culture along with the nestin staining in Figure 6.13, this is unlikely. Therefore it would appear that although the method for co-culturing the cells could be improved upon the Tera2.cl.SP12 cell line is seemingly as or more potent than the Ntera2 cell line in terms of its ability to express markers of a dopaminergic phenotype. It also seems to be the case that PA6 cells are more able inducers of dopaminergic marker expression in relation to mesenchymal stem cells, although these cells may possess some potential to do this in more refined culture conditions.

Figure 6.7: Staining of Ntera2 EC cells for TH & Beta III tubulin

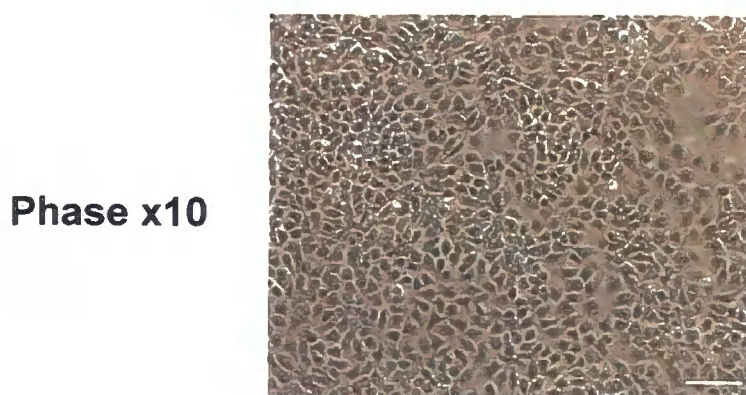
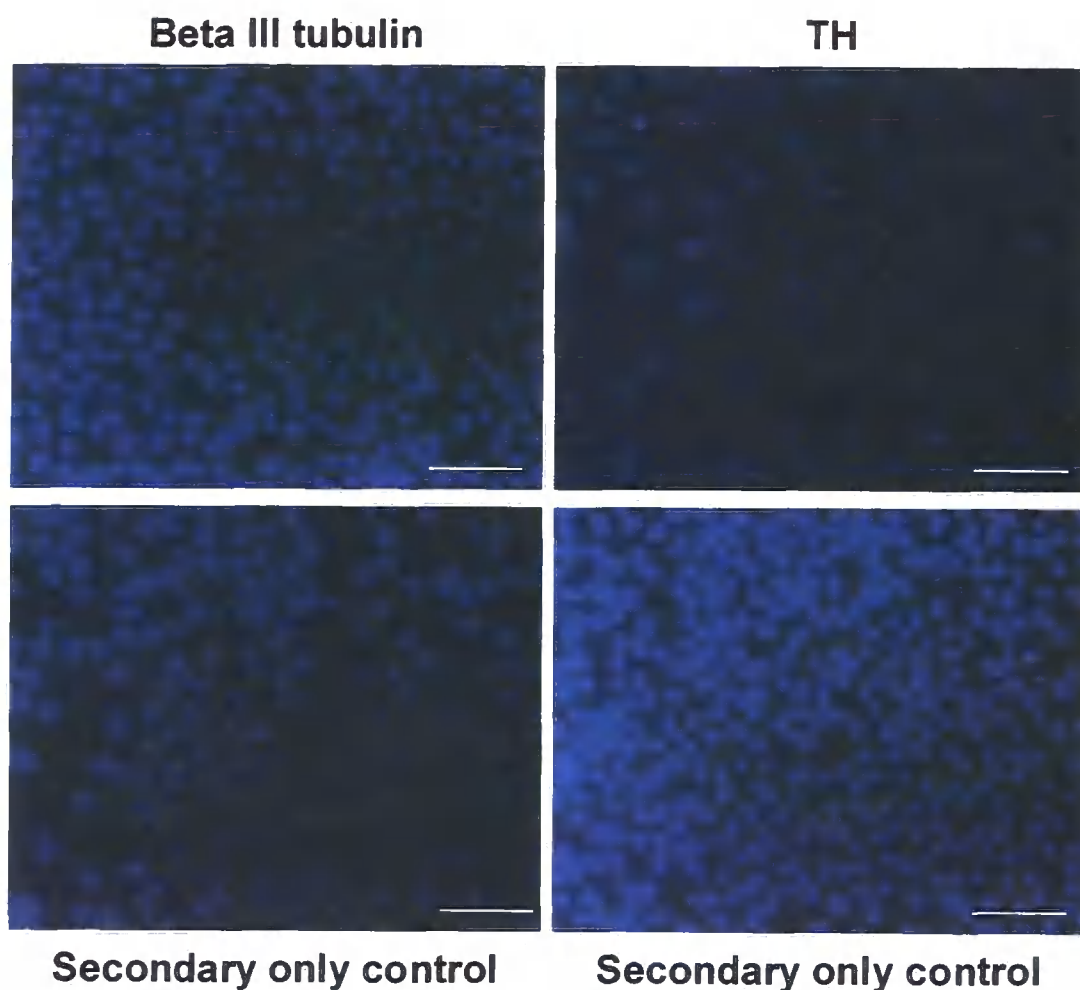
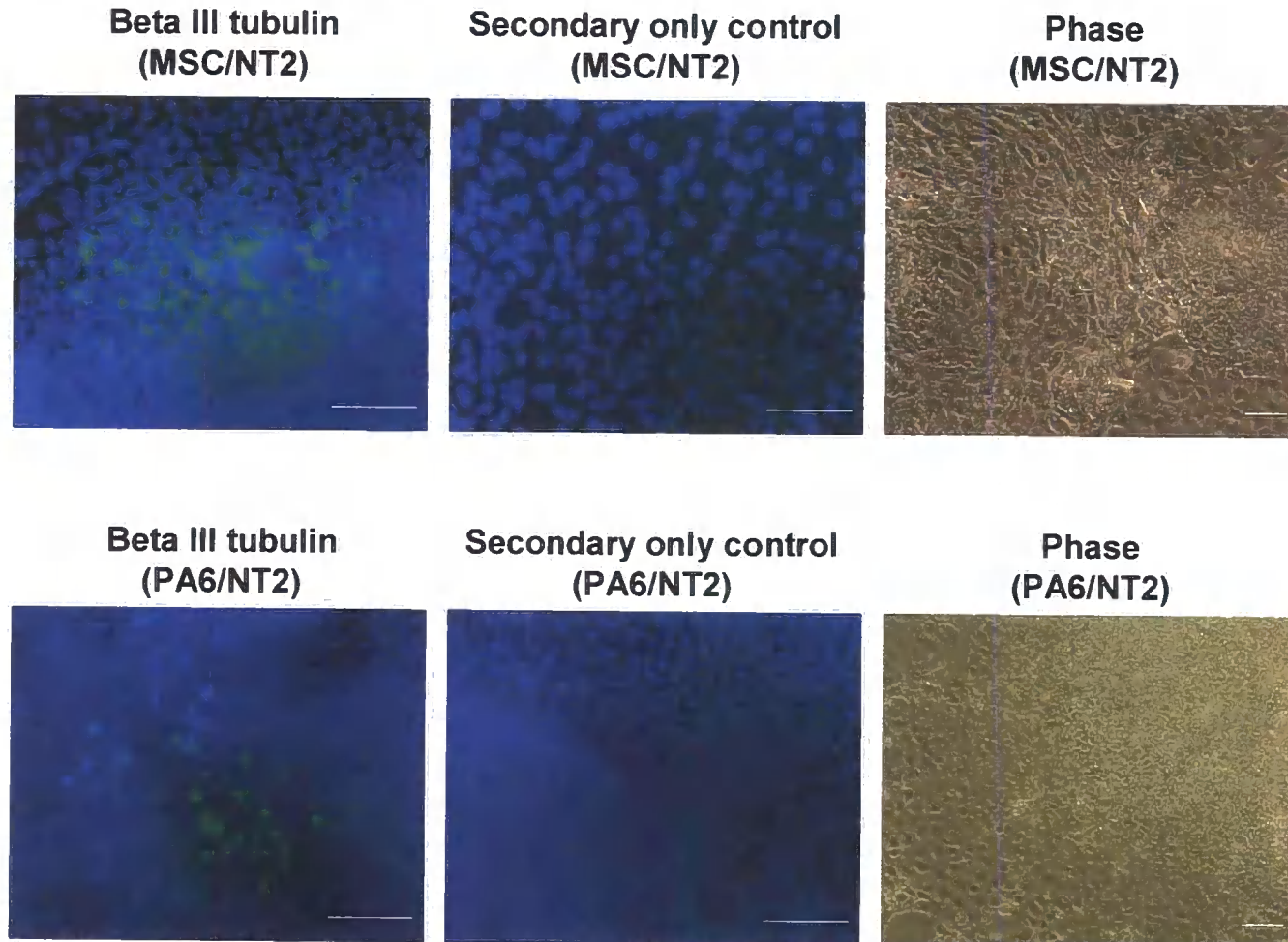


Figure 6.7: Immunostaining of the Ntera2 cell line for Beta III tubulin and TH. There is no real detection of tyrosine hydroxylase and a faint diffuse background level of detection for Beta III tubulin. This is very hard to detect in the merged image produced in Adobe Photoshop. The secondary only control conditions were not treated with any primary antibody just the vehicle in which antibodies were diluted (SBP), prior to treatment with the appropriate secondary antibody for either TH or Beta III tubulin. As expected there is an absence of any detection in the secondary only controls which are merged with the corresponding Hoescht images to confirm the presence of cells by staining their nuclei. The phase image displays the morphology of the cells after fixation and the staining process. Scale bars are approximately equal to 100 μ m. Representative images of n=3 repeats are shown. Given that the TH and Beta III tubulin staining are so weak it is probably safe to conclude that there is no true detection.

