Durham University

Durham E-Theses

"Developmental studies of the murine homeobox gene - hoxa-9"

Joy Lincoln

How to cite:

Lincoln, Joy (2002) "Developmental studies of the murine homeobox gene - hoxa-9". Doctoral thesis, Durham University.

Use policy

The full-text may be used and/or reproduced, and given to third parties in any format or medium, without prior permission or charge, for personal research or study, educational, or not-for-profit purposes provided that:

- a full bibliographic reference is made to the original source
- a https://etheses.durham.ac.uk/id/eprint/4145/ is made to the metadata record in Durham E-Theses
- the full-text is not changed in any way

The full-text must not be sold in any format or medium without the formal permission of the copyright holders.

Please consult the full Durham E-Theses policy for further details.

Academic Support Office, The Palatine Centre, Durham University, Stockton Road, Durham, DH1 3LE
e-mail: e-theses.admin@durham.ac.uk Tel: +44 0191 334 6107
https://etheses.durham.ac.uk

"Developmental Studies of the Murine Homeobox Gene - *Hoxa-9*"

by

Joy Lincoln
(BSc. (Hons) University of Durham)

Thesis submitted for the degree of Doctor of Philosophy
University of Durham
Department of Biological Sciences

October 2002

The copyright of this thesis rests with the author. No quotation from it should be published without his prior written consent and information derived from it should be acknowledged.

2 4 MAR 2003

This thesis is entirely the result of my own work. It has not been accepted for any other degree and is not being submitted for any other degree.

Joy Lincoln

October 2002

Abstract

Cell patterning during embryogenesis is essential for establishing the identity of the developing body plan. *Hox* genes are fundamental regulators of tissue organisation along the anterior-posterior body axis of the developing embryo. These homeodomain-containing proteins act as transcription factors during normal development. The function of the homeodomain is to bind sequence-specific DNA motifs which allows either activation or repression of downstream effector genes, which consequently results in the control of tissue-specific determination and differentiation.

Aberrant expression of such *Hox* genes, including *Hoxa-9* can result in homeotic transformations leading to phenotypic malformations and oncogenesis. However the normal function of *Hoxa-9* is poorly understood. This study explored the potential role for *Hoxa-9* in normal development and differentiation. An *in situ* hybridisation approach was taken to define the expression of *Hoxa-9* in the developing mouse. *Hoxa-9* was found to expressed in a temporarily and spatially regulated manner, in particular being detected in the developing cardiac atria, ventricles and cardiac vessels during E9.5-E12 stages of development. The expression of this homeotic gene during *in vitro* differentiation of embryonic stem cells into cardiomyocytes and haematopoietic cells demonstrated a profile that correlated with the emergence of these cell types. The functioning relationship between *Hoxa-9* expression and lineage commitment was further explored using over-expression in embryonic stem cells. A potential role for *Hoxa-9* in normal development is discussed.

Acknowledgements

There are so many people to thank, all of which have contributed in such a way that without them, things would have been a bit more of a struggle. I would particularly like to thank my supervisor Dr. Nicholas Hole for his continued, invaluable support throughout the sweat and tears of my Ph.D years, Dr. Colin Jahoda and Dr. Paul Hunt for their expertise. I also owe many thanks to several other people at the University of Durham including Dr. Majlinda Lako and Dr. Lyle Armstrong who have not only been true friends, but have supported me throughout my Ph.D in every way! I would also like to thank Dr. Jane Ives, as without her support, tissues, coffee and chocolate things may have seemed a little less possible, and Dr. Jenna Whitehouse for donating her reading skills. I am particularly grateful to Ms. Trudy Horton for all her excellent technical skills in generating the flow cytometry data in chapter four, Mrs. Christine Richardson for her assistance with wax embedding and tissue sectioning and Dr. Heiko Peters, Dr. Deborah Henderson, Dr. Lorraine Eley, Helen Imrie and Darren Key from the International Centre of Life, Newcastle, for all their help with the wholemount *in situ* hybridisation data in chapter two.