Figure 6.8: Beta III tubulin staining in EC/Stromal cell co-cultures



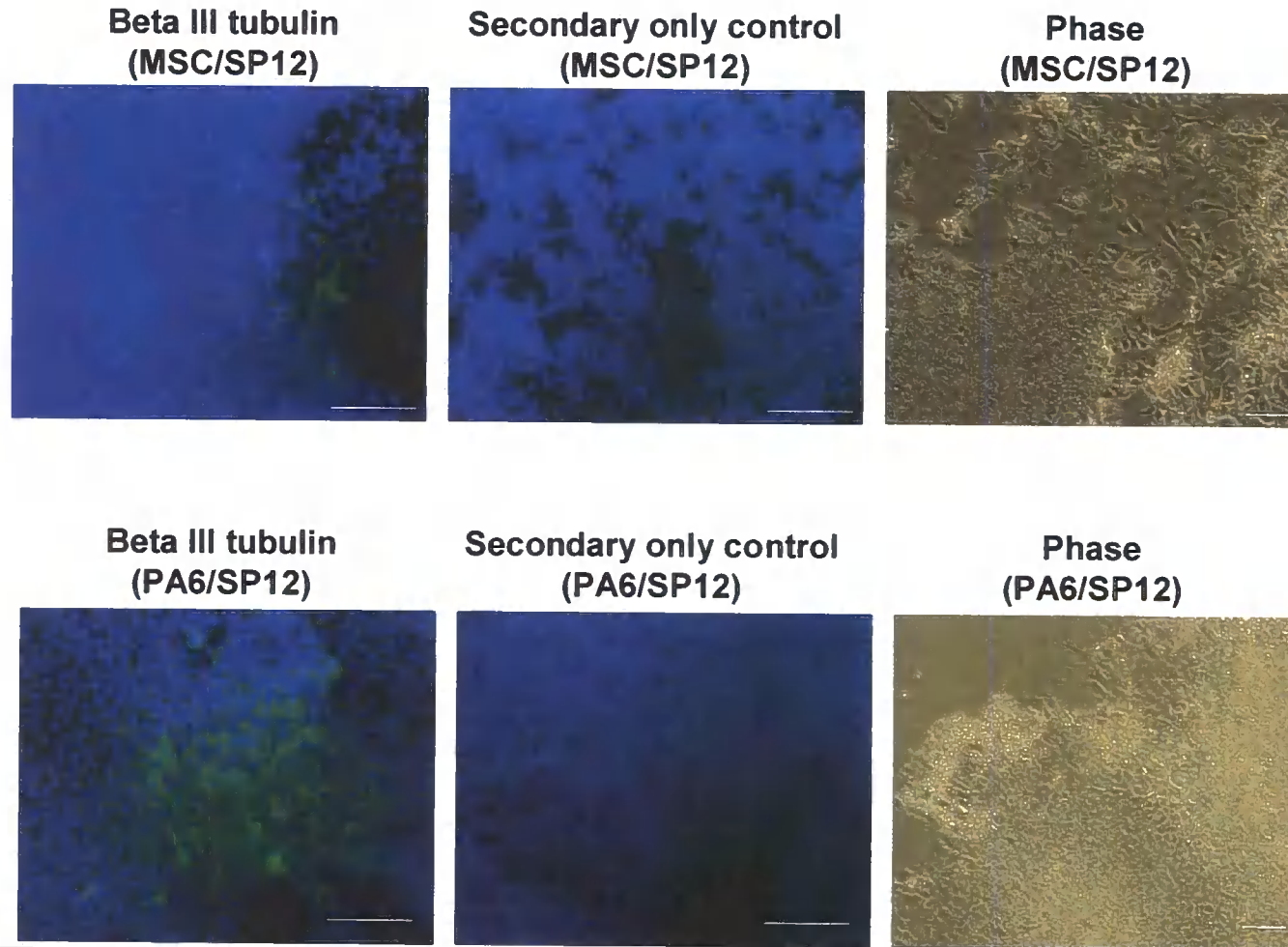
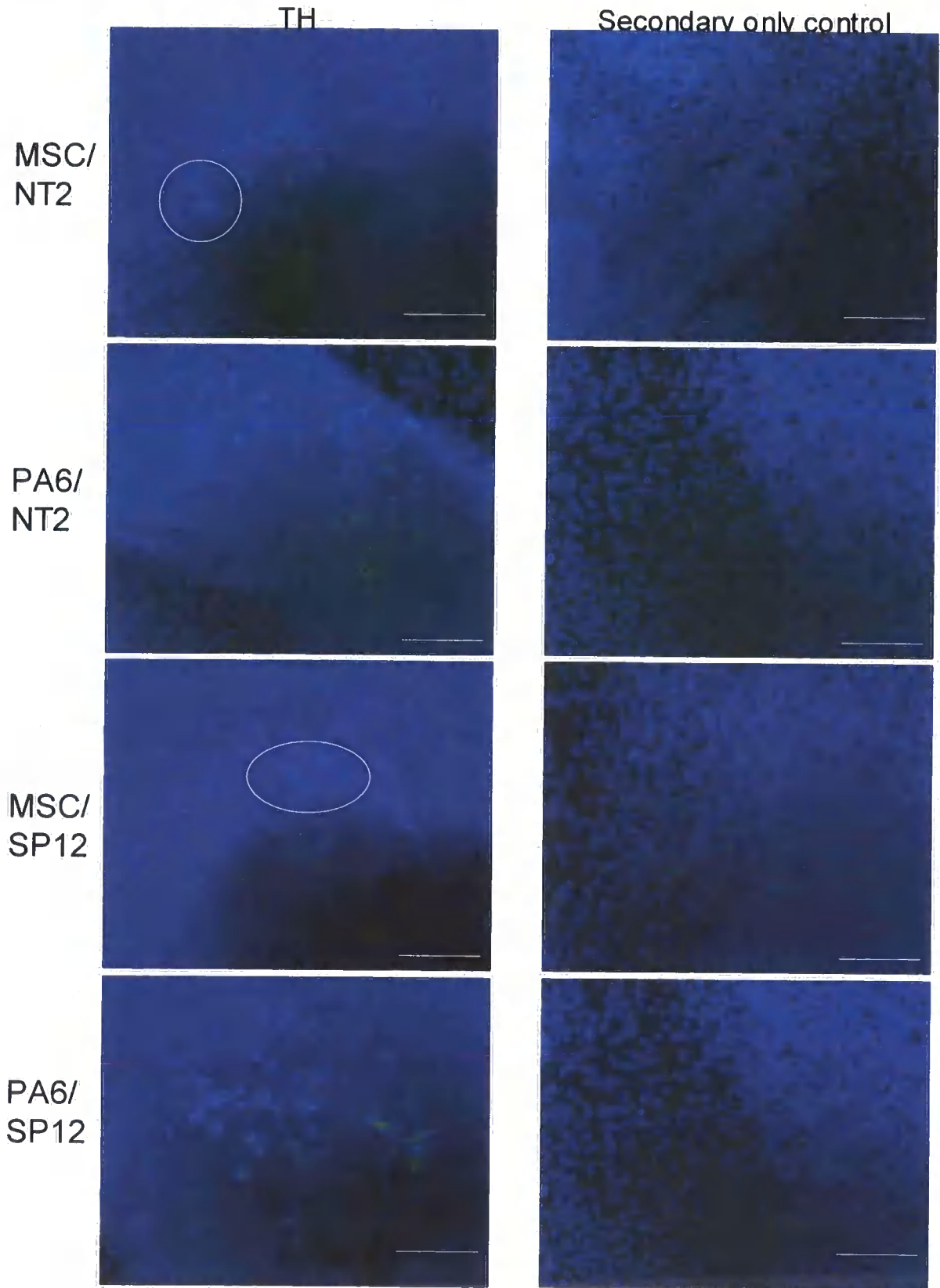


Figure 6.8: Merged images (produced in Adobe Photoshop) of Beta III tubulin immunostaining (FITC) against a background of Hoescht stained nuclei in 17 day old co-cultures of stromal and embryonal carcinoma cells. The conditions represent Ntera2 (NT2) cells in co-culture with MSCs or PA6 cells and Tera2.cl.SP12 (SP12) cells in comparable co-culture conditions. The secondary only control conditions were not treated with any primary antibody just the vehicle in which antibodies were diluted (SBP), prior to treatment with the appropriate secondary antibody for Beta III tubulin, these images were also merged with the corresponding Hoescht image to show cells were detectable. As expected there is an absence of any FITC detection in the secondary only controls. The phase images show the confluent nature of the cultures at the point of fixation and staining. Scale bars are approximately equal to 100 μ m. Representative sample images of n=3 biological repeats are shown. The merged images highlight that the cultures are very confluent and that only some areas of the cultures imaged were positive for Beta III tubulin expression.

Figure 6.9: TH staining in EC/Stromal cell co-cultures



To try and overcome the problem of the embryonal carcinoma cells predominating in the co-cultures, retinoic acid was used to try and direct their differentiation for 3 days, prior to subsequent co-culture for 17 days. In these experiments only the Tera2.cl.SP12 cell line was used to make the comparison as it had shown in broad terms the greatest capacity to up regulate marker expression and as such seemed the most promising vehicle to identify any differences that retinoic acid pre treatment of the embryonal carcinoma cells may bring about. Figure 6.10 displays phase images that reveal the co-cultures of retinoic acid pre treated embryonal carcinoma cells with PA6 or mesenchymal stem cells are not heavily overgrown. This is not observed when using untreated embryonal carcinoma cells which produce very cluttered cultures. However in the mesenchymal stem cell cultures at 7 days there are signs of floating, probably dead, cells. Even at day 1 the mesenchymal stem cells do not show the expected morphology and as such may be undergoing a stress response, prior to possible death. This is unsurprising given that the protocol has previously been used/refined for use with PA6 cells which respond seemingly more favourably to it. Although this may be the case even when using PA6 cells with retinoic acid pre treated embryonal carcinoma cells, one passage of such cells produced very confluent cultures, so their proliferative nature had not in 3 days been fully brought under control, the result being one sample that appeared over grown in some areas, with clear signs of dead cells floating in the media.

Immunocytochemistry was again used to assess Beta III tubulin and tyrosine hydroxylase expression in co-culture samples. Retinoic acid treated embryonal carcinoma cells following co-culture with PA6 cells or mesenchymal stem cells show Beta III tubulin expression (Figure 6.11) and a faint but detectable level of tyrosine hydroxylase (Figure 6.12). There appear to be no real differences in these samples relative to comparable untreated embryonal carcinoma cell co-cultures. The number of tyrosine hydroxylase positive cells is a tiny proportion of the entire culture, with more cells being broadly neuronal (Beta III tubulin positive) as might be expected and as was found previously. The same technique was also employed to address the question as to whether the co-cultures contained any stromal cells at 17 days. A human specific nestin antibody was used that would identify the vast majority/possibly the whole embryonal carcinoma cell based population. Figure 6.13 and supporting Appendix E show that the bulk of the co-cultures were heavily nestin

positive (indicated by the upper heavy condition images), where as in less confluent areas there appeared in PA6/Tera2.cl.SP12 co-cultures to be nestin negative cells, indicated by the presence of positive Hoescht stained nuclei but a lack of nestin staining that morphologically in the phase images are disparate from what one would expect for an embryonal carcinoma cell or a derivative of it. Mesenchymal stem cell co-cultures though even in the less confluent areas show nestin staining that correlates with the presence of Hoescht stained nuclei, the supporting phase image also shows the samples to have observable characteristic embryonal carcinoma cell type morphologies. Therefore it would appear there may be a small number of stromal cells left in the co-cultures at 17 days or possibly particularly in the case of the mesenchymal stem cells none at all. Even with the retinoic acid pre treatment the embryonal carcinoma cells still seem to predominate to the detriment of the stromal cell types.

Figure 6.10: The morphology of R.A. treated EC/Stromal cell co-cultures

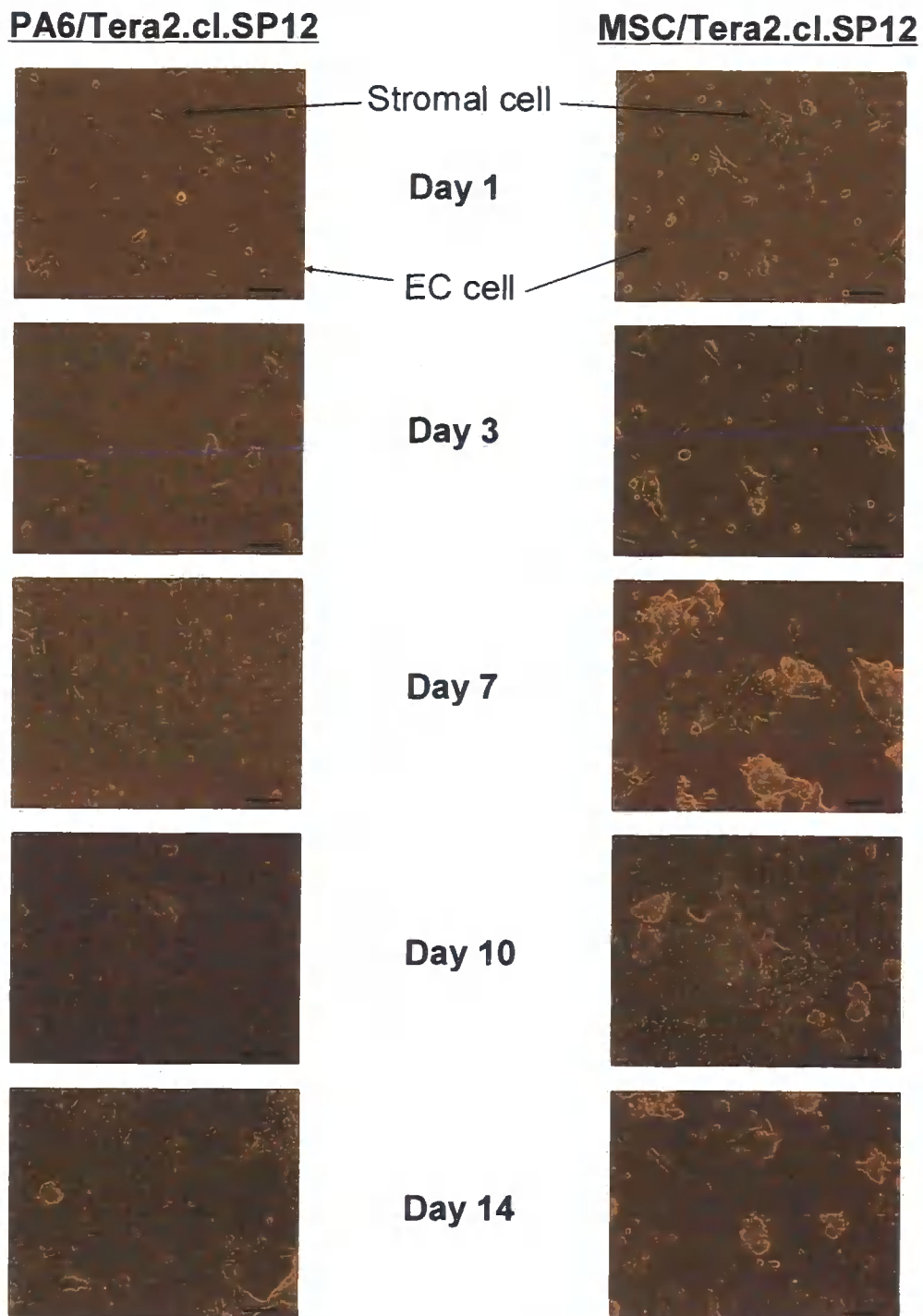


Figure 6.10: Phase images of Tera2.cl.SP12 cells (pre treated for 3 days with 10 μ M retinoic acid) in co-culture with either murine PA6 or rat mesenchymal stem cells at different time points. The bright clumps of cells/debris clearly present at 7 days in MSC/Tera2.cl.SP12 may represent dead cells. When retinoic acid is used the co-cultures appear far less confluent than when it is omitted from the differentiation strategy. Scale bars are approximately equal to 100 μ m.