Finally, I owe this thesis to my family, in particular my husband Derek, who is my whole inspiration behind my intentions in life, and without him my life wouldn't be full of so much sunshine! Also my Mam, who has supported me continuously throughout all my decisions in life, and my brother Paul, who sadly couldn't see this through with me, but he taught me to keep smiling and live life to the full, so here I am!!

Abbreviations

Abd	*Abdominal*
AFP	Alpha feta protein
AGM	Aorta-gonad-mesonephros
AML	Acute myeloid leukaemia
ANF	Atrial natriuretic factor
Antp	*Antennapedia*
Bp	base pair
BCIP	5-bromo-4-chloro-inodyl-phosphate
bFGF	basic Fibroblastic growth factor
BMP	Bone morphogenic protein
BX-C	*Bithorax*
CD	Cluster differentiation
CFU-A	Colony forming unit-Assay
CFU-B	Colony forming unit-basophil
CFU-Eo	Colony forming unit-eosinophil
CFU-E	Colony forming unit-erythrocyte
CFU-G	Colony forming unit-granulocyte
CFU-M	Colony forming unit-macrophage
CFU-Meg	Colony forming unit-megakaryocyte
ddH_2O	Double distilled water
DEPC	Diethyl Pyrocarbonate
Dfd	*Deformed*
DIG	Digoxigenin
DMSO	Dimethylsulfoxide
ds	Double stranded
EB	Embryoid body
ES cell	Embryonic stem cell
Exd	*Extradenticle*
FGF	Fibroblastic growth factor
GM-CSF	Granulocyte macrophage – Colony stimulating factor
GMEM	Glasgow Modified Eagle Media
HTH	Helix-turn-helix

HSC	Haematopoietic stem cell
ICM	Inner Cell Mass
IL	Interleukin
l	litre
Lab	*Labial*
LIF	Leukaemia Inhibitory Factor
LTR	Long Term Reconstitution
m(RNA)	messenger (RNA)
m	milli-
M	Molar
Mef2	Myosin enhancing binding-factor 2
Mesp2	Mesoderm enhanced specific protein
MoMLV	Molony-Murine-Leukaemia-Virus
MyHC	Myosin heavy chain
NBT	Nitro blue tetrzolium
Shh	Sonic Hedgehog
PBS	Phosphate Buffered Saline
Pc-G	Polycomb group
PFA	Paraformaldehyde
RA	Retinoic acid
RARE	Retinoic acid response element
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SV40	Simian virus 40
TAE	Tris-Acetic-EDTA (buffer)
TE	Tris-EDTA (buffer)
Trx	*Trithorax*
U	units
μ	micro
Ubx	*Ultrabithorax*
UTP	Uridine-5-Triphosphate
UV	Ultra Violet
vMLC	ventricular Myosin Light Chain

Contents

Chapter one - Introduction

Chapter two – Wholemount and *in situ* hybridisation

Chapter four – Gene overexpression

Chapter five – Final Discussion

Figures and tables

Chapter one – Introduction

Chapter two – Wholemount and *in situ* hybridisation

Chapter three Embryonic Stem Cell Differentiation *in vitro*

Chapter four – Gene Overexpression

Chapter five – Final Discussion

Chapter One - Introduction

1.1 Introduction

During embryogenesis, one of the initial tasks is to lay down the overall body plan of organisation followed by the development of individual organs such as the limbs, eye, and nervous system. Much of the evidence for the genetic control of these developmental events has been found in *Drosophila* mutants.

Mutant phenotypes can reveal the hidden logic of the genetic control of development. The *Drosophila* maternal effect mutant *torso* may serve as an example. Females homozygous for loss-of-function alleles of *torso* provide nonviable embryos that are deficient in their anteriormost as well as their posteriormost body parts (Schüpbach and Wieschaus, 1986). In contrast, females with at least one gain-of-function allele of *torso* have offspring with enlarged terminal regions and little in-between. Similar unexpected phenotypes were found for a group of genes termed *pair-rule* genes because they affect the body pattern with a bisegmental periodicity. For example *Drosophila* embryos homozygous for null alleles of the *fushi tarazu* gene show half the normal number of segments, with each of the segments being longer than normal, suggesting that *fushi tarazu* and other *pair-rule* genes are involved in bisegmental periodicity (Duncan, 1986).