Figure 6.11: Beta III tubulin staining in R.A. treated EC/Stromal cell co-cultures

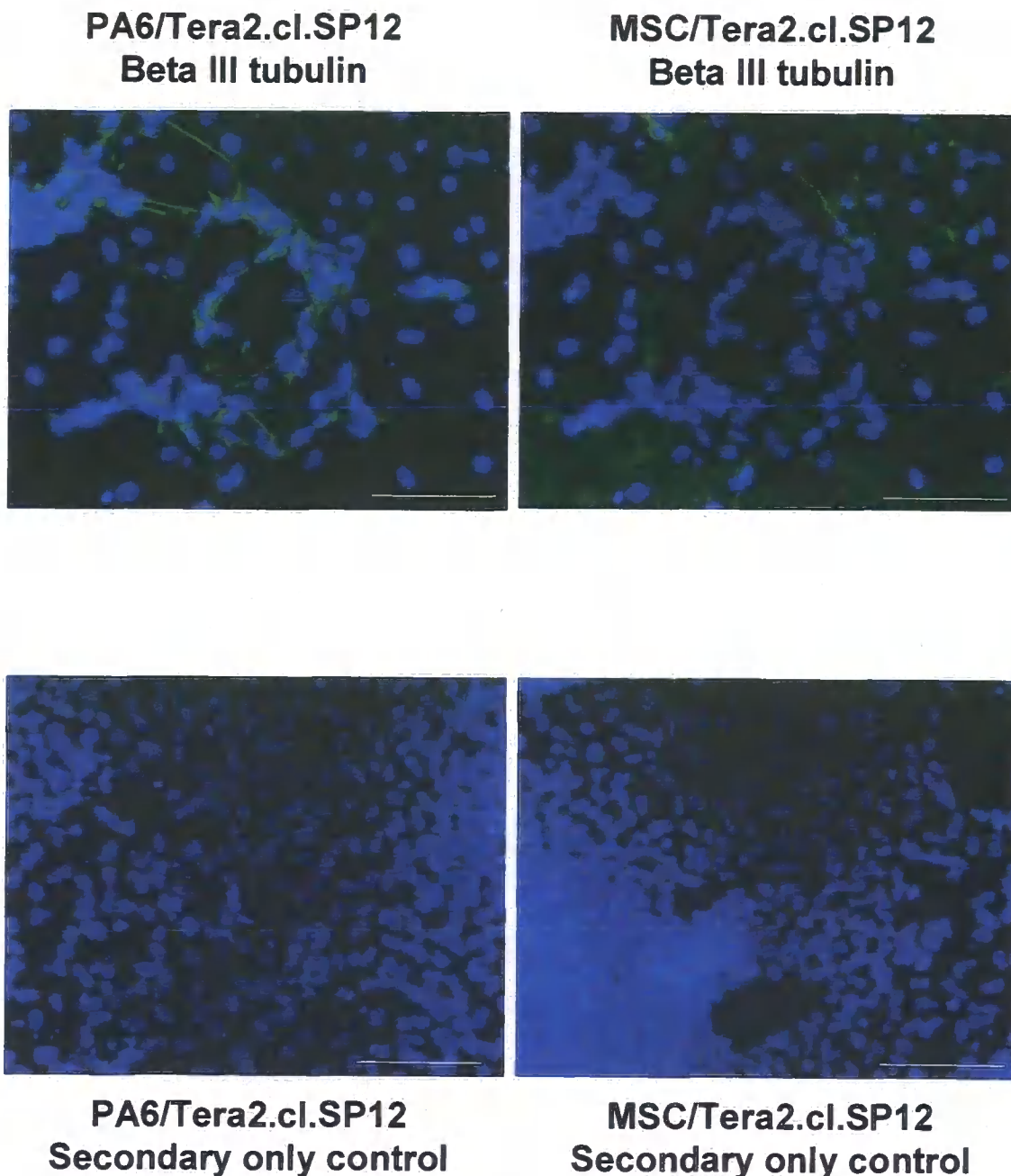


Figure 6.11: Merged FITC/Hoescht images (produced in Adobe Photoshop) showing Beta III tubulin immunostaining in Tera2.cl.SP12 cells (pre treated for 3 days with 10 μ M retinoic acid) then co-cultured with either murine PA6 or rat mesenchymal stem cells for 17 days. The secondary only control conditions were not treated with any primary antibody just the vehicle in which antibodies were diluted (SBP), prior to treatment with the appropriate secondary antibody for Beta III tubulin. As expected there is an absence of any detection in the secondary only controls, the merge with the corresponding Hoescht image confirms the presence of cells by staining their nuclei. Scale bars are approximately equal to 100 μ m. Representative sample images of n=3 biological repeats are shown. It is clear that only some of the total cellular population are positive for Beta III tubulin, areas were imaged that were relatively sparsely populated to try and alleviate the problem of the Hoescht background drowning out the FITC detection when the images were merged.

Figure 6.12: TH staining in R.A. treated EC/Stromal cell co-cultures

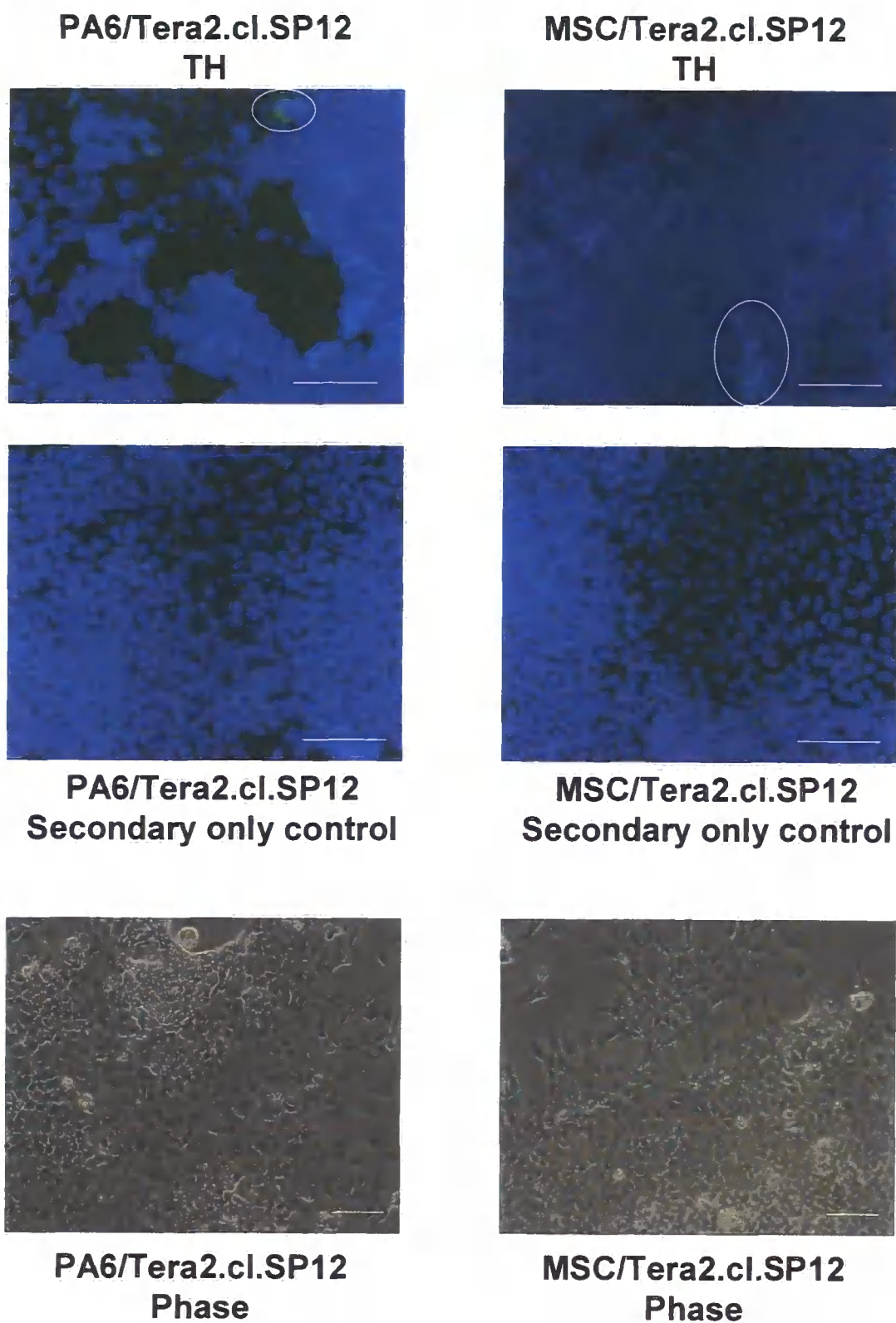
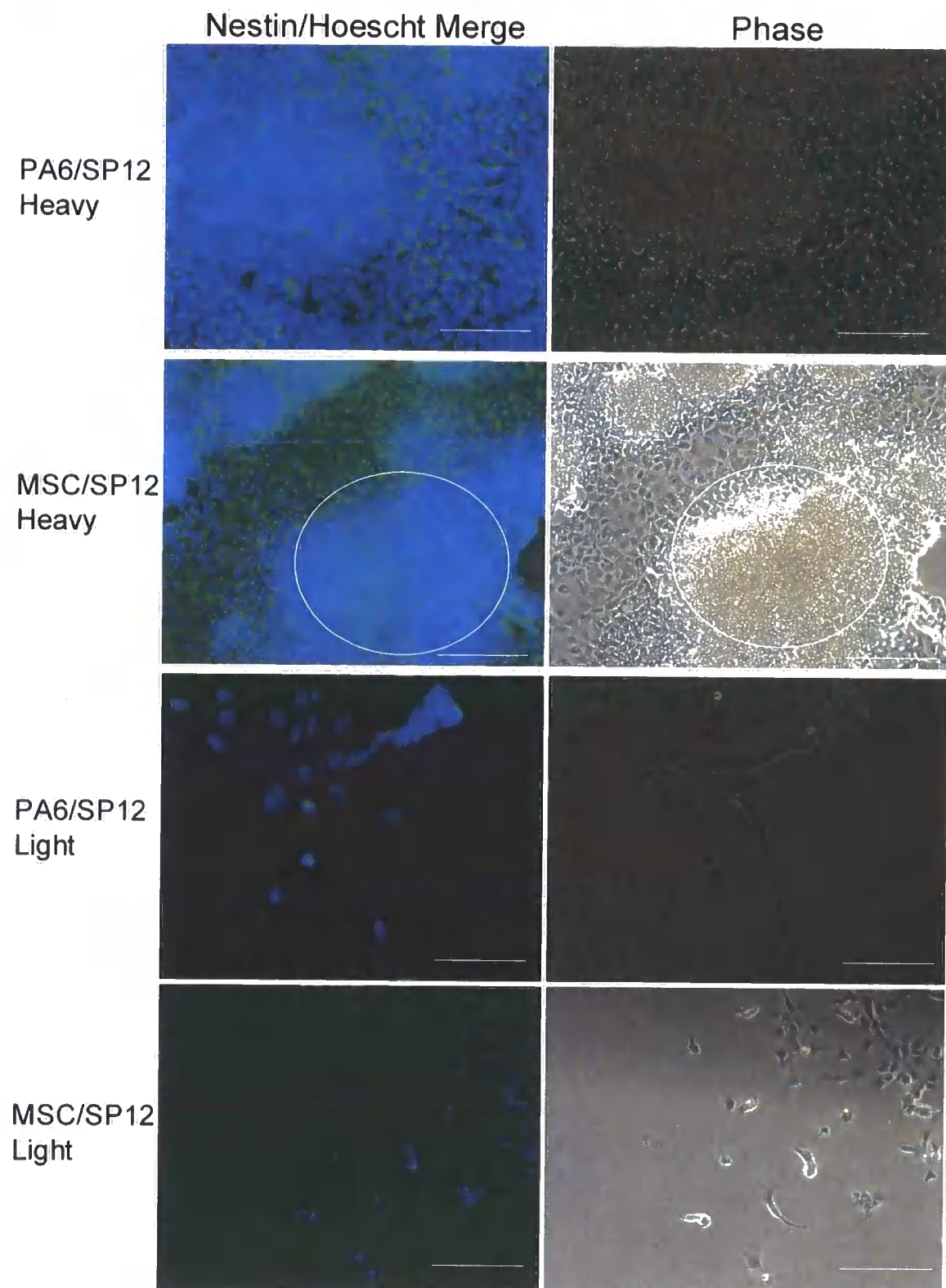


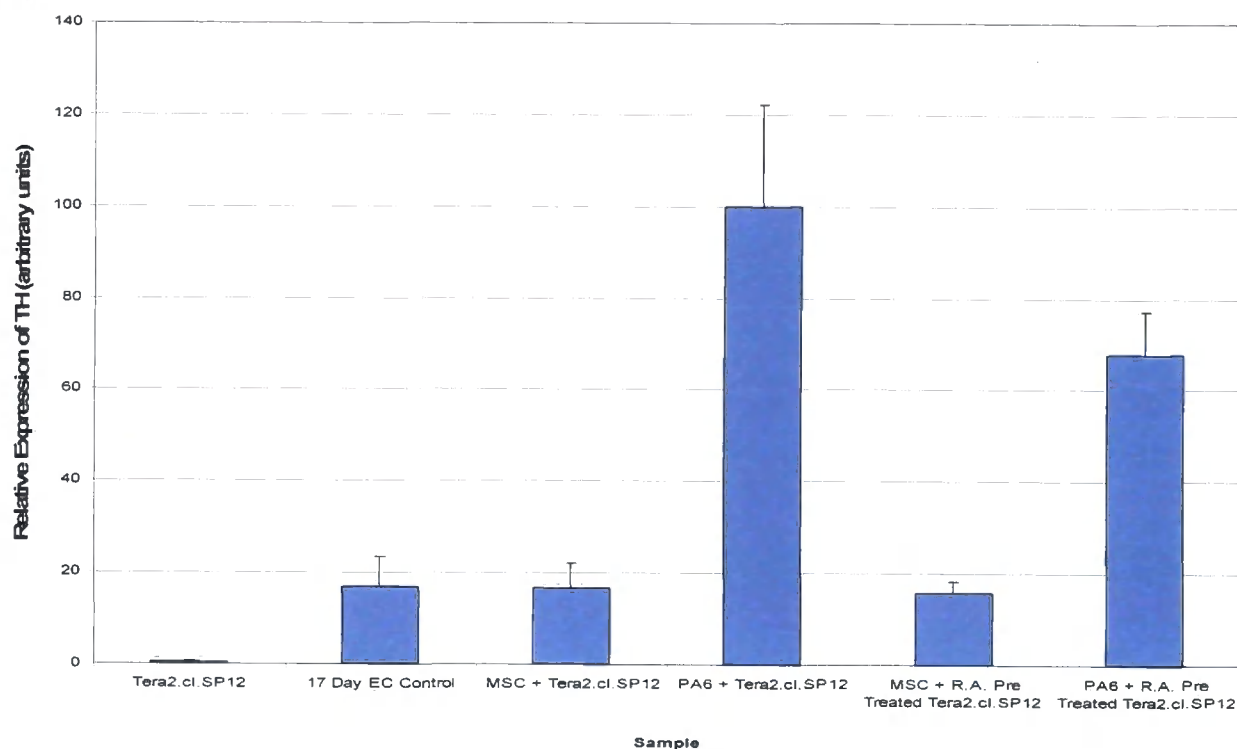
Figure 6.13: Nestin staining in R.A. treated EC/Stromal cell co-cultures



One final real time PCR screen was carried out to compare mRNA levels of neuronal and dopaminergic markers in untreated and 3 day retinoic acid pre treated Tera2.cl.SP12 cells co-cultured for 17 days with PA6 cells or mesenchymal stem cells. A comparison was also made to Tera2.cl.SP12 embryonal carcinoma cells left to grow for 17 days in the medium used for the co-culture step. Figure 6.14A shows that for tyrosine hydroxylase there is no real difference between retinoic acid pre treated or untreated embryonal carcinoma cells following the co-culture step. The mesenchymal stem cell co-cultures appear almost identical to the 17 day embryonal carcinoma control suggesting that the mesenchymal stem cells may well be under stress and as such exert less of an effect prior to potentially dying off. Figure 6.14B shows that for Beta III tubulin the 17 day embryonal carcinoma control samples actually display the highest level of mRNA expression for this neuronal marker, though there is a noticeable variation in the results probably due to the total absence of any control over the differentiation process. Within the context of the experimental error there is no real difference between the co-culture samples, regardless of whether or not the embryonal carcinoma stem cells are given a retinoic acid pre treatment. Figure 6.15A shows that in terms of dopamine receptor 2 mRNA expression, retinoic acid pre treatment of the embryonal carcinoma cells has no real effect in comparison to using their untreated counterparts. In this instance expression in the control is greater than in the mesenchymal stem cell co-culture conditions supporting the concept that the conditions are sub optimal for using this cell type. Figure 6.15B demonstrates similar trends for Lmx1a, suggesting once again that retinoic acid pre treatment offers no real advantages and that mesenchymal stem cell co-culture of embryonal carcinoma cells is not adequately facilitated using the methods followed in this work. Overall therefore exposing the Tera2.cl.SP12 cell line to retinoic acid prior to co-culturing it with PA6 cells or mesenchymal stem cells for 17 days using the protocol outlined appears to offer no advantages in driving it towards the acquisition of a dopaminergic phenotype.

Figure 6.14: TH & Beta III tubulin expression in R.A. treated EC/Stromal cell co-cultures

A.



B.

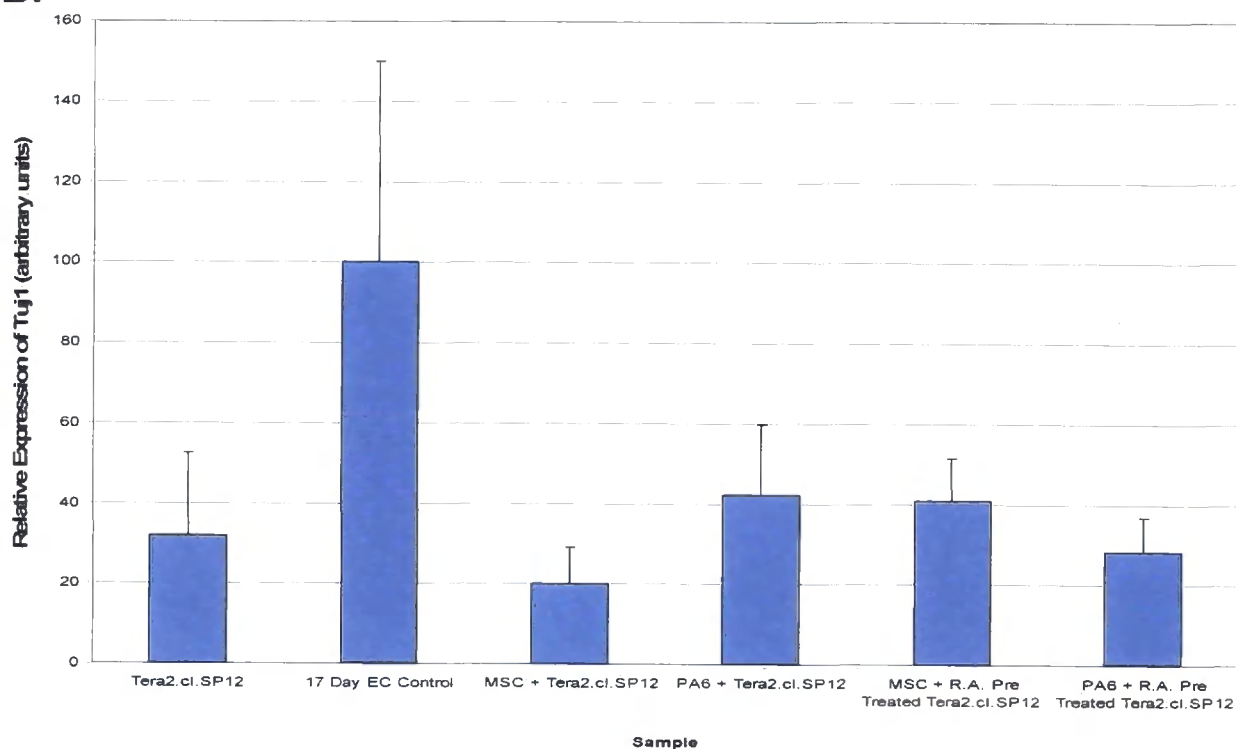


Figure 6.14A: Relative expression of human TH mRNA in either undifferentiated or 3 day $10\mu\text{M}$ retinoic acid pre treated Tera2.cl.SP12 cells co-cultured with rat MSCs or murine PA6 cells for 17 days. The 17 day EC control samples represent Tera2.cl.SP12 cells left to grow for this duration in just the media used for the co-culture step in the absence of any other cell type. The Tera2.cl.SP12 condition represents the EC cell starting material. Although by eye there appears to be the possibility of statistically significant differences between the starting material and the test conditions in particular the PA6 + Tera2.cl.SP12 samples this is not the case. **B:** As above but for human Beta III tubulin. In all cases error bars represent the standard deviation of the mean; $n=3$.

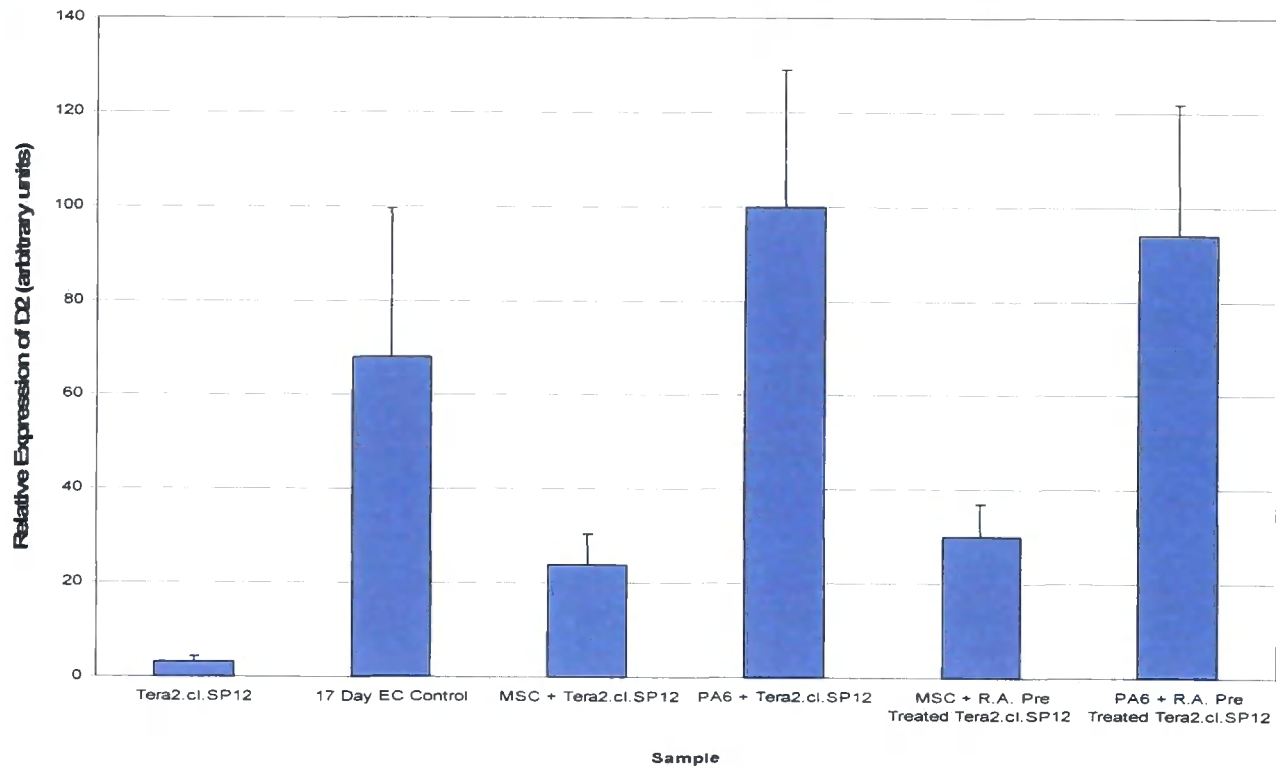
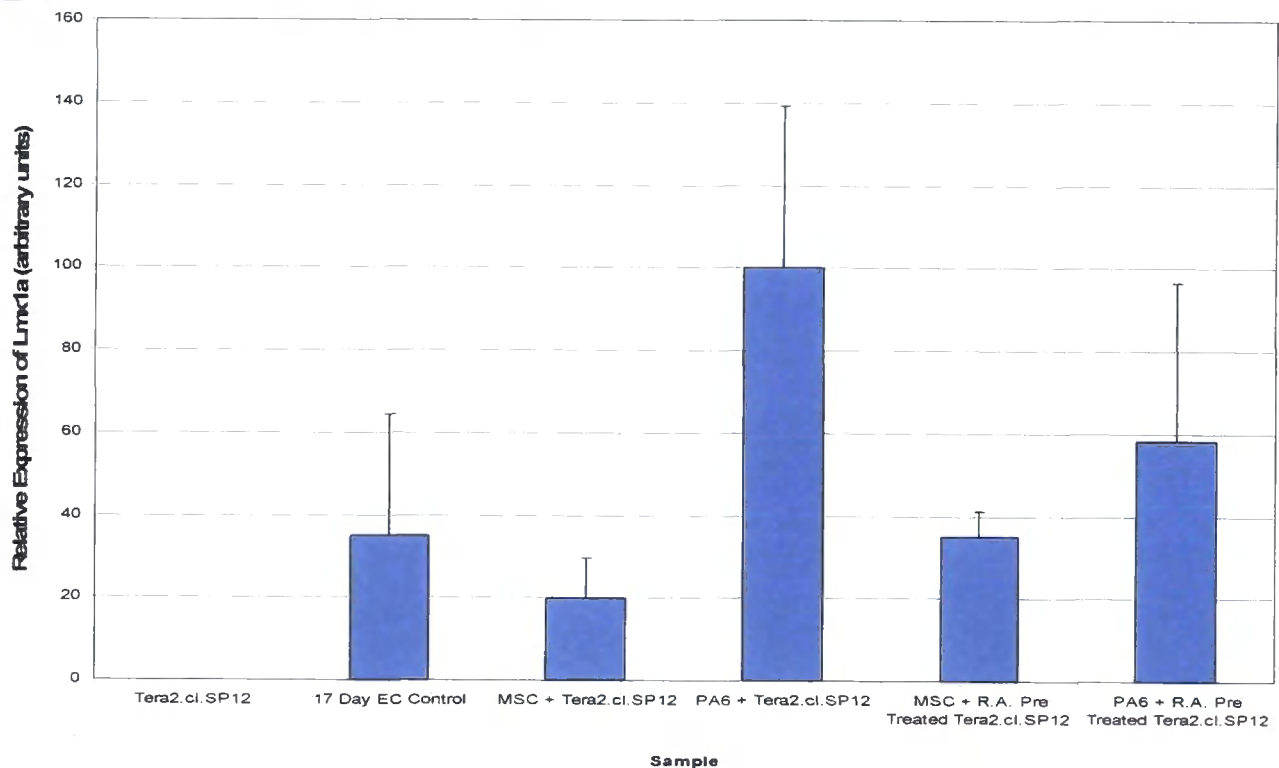
Figure 6.15: D2 & Lmx1a expression in R.A. treated EC/Stromal cell co-cultures**A.****B.**