Pattern formation in living organisms can be recognised at different levels of organisation, for example the body of the fruit fly is subdivided into the head, thorax, and abdomen, and thus each subdivision can be divided further. The rich, detailed body patterns of *Drosophila* make it a favourable object for many investigations of pattern formation (Sander, 1976). There is an abundance of known genes affecting every aspect of the body pattern, ranging from maternal effect genes that affect the overall body plan,

such as *bicoid* (Frohnhöfer and Nüsslein-Volhard, 1986), to cell-autonomous genes, such as *multiple wing hairs,* which control the number and shape of hairs on single cells.

Mutations in zygotic genes can also have profound effects on the embryonic body pattern. For example, in *Drosophila* adults with mutations in *Ultrabithorax (Ubx),* the third thoracic segment (T3) looks like another copy of the second thoracic segment (T2). Normally, T2 carries a pair of wings while T3 carries a pair of balancer organs. In *Ubx* mutants, as part of the transformation of T3 into T2, the balancing organs have been replaced with another pair of wings (Akam, 1987). Mutations that transform certain body regions into the likeness of another body regions are known as homeotic transformations, and the genes that have this kind of mutant alleles are known as homeotic genes (Lewis, 1978). A large group of homeotic genes which are of particular importance are the homeobox genes.

1.1.1 The conservation of the homeobox genes

The homeobox encodes a helix-turn-helix motif consisting of a 60 amino acid residue polypeptide (Laughon and Scott, 1984, Shepherd *et al.*, 1984), also called the homeodomain. It was found that this domain directly binds to DNA (Johnson and Herskowitz, 1985, Desplan *et al.*, 1985, 1988, Fainsod *et al.*, 1986, Hoey and Levine, 1988, Cho *et al.*, 1988, Müller *et al.*, 1988, Beachy *et al.*, 1988, Laughton *et al.*, 1988).

It was discovered that binding to DNA was specific and the target sequences were very similar to each other. It was suggested that several distantly related homeodomain proteins recognise the same sequence element in vitro (Hoey and Levine, 1988, Desplan, 1988).

As more and more homeobox-containing genes were isolated and sequences were compared, it became clear that for closely related homeodomains the sequence similarity could be extended upstream and/or downstream of the core 60 amino acids. In contrast, highly divergent homeodomain sequences have been identified for which the sequence similarity is shorter than the original defined 60 amino acids.

These homeodomain containing proteins have been found within members of the plant, fungi and animal kingdoms, but not in the bacterial kingdom. It is therefore thought that such genes came early in the evolution of eukaryotes. Over subsequent evolution the amino acid sequence of the homeodomain has remained highly conserved, and sequence preservation maintained (Qian *et al.*, 1992) This is demonstrated in figure 1 comparing the homeodomain sequence of *Hoxa-9* amongst species. The discovery of a large number of *Hox* genes that were highly conserved between species suggested ancestral gene duplication. To better study this, the genomic organisation was investigated.

1.1.2 The mapping of chromosomal organisation of *Hox* genes

Mapping of the nine *Drosophila* homeobox genes demonstrated that they were co-localised to two complexes (Antp and BX-C) on one chromosome. Subsequent mapping of the 39 murine homeobox genes revealed the presence of four complexes, showing differing levels of synteny between species (Rabin *et al.*, 1986), shown in figure 2.