Figure 6.15A: Relative expression of human D2 mRNA in either undifferentiated or 3 day 10 μ M retinoic acid pre treated Tera2.ci.SP12 cells co-cultured with rat MSCs or murine PA6 cells for 17 days. The 17 day EC control samples represent Tera2.ci.SP12 cells left to grow for this duration in just the media used for the co-culture step in the absence of any other cell type. The Tera2.ci.SP12 condition represents the EC cell starting material. **B:** As above but for human Lmx1a. In all cases error bars represent the standard deviation of the mean; n=3. Again although by eye there appear to be some conditions which show variations which could be viewed as potentially significant no statistically relevant differences were found.

6.4 Discussion

The main focus of this Chapter was to compare and contrast the Tera2.cl.SP12 cell line to its Ntera2 counterpart to try and gauge its potential for dopaminergic differentiation; given that the Ntera2 system has been used previously in the published literature in the context of studying dopaminergic neurogenesis via a number of established protocols such as those used in Ravindran & Rao, 2006, and Schwartz et al., 2005. A PA6 co-culture approach similar to that of Schwartz et al., 2005 was adopted for use and in the initial investigations, a number of markers associated with a neuronal or dopaminergic phenotype displayed an apparent mean up regulation in their expression that was particularly marked in PA6/Tera2.cl.SP12 co-cultures, however only Beta III tubulin, Lmx1a and Msx1 showed a statistically significant increase in their mRNA expression relative to the starting material. In contrast PA6/Ntera2 co-cultures displayed statistically significant increases in tyrosine hydroxylase, dopamine receptor 2, Lmx1a, Msx1 and Nkx 6-1 mRNA expression relative to the Ntera2 starting material. These samples though, on average, showed a relatively low expression of these markers in contrast to their Tera2.cl.SP12 counterparts. Therefore it would appear that although these may be statistically relevant differences in some cases they might not represent biologically meaningful variations. It does though suggest that the Ntera2 cells offer a more reproducible system that may give more results to which a statistical degree of significance can be attributed. In contrast the Tera2.cl.SP12 cells appear to offer a system that shows slightly less reproducibility but in absolute terms a greater ability to up regulate dopaminergic markers. In essence the two cell lines are probably both suitable for use but offer different advantages and disadvantages.

The Tera2.cl.SP12 cells appear more able to up regulate Beta III tubulin mRNA showing a statistically significant difference in this initial screen (Figure 6.3B) to both their Ntera2 counterparts when the two cell lines are co-cultured with PA6 cells and to the Tera2.cl.SP12 starting material. However in a later screen (Figure 6.14B) comparable samples of PA6 co-cultured Tera2.cl.SP12 cells do not differ to such a degree in relation to the starting material. This highlights neatly the problem of reproducibility found when using the Tera2.cl.SP12 system. This is not dissimilar to

the scenario seen in earlier work where in Chapter 4 Wnt1 in the presence of retinoic acid had an effect on dopaminergic and neuronal marker expression at 7 days in adherent cultures, whilst in Chapter 5 it exerted little or no impact in comparable conditions for no apparent reason. Despite this though, it is not possible to ignore the often strong trends seen when using Tera2.cl.SP12 cells and ultimately if up regulation of markers is a positive which it may well be in a simple differentiation model then such cells are well suited to this purpose. In comparison it would appear that Ntera2 embryonal carcinoma stem cells offer a more reproducible system for the purpose investigated, but they are seemingly less able to produce such striking differences in relation to those seen for the Tera2.cl.SP12 cells.

Primary rat stromal cells were also tested for their ability in co-culture to induce dopaminergic marker expression. Only Msx1 was up regulated to a statistically significant degree in Tera2.cl.SP12 co-cultures, but 6 markers were up regulated to such an extent in Ntera2 co-cultures. Subsequent data make it questionable if any of these differences were of biological importance but the statistics do reinforce the concept that the Ntera2 system is more measured, controlled and possibly less aberrant than the Tera2.cl.SP12 cell line as Ntera2 samples consistently give rise to more reproducible results that allow statistical relevance to be more often attributed. To some extent this could be an advantage but it is also a risk in that it may give some results that are biologically of little value a statistical meaning and this should be guarded against.

In this regard the most interesting observations from the initial real time PCR data are the concomitant rises in Msx1 and Nkx 6-1 mRNA expression levels. Msx1 is thought to be a suppressor of Nkx 6-1 (Abeliovich & Hammond, 2007) so if Msx1 is up regulated to a statistically significant degree, one would predict Nkx 6-1 levels would show a reduction, but this is not the case. This suggests that different cells within the culture are up regulating the markers independently or there is a lack of control over the system. This concept (of a lack of control) is supported by the total lack of tyrosine hydroxylase protein expression and the lack of induction (which might be expected) of Beta III tubulin at this level. Ultimately if protein expression is not correctly monitored then as proteins are the functional components of a cell, it is highly unlikely that the expected behaviour of the cells under investigation will occur.

In the context of the Msx1 observation if the mRNA for Msx1 is not translated into protein (as appears to be the scenario for tyrosine hydroxylase) then Nkx 6-1 levels are unlikely to be repressed at any level and up regulation of mRNA for markers can take place at will. To extend this notion it appears that especially in relation to 17 day embryonal carcinoma cell only controls that markers are being up regulated over time and not in a controlled fashion due to the co-culture. This is not true in all cases but it is clear from the amount of nestin expression and seemingly total absence or at best minimal presence of stromal cells at 17 days that there is insufficient control over the embryonal carcinoma cells. It would appear that given that using retinoic acid to try and gain control over the embryonal carcinoma cells helps very little, evidenced by the slight drop in marker expression, that perhaps the mitotic inactivation of a confluent monolayer of PA6 cells as in Schwartz et al., 2005, is the best way forward, to achieve control over the co-culture system. Alternatively as in Hayashi et al., 2008 key components such as Wnt5a that constitute the stromal derived inducing activity need to be defined and then if possible applied in an exogenous fashion in a controlled way, in an analogous manner to that by which Sonic hedgehog and Fibroblast growth factor 8 are often effectively used for example in Yan et al., 2005. Co-culture could also be carried out in the presence of Fibroblast growth factor 20 as in Correia et al., 2008, application of this molecule increased the percentage of tyrosine hydroxylase positive neurons obtained from human embryonic stem cells five fold from 3 to 15%.

The somewhat cluttered nature of the co-cultures at 17 days also detracts from the immunocytochemical results, because the presence of Beta III tubulin and tyrosine hydroxylase although detectable is only observed in discrete areas, there is no consistency or uniformity to the cultures. As such any comparison between conditions is difficult to make especially for the more sparsely detectable tyrosine hydroxylase. The only real observation that can be made is that there are neuronal like structures with cell bodies extending thin neuritic processes that stain positively for Beta III tubulin and tyrosine hydroxylase, this at least supports the concept that the PA6 cells and mesenchymal stem cells can secrete neurotrophic factors that may cause some differentiation amongst the melée of proliferating embryonal carcinoma cells. In addition the high level of nestin expression in the co-cultures is indicative of the presence of relatively immature neural cells and as such supports the idea that

more stromal cells are necessary to drive the process of embryonal carcinoma cell differentiation, this would be a necessity in future work.

Other aspects to consider in any additional studies building on this Chapter would be is the effect of treating the cells with retinoic acid for 3 days undone or undermined by disrupting them when seeding them out a second time for the co-culture step? It may be better to treat the embryonal carcinoma cells with retinoic acid (possibly for longer than 3 days) then seed the stromal cells into the vessel in which the retinoic acid treated embryonal carcinoma cells already reside. In addition the protocol used is refined for use with the PA6 cells, it may well be the mesenchymal stem cells are dying off in the conditions used. Therefore if extending this work, it would be advisable to try and define more optimal conditions in which to grow the mesenchymal stem cells in conjunction with the embryonal carcinoma cells. Both mesenchymal stem cells and embryonal carcinoma stem cells grow in DMEM but when seeded out together in this medium (supplemented as it would be for mesenchymal stem cells) neither cell type showed any sign of adhering. This was just a small test study and the problem may have been with the plastic ware. Alternatively it may be the two cell types do not directly interact well or that one or both cell types have a negative effect on each other via the factors they may secrete. Under the conditions tested here though it is quite clear that the mesenchymal stem cell co-cultures are seemingly sub optimal not just for use as a means to try and produce dopaminergic neurons but also as a means to study the process. The PA6 based co-cultures are less sub optimal but the method needs further refinements to try and produce more conclusive results. It may be that co-culture methods involving mesenchymal stem cells or PA6 cells are best combined with the use of other dopaminergic inducing agents such as Sonic hedgehog and Fibroblast growth factor 8 as in the work of Shintani et al., 2008 using mesenchymal stem cells, or even the use of such molecules in conjunction with ascorbic acid application and Nurr1 over expression as in the work of Kim et al., 2006, using PA6 cells. In the light of the fact embryonal carcinoma cells are highly proliferative they may also be viewed as not well suited to co-culture based approaches that in themselves by combining the use of two cell types potentially create a greater burden on the culture media and possibly a more stressful cellular environment. The use of conditioned media may help in this

regard and would also eliminate the possibility of embryonal carcinoma cells having inductive effects on their stromal cell counterparts.

In summary it is not possible to make any inference as to whether highly conserved factors between species can drive the dopaminergic differentiation of human embryonal carcinoma cells as the range of molecules that could be secreted by the two stromal cell types is too vast and the method employed in this study too sub optimal especially when using the mesenchymal stem cells. In turn it is hard to make any conclusive remarks as to whether mesenchymal stem cells can direct the dopaminergic differentiation of human embryonal carcinoma stem cells in the conditions tested as the mesenchymal stem cells are clearly not behaving in a characteristic fashion, exemplified by the observation that they do not show an expected morphology even after just 1 day of co-culture. PA6 cells as would be expected do seem to be able to cause an induction of dopaminergic markers, especially in Tera2.cl.SP12 cells at least at the level of the mRNA. Retinoic acid pre-treatment of such embryonal carcinoma cells prior to co-culture appears to offer no advantage in terms of inducing dopaminergic or neuronal marker expression relative to the use of untreated Tera2.cl.SP12 cultures as the starting material. Therefore it would appear that the most balanced view of this work is that mesenchymal stem cells in more optimised conditions may be able to show positive effects on the dopaminergic differentiation of embryonal carcinoma cells, although they appear to exert a minimal effect in the conditions tested here. PA6 cells are perhaps more able to function in the context of the experimental protocol used but they could possibly have a greater effect if more of them were used. In addition it would appear that using the method outlined earlier in this chapter that Tera2.cl.SP12 embryonal carcinoma stem cells are as viable and potent a model for the study of dopaminergic neurogenesis as the Ntera2 cell line.

Chapter 7

Final Discussion

The investigations undertaken in this thesis have attempted to assess the capacity for dopaminergic differentiation of the human embryonal carcinoma cell line Tera2.cl.SP12. The findings presented offer an insight into the potential of this model as a research tool but further work is required to give a broader perspective.

To begin with it would appear that when treated with retinoic acid the Tera2.cl.SP12 cell line is able to up regulate the expression of markers associated with a dopaminergic phenotype over time. However the dopaminergic differentiation of the system is seemingly unaffected by the use of more physiologically relevant oxygen pressures, in contrast to work in rat mesencephalic precursor cultures (Studer et al., 2000). This is most probably due to embryonal carcinoma cells not being derived from the brain and as such *in vivo* mimicry of the environment in this organ is less likely to exert an effect on them in comparison to that seen with cells of midbrain origin. There is also the possibility that 5% oxygen is not a low enough pressure to produce a clear effect as there may be a threshold level above which no tangible difference is observed. If such a situation exists and the 5% oxygen condition lies above the critical point for an effect to be detectable then this may explain these observations. Alternatively it may be that certain technologies such as lowered oxygen culture are more optimal in their usage where they offer the opportunity for recapitulation of the *in vivo* environment, rather than being looked upon as a means to induce a dopaminergic phenotype. The finding that when using embryonic stem cells 3.5% oxygen culture exhibited only a small but positive effect on dopaminergic differentiation (Kim et al., 2008) adds support to this concept. It may be that the main purpose of physiological oxygen culture is to reduce the oxidative stress that standard (21%) oxygen culture may engender. This though would still be of value if a relatively pure population of dopaminergic neurons could be generated.

In the pursuit of an enriched population of potential neurons displaying this particular neurotransmitter phenotype one molecule that may be of use is Wnt1. Although additional experiments to elucidate the mechanism by which it acts in synergy with retinoic acid are required, the findings in Chapter 4 are consistent with it having a positive effect on the production of Beta III tubulin positive cells that are potentially neurons. In addition it appears to possess the ability to up regulate dopaminergic marker expression after a relatively short period of exposure to it in conjunction with

retinoic acid in the Tera2.cl.SP12 cell line. Both of these elements are of appeal, in that, in an *in vitro* system where there are mixtures of cell types, the ability to first and foremost increase the neuronal population offers a starting point from which further more specific differentiation strategies can be focussed. Also in the context of creating screening assays, protocols are required that provide temporal efficiency; Wnt1 by displaying an early mode of action may help meet this requirement.

It could have been postulated that Wnt1 may have influenced Nurr1 expression (Castelo-Branco et al., 2003). In Chapter 3 retinoic acid was seen to have positive effects on the up regulation of the mRNA for this marker after just one day of differentiation, in a similar manner to that seen in Misiuta et al., 2006 for Nurr1 protein. Therefore one area for advancement of this study would be to over express Nurr1 to attempt to efficiently produce more neuronal cultures in a manner analogous to that in Hara et al., 2007. If this could be combined with the use of Wnt1 then potentially a very large number of potential neurons could be generated.

It has also been proposed that Wnt1 creates a permissive environment for inductive signals such as Sonic hedgehog and Fibroblast growth factor 8 to act (Burbach & Smidt, 2006). These molecules may offer the ability to produce seemingly highly neuronal cultures derived from the Tera2.cl.SP12 cell line, as demonstrated by immunostaining and a clear induction of Beta III tubulin mRNA. However Western blotting displays a less apparent induction, in contrast to the situation for dopaminergic markers which appear to be down regulated at the mRNA level by the terminus of the 17 day differentiation protocols outlined in Chapter 5, despite expression of tyrosine hydroxylase protein being clearly induced. The results obtained are therefore of interest as they indicate not only the need for further experimentation including functional testing but also that there is a complex series of interactions that lead to the specification of particular cellular fates that cannot simply be recognised or understood via the assessment of rising or falling levels of certain phenotypic indicators. Standard reverse transcription PCR is often used to display the induction of dopaminergic markers but this relatively simple on/off or at best weak to strong based expression analysis is a limiting factor in furthering our knowledge and understanding of the process of dopaminergic neurogenesis *in vitro*, as it does not account for the clear idiosyncrasies observed when using real time reverse

transcription PCR. The more widespread use of this technology could potentially be of benefit in broadening our grasp of how the process of generating dopaminergic neurons *in vitro* is regulated by certain molecules. It may be though in the work presented in Chapter 5 that the unexpected and varying observations arise from the production of other neuronal subtypes.

The situation is complicated still further in that the high level expression of a particular mRNA may reflect a high rate of turnover of the associated protein, which might correlate with less protein being detected in relative terms at the point when the sample is collected. A cellular system is essentially like a living organism in that it is in a state of constant flux. Therefore as such methods that just detect mRNA or protein levels for a particular marker are only of value as a starting point. In many respects techniques such as immunocytochemistry that can demonstrate the distribution of a given protein of interest are of more value, as they at least give an insight into the morphology of a culture. Methods that assess overall expression of a protein, for example Western blotting are perhaps more prone to error in the interpretation of the results they produce. This is exemplified by embryonal carcinoma cells appearing to express Beta III tubulin; it may be that the sensitivity of detection is so high that if a cell line with a propensity for neural differentiation is used then any more generic marker can be detected. This may also be influenced by loading volume with an excess of protein essentially causing saturation and a signal that gives an output of bands on the blot that are essentially indistinguishable. Great care therefore is required when putting expression data into context and whenever possible some aspect of functional testing is desirable.

The co-culture study in Chapter 6 reinforces this concept as clearly there is a lack of control over the Tera2.cl.SP12 cell line when using this differentiation strategy. This is evidenced by the concomitant rise in expression of mRNA for both Msx1 and Nkx 6-1. It would be expected from the model presented in Figure 4.2 that Nkx 6-1 would be repressed with rising levels of Msx1; that is of course if Msx1 mRNA is translated into functional protein. The rise in levels of Msx1 mRNA does not correlate with a fall or even low levels of Nkx 6-1 mRNA. In addition the rise in mRNA levels for dopaminergic markers like Msx1 and tyrosine hydroxylase is not reflected at least for tyrosine hydroxylase at the protein level. If Msx1 protein is similarly not present then

normal development cannot occur. Therefore at a number of stages the dopaminergic differentiation observed in the Tera2.cl.SP12 cell line using this method is not just uncontrolled but unrepresentative of what would be expected in the course of the regular development of this neuronal subtype as it is currently understood. The use of retinoic acid to try and gain control over this process also exhibits no positive effects. Indeed its usage appears to have negative implications for the dopaminergic differentiation of the Tera2.cl.SP12 cell line in co-culture with PA6 stromal cells, in a similar fashion to that reported for mouse embryonic stem cells in Kawasaki et al., 2000 though this data was not shown, merely given mention.

The main question to arise therefore is can a cancerous cell line be used as a developmental model? If cancer is seen simply as the result of an abnormality or abnormalities in development then ultimately the answer is no. There is no means to justify studying developmental processes in a system that is aberrant in its regulation of at least some of them. However the situation is not so simple in the broader context. Culture of cells *in vitro* may result in them evolving genetic and possibly other, for example morphological abnormalities. If animal models are used even though certain pathways may be conserved there could be species specific differences. Therefore in the light of the alternatives, cell lines like Tera2.cl.SP12 offer a human system, that although flawed, if used in conjunction with other models and in a suitably selective manner may be of some value. This is seen in the work that combines the use of Wnt1 and retinoic acid that could perhaps be translated into embryonic stem cells for which embryonal carcinoma cells are seen as a model. To this end, perhaps embryonal carcinoma cells would be better viewed in the context of dopaminergic differentiation strategies as a cell line with a propensity for neural differentiation as it is this feature that is being put to use not their more pluripotent stem cell like characteristics. In addition any model is essentially, even if only in a very minor way incorrect; an embryonic stem cell *in vitro* is a model of development for such a cell *in vivo*, and if an embryonal carcinoma cell is viewed as a model of an embryonic stem cell then it is essentially becoming a model of a model. Given the cancerous origins of an embryonal carcinoma cell it is unlikely to compensate for the flaws in the *in vitro* embryonic stem cell model and as such would occupy a very restricted and questionable niche, which would short sell such cells as they are robust, inexpensive to culture and can display quite rapid differentiation. Also dopaminergic

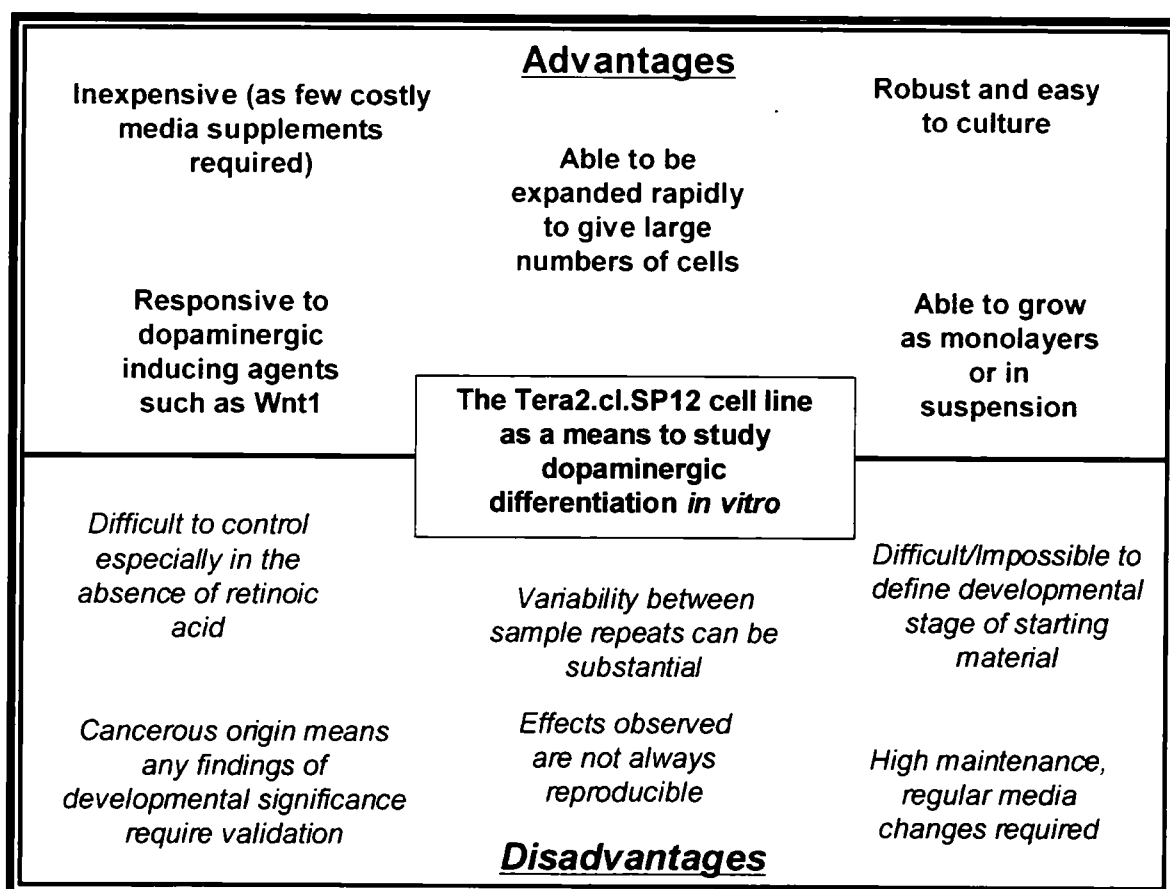
differentiation *in vitro* rarely takes place on the time scales seen *in vivo*, demonstrated in the work of Kawasaki et al., 2002 using primate embryonic stem cells. It would thus appear that although idealistic positions are in theory desirable, in practice the reality is very different and a compromise is required that encompasses balanced experimentation across a range of systems.

In the light of this the Tera2.cl.SP12 cell line is from the findings presented here most effectively utilised in approaches that are more highly controlled and make use of exogenously applied molecules such as Wnt1, Sonic hedgehog, Fibroblast growth factor 8 and ascorbic acid. Any further work in this system may well try to expand on the methods tested here, for example ascorbic acid could be investigated as an agent to induce differentiation and if viable for this task could be a replacement for retinoic acid. The effect of varying the concentrations of such agents is an additional approach not assessed here that could also be beneficial in deepening our understanding of the process. There is also the possibility that over expression of key determinants of a dopaminergic phenotype such as *Lmx1a* and *Msx1* could help provide the foundations for producing a highly enriched population of dopaminergic neurons. If this is possible, functional testing is required as the current findings are not sufficient to make any judgement on the ability of this cell line to act as a source of cells for application in screening assays. In the light of the variability sometimes seen and the lack of reproducibility observed with the Tera2.cl.SP12 cell line, it may be that rather than act as a model for embryonic stem cells, embryonal carcinoma cells provide a system that is complimentary to the development of strategies for the dopaminergic differentiation of embryonic stem cells.

Indeed if the transplantation of dopaminergic neurons derived *in vitro* is seen as desirable in the context of trying to remedy the loss of such cells in Parkinson's disease patients, then in keeping with the concept that the implantation of cells from a cancerous source is now deemed too precarious to contemplate, it is safe to say that embryonal carcinoma cells are never going to be a source of material for use in such treatment approaches. It may also be viewed as unlikely that they could provide a robust enough system in terms of producing suitably consistent results if used in screening assays, although as part of a range of assays, and given an awareness of their inherent limitations, the use of embryonal carcinoma cells in this setting is not so

far removed. It may well be that other cellular models are just as variable but this could only be assessed if in future a direct comparison was made. If this was unfavourable with respect to embryonal carcinoma cells then they may be limited to basic investigations. At the level of a fundamental research tool though they compare reasonably well against another embryonal carcinoma cell line Ntera2 that features in the literature and from the findings in this thesis may be a suitable means to investigate new and refine current methods aimed at the *in vitro* generation of dopaminergic neurons. If embryonal carcinoma cells are put to use in this manner and if any findings from their utilisation can be translated into embryonic stem cell or other model systems that are then used in an application that may help improve current therapies for Parkinson's disease then they are of undoubted value. Figure 7.1 summarises the potential advantages and disadvantages of the Tera2.cl.SP12 cell line as a means to study dopaminergic differentiation *in vitro*.