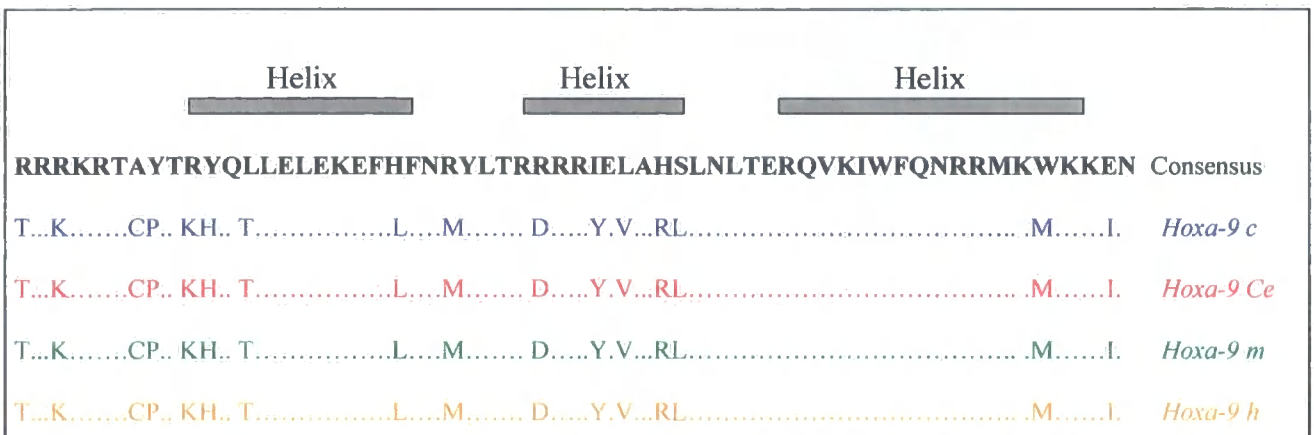


***Figure 1* - The conservation of the *Hoxa-9* sequence amongst species**

A consensus sequence based on 346 of the most frequently encountered 60 amino acid of the *Hox* gene homeodomain is shown on the top line. Grey boxes highlight the positions of the three helices. One *Hox* gene, *Hoxa-9* is shown for four different species.

Hoxa-9 c denotes *Gallus gallus* (chicken), *Ca ~ Cavia sp* (guinea pig), *m ~ mus musculus* (mouse), *h ~ homo sapiens.* It is important to note that within *Hoxa-9,* there is 100% conservation in the homeodomain for these species.

Lines below show identity (dots) or variation in sequence (amino acid codes) between species.