Figure 7.1: The advantages and disadvantages of the Tera2.cl.SP12 cell line as a model to study dopaminergic differentiation *in vitro*



Summary of key findings

- Retinoic acid treatment of Tera2.cl.SP12 cultures can induce expression of a range of dopaminergic markers over time.
- Lowered oxygen culture appears to have little detectable effect on the ability of the Tera2.cl.SP12 cell line to acquire a dopaminergic phenotype.
- Expression of Otx2 a potential determinant of a dopaminergic fate is down regulated rapidly by exposure of embryonal carcinoma cells to retinoic acid.
- Wnt1 shows a minimal ability to rescue the drop in Otx2 expression caused by retinoic acid.
- The application of Wnt1 can approximately double the number of Beta III tubulin positive cells obtained from Tera2.cl.SP12 cultures exposed to retinoic acid. It may also augment dopaminergic differentiation as well.
- Suspension culture of embryonal carcinoma cells may offer advantages when trying to promote the acquisition of a neuronal cell fate.
- Sonic hedgehog may be able to induce dopaminergic marker expression via a mechanism congruous with that observed in other experimental systems.
- The application of retinoic acid, Sonic hedgehog, Fibroblast growth factor 8 and ascorbic acid to embryonal carcinoma cell derived neurospheres treated with retinoic acid and Wnt1 appears to facilitate the production of cultures that are highly positive for the potential neuronal marker Beta III tubulin.
- The use of this cocktail of signalling molecules causes differential regulation of the mRNA and protein for dopaminergic markers such as tyrosine hydroxylase.

- PA6 co-culture can induce detectable expression of neuronal and dopaminergic markers in embryonal carcinoma cells.
- Co-culture driven differentiation of embryonal carcinoma stem cells does not appear to occur in a highly regulated or predictable fashion.
- Pre treatment of Tera2.cl.SP12 cultures with retinoic acid does not appear to increase their potential for subsequent co-culture induced dopaminergic differentiation.

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Appendix A

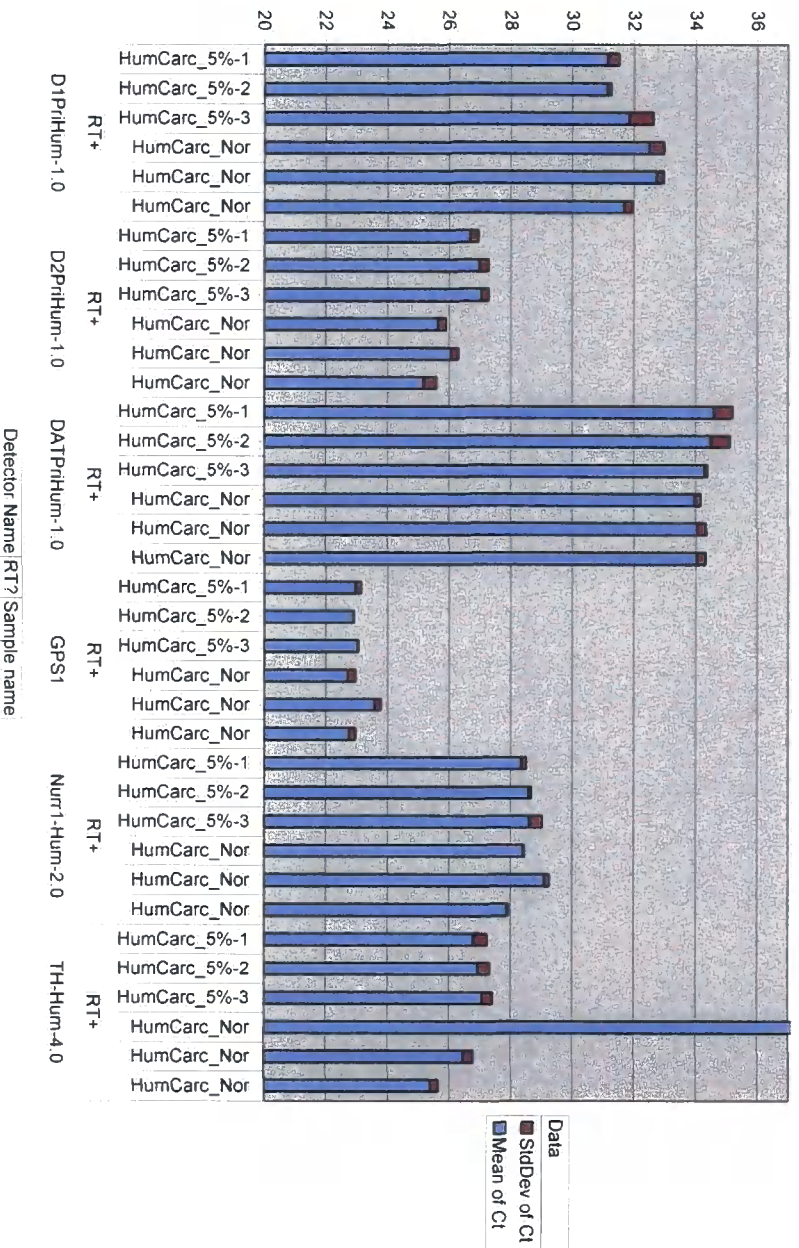
Buffer compositions for Western blot analysis

- 10x TBS – 6.1g Tris base (Fisher Scientific) and 43.8g Sodium Chloride (BDH Biosciences), dissolved in 500ml of distilled water, (adjust pH to 7.5 and autoclave).
- 1x TBS – dilute 100ml of TBS (10x) with 900ml of distilled water.
- 1x TBS-T – TBS (1x) with 0.2% Tween 20 (Sigma).

Appendix B

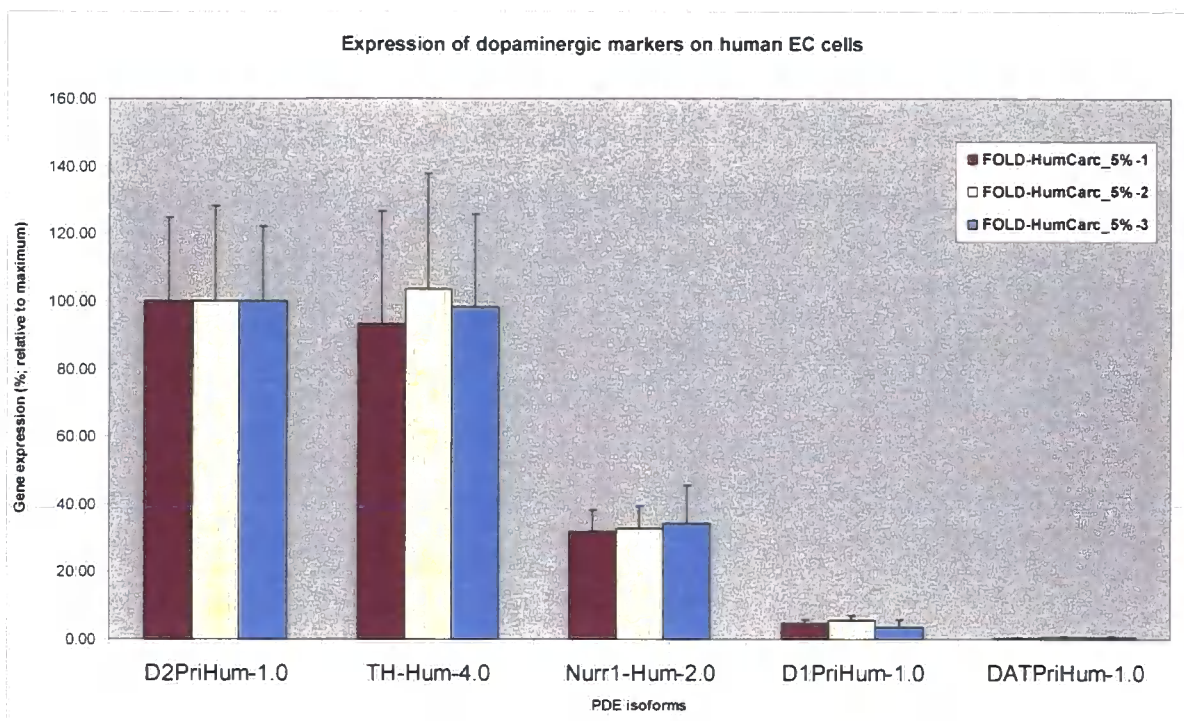
Supporting real time PCR data (courtesy of Dr. V. Lakics)

DAerg marker expression on human EC cells (4weeks RA) – raw Ct data (exponential scale) (note: the lower the value, the higher the expression)



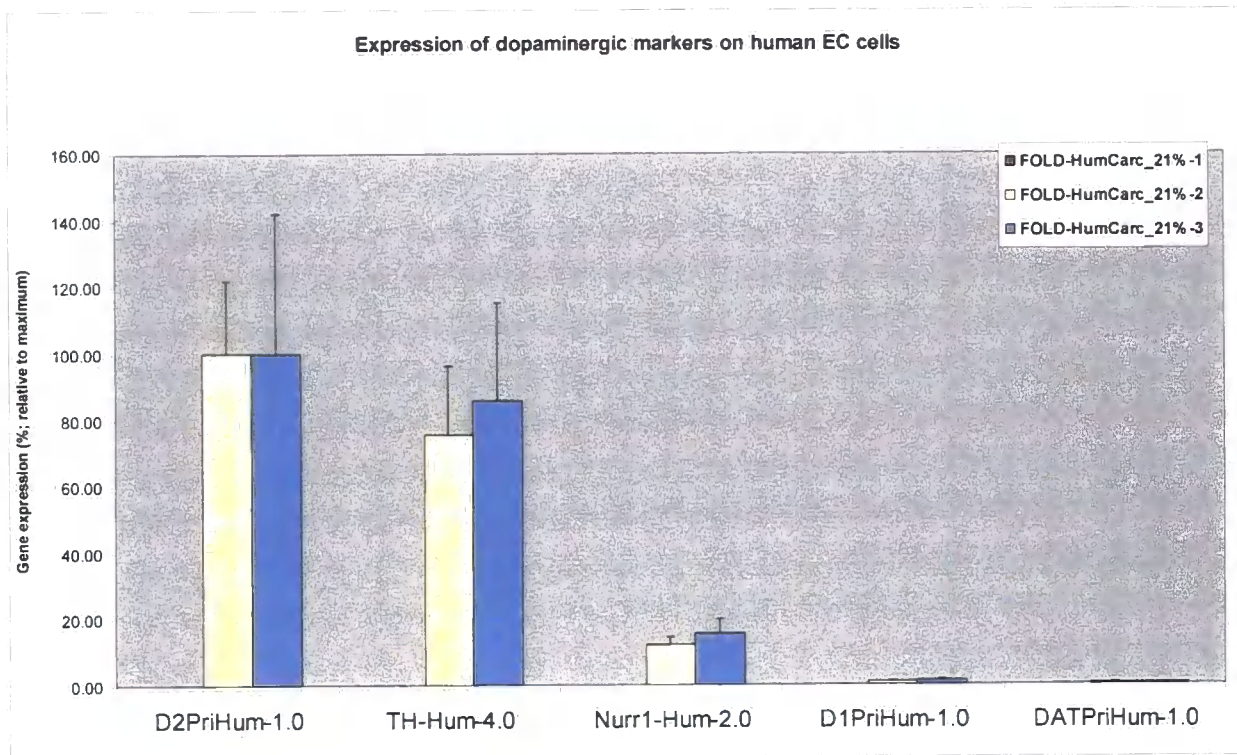
Appendix B: Dopaminergic marker expression in Tera2.cl.SP12 cells exposed to 10µM retinoic acid for 4 weeks in either 5% or atmospheric (normal, 21%) oxygen conditions.

Relative expression (linear scale) of DAerg markers on EC cells (5% oxygen)

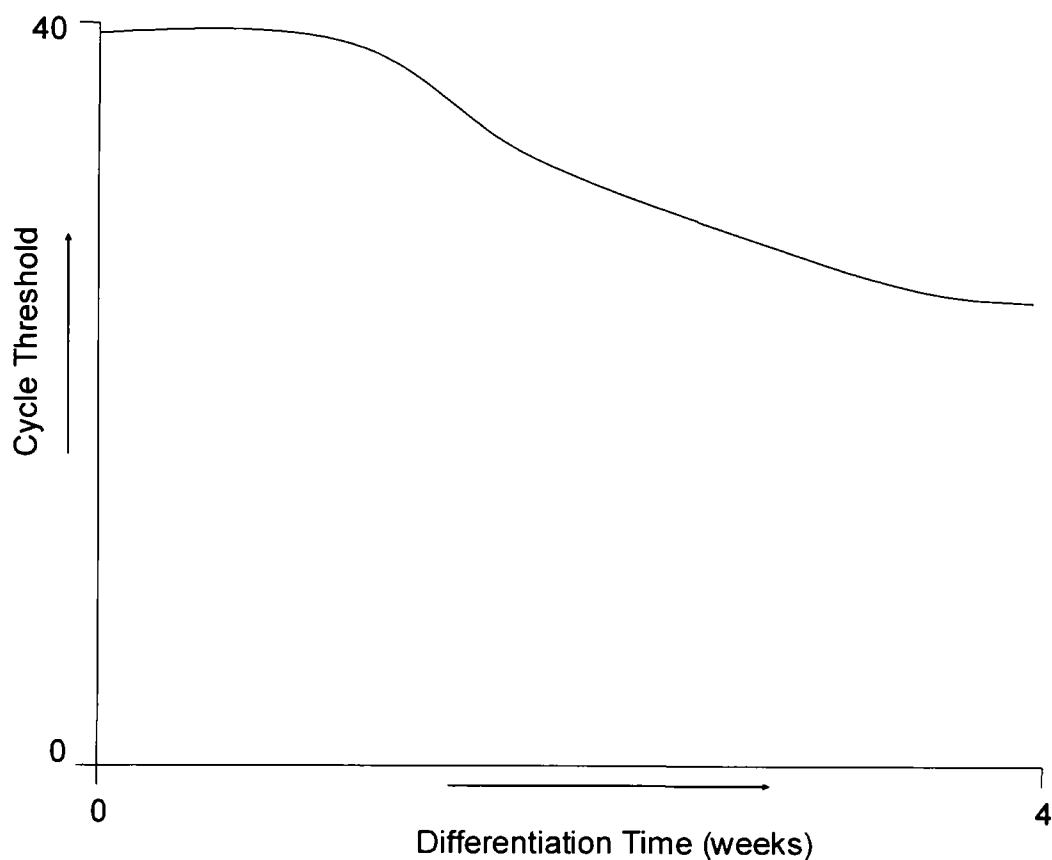


Appendix B: Dopaminergic marker expression in Tera2.cl.SP12 cells cultured for 4 weeks in the presence of 10 μ M retinoic acid in a 5% oxygen incubator.

Relative expression (linear scale) of DAergic markers on EC cells (21% oxygen)



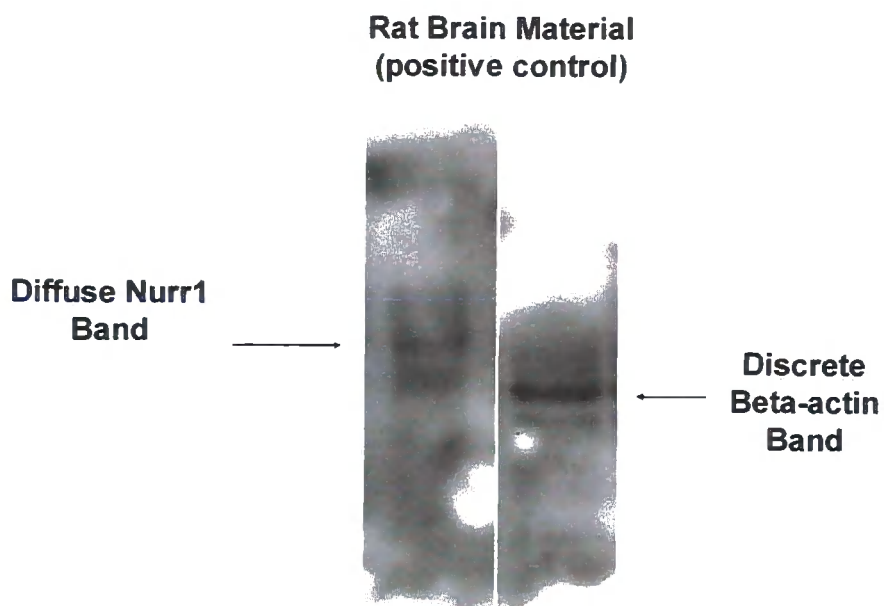
Appendix B: Dopaminergic marker expression in Tera2.cl.SP12 cells cultured for 4 weeks in the presence of 10 μ M retinoic acid in a 21% (atmospheric) oxygen incubator.



Appendix B: A schematic summary of the trends in dopaminergic marker expression for Tera2.cl.SP12 cells treated with 10 μ M retinoic acid for varying periods up to 4 weeks. Most markers are barely detectable or absent in the starting material and are induced by increasing the duration of retinoic acid exposure. Some markers such as DAT and D1 are more weakly expressed and are detected at higher Ct values than those such as D2 and TH that are more strongly detectable. Therefore the line position is only a guide as to the trend and will vary for each specific marker investigated.

Appendix C

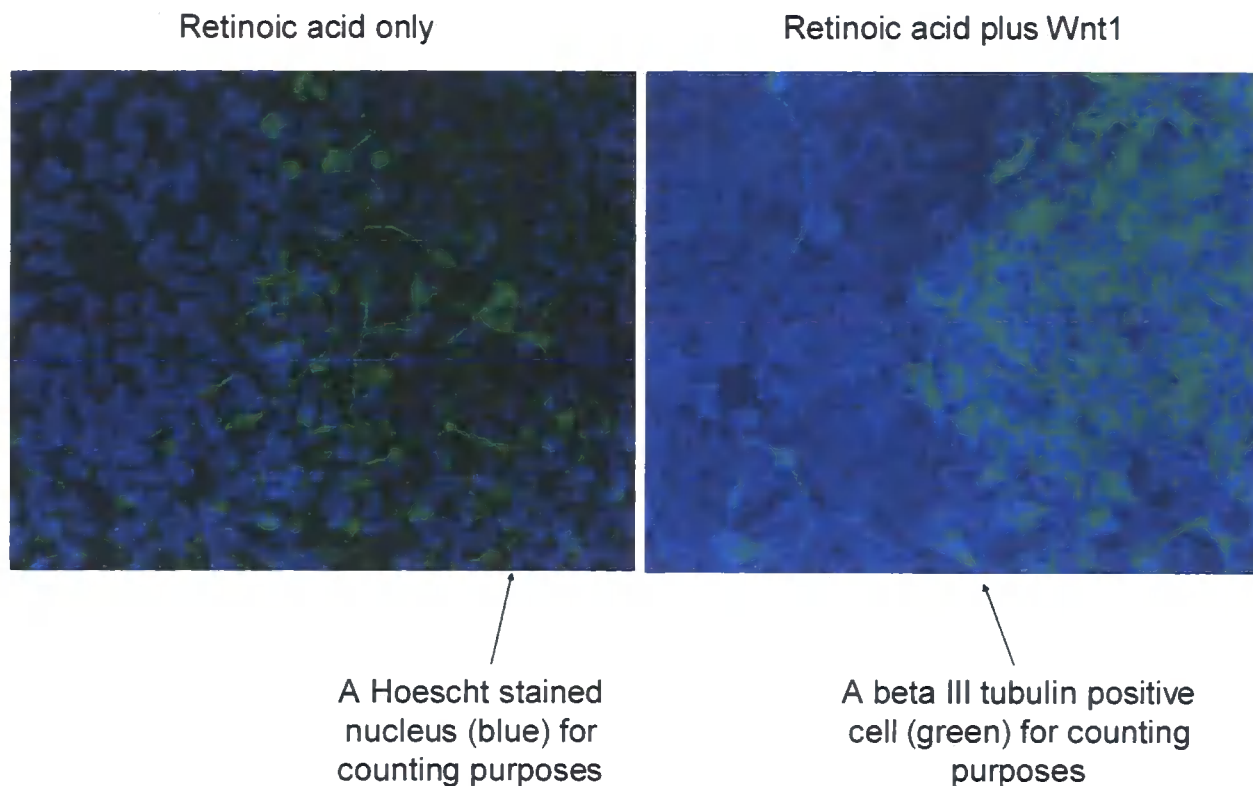
Western Blot - Nurr1 Positive Control



Appendix C: Post natal adult rat brain material (courtesy of the lab of Dr. P. Chazot, Durham University) thought to be positive for expression of Nurr1 was used as a positive control to test the Nurr1 antibody (obtained from Cambridge Bioscience) used in the investigations outlined in this thesis.

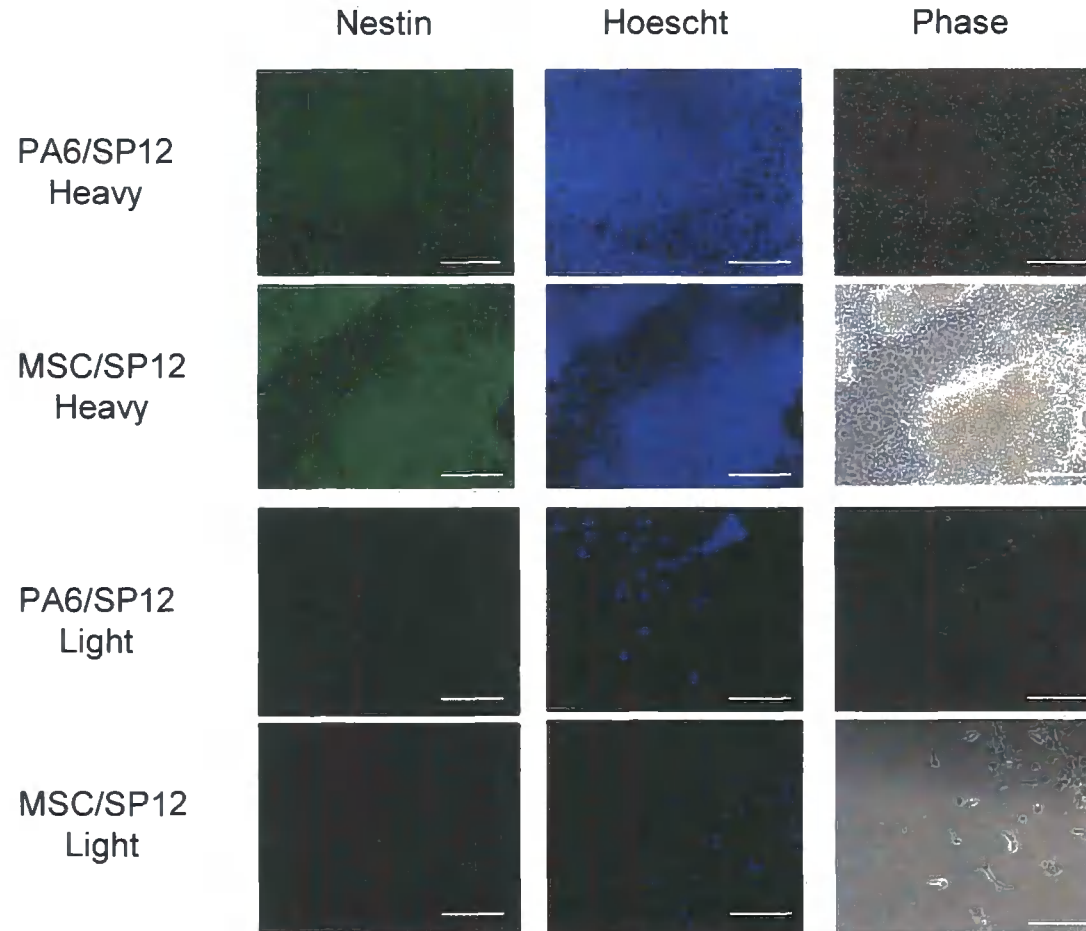
Appendix D

Cell count merged images



Appendix D: A merge of FITC and Hoescht images performed using Adobe Photoshop to show the expression of Beta III tubulin (FITC detection – green in the images above) in some cells against a background of total cell number (defined by the presence of Hoescht stained cell nuclei (blue in the images above)). The cell count data in Figures 4.28 and 4.29 were based on such immunocytochemical observations. The intensity of staining was generally greater in the Wnt1 plus retinoic acid condition, this is reflected in these images but some of the difference arises from a stronger background on the Wnt1 plus retinoic acid image shown here, the difference although apparent was not observed to be this striking in practice and may in part arise from the merging process.

Appendix E: Nestin staining in R.A. treated EC/Stromal cell co-cultures



Appendix E: Human specific nestin immunostaining in Tera2.cl.SP12 cells (pre treated for 3 days with 10 μ M retinoic acid) then co-cultured with either murine PA6 or rat mesenchymal stem cells for 17 days. The upper two rows of images (termed Heavy) display the strong nestin expression seen in the bulk of most cultures, that are highly confluent as seen in the corresponding Hoescht and phase images. The two lower rows of images (termed Light) are representative of less confluent areas of such cultures. In the case of the PA6/Tera2.cl.SP12 condition it would appear that there are cells (evidenced by their Hoescht stained nuclei) present that are not EC cell like in morphology that are nestin negative and as such may well be PA6 cells confirming that some stromal cells are still present in these co-cultures at the terminus of the differentiation protocol. However even in the less confluent areas of MSC/Tera2.cl.SP12 co-cultures all the cells appear to be nestin positive and morphologically characteristic of EC cells, suggesting few if any of the original stromal cell population remain at this point. Scale bars are approximately equal to 100 μ m.