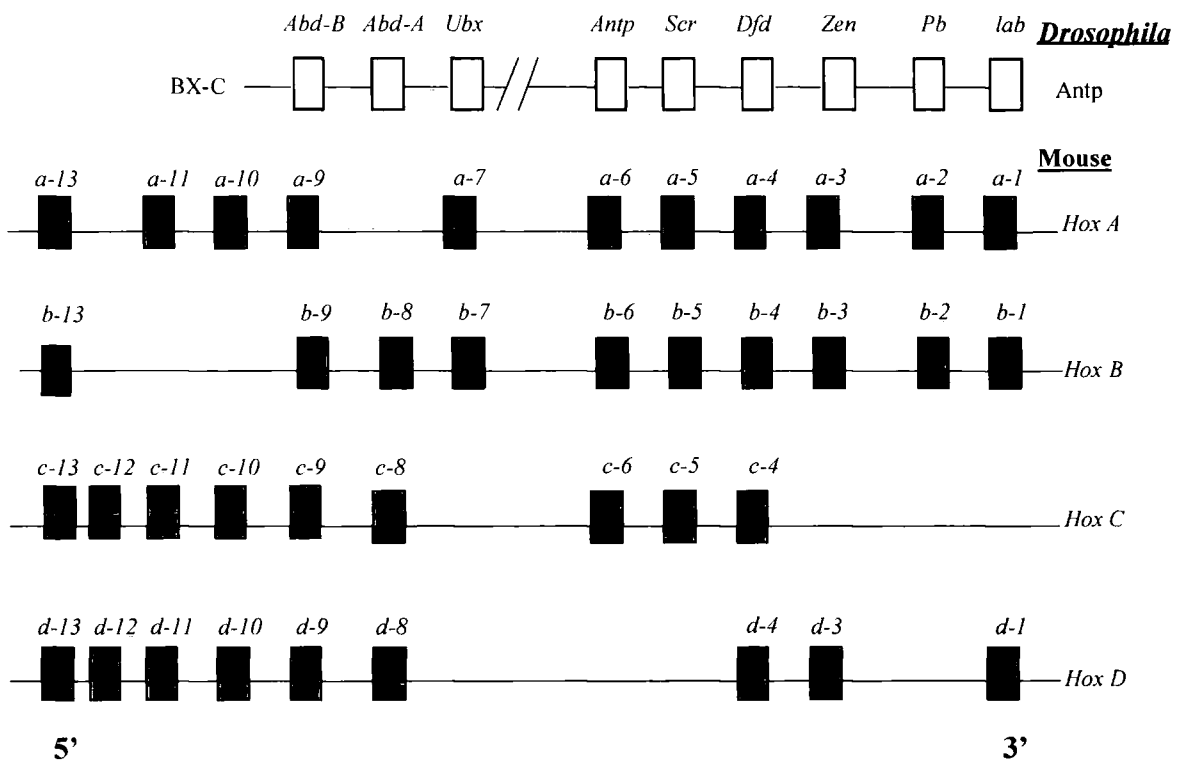


***Figure 2* - The nomenclature of vertebrae *Hox* genes** (Scott, 1992)

Each *Hox* gene is shown by black filled boxed regions on four separate chromosomes and are named. The unfilled boxes show the *Drosophila* genes of the *Antennapedia (ANT-C)* and *Bithorax (BX-C)* complexes.

The 5' and 3' chromosomal positioning has been indicated above, and will be referred to during this manuscript.

The genomic and organizational similarities between vertebrae *Hox* and *Drosophila* HOM-C complexes (the complex of homeobox genes that encode *Antp* class homeodomain proteins in the insect, Lewis 1978) suggest that both families arose by duplication and divergence from a common ancestral cluster. This ancestral clustering is believed to have been conserved in many other animal species (Akam 1989, Scott *et al.*, 1989, Kessel and Gruss 1990, Boncinelli *et al.*, 1991, Duboule 1992, Krumlauf 1992, McGinnis and Krumlauf 1992). Although the complexes are likely to have arisen from duplication (Scott *et al.*, 1992), not all four loci have the same number of paralogue members, so some duplicated genes may have been lost or not duplicated. Each cluster has selectively retained different subsets of these paralogous genes. These subsets have been maintained within vertebras.

In vertebrates, *Hox* gene organisation is distinctive in that the 39 family genes are clustered in four chromosomal loci, the *Hox a, b, c,* and *d* complexes (reviewed by McGinnis and Krumlauf 1992). The similarities in amino acid homeodomain sequence between Hox genes led to a classification into 13 subfamilies or homology groups (Scott *et al.*, 1984). Each sub-family contains 2-4 highly related paralogue genes; of tremendous interest is that within each of the four clusters the position of each paralogue is maintained.

1.1.3 The relationship between the *Hox* and *HOM-C* complexes

Although the *Hox* and *HOM-C* complex are very similar, there are some features that are not shared. For example in *Drosophila* the *HOM-C* complex is comprised of two separate clusters– *bithorax* (BX-C (Lewis 1978)) and *Antennapedia* (Antp) complex (Kaufman *et al.*, 1980, 1990), as oppose to the four clusters of vertebrae *Hox. The HOM-C* cluster also

contains genes encoding *Scr* implicated in segmental identity and two *zen* homeobox genes involved in dorsoventral patterning (Kaufman *et al.*, 1990).

The presence of a single *HOM-C* cluster in *Drosophila* is consistent with the concept of there being a single ancestral cluster, (Beeman *et al.*, 1989, Kenyon and Wang 1991, Salser and Kenyon 1994, Averof and Akam 1995, Pendleton *et al.*, 1993, Holland *et al.*, 1994). Genes that show high levels of conservation between species suggest a conservation of function. However, for *Hox* genes there is an additional level of conservation; position within the cluster which may be of functional significance. Evidence for this emerged when early studies examined the pattern of expression of each paralogue during embryogenesis.

1.2 Colinear expression patterns of *Hox* genes

In situ hybridisation studies in *Drosophila* revealed that the genomic organisation of *Hox* genes is reflected by their expression pattern along the anterior-posterior axis.

Figure 1.1c, shows how *Hox* gene expression is related to chromosome position within the complex, in that 3' genes are expressed in the anterior region of the embryo (reviewed by Dollè and Duboule, 1993).

The molecular mechanisms that determine the anterior-posterior axis appear to be conserved through evolution. The importance of this characteristic colinearity, and how it is achieved to formulate *Hox* gene function and regulation is not completely understood, but is thought to lie in *Hox* gene regulatory mechanisms.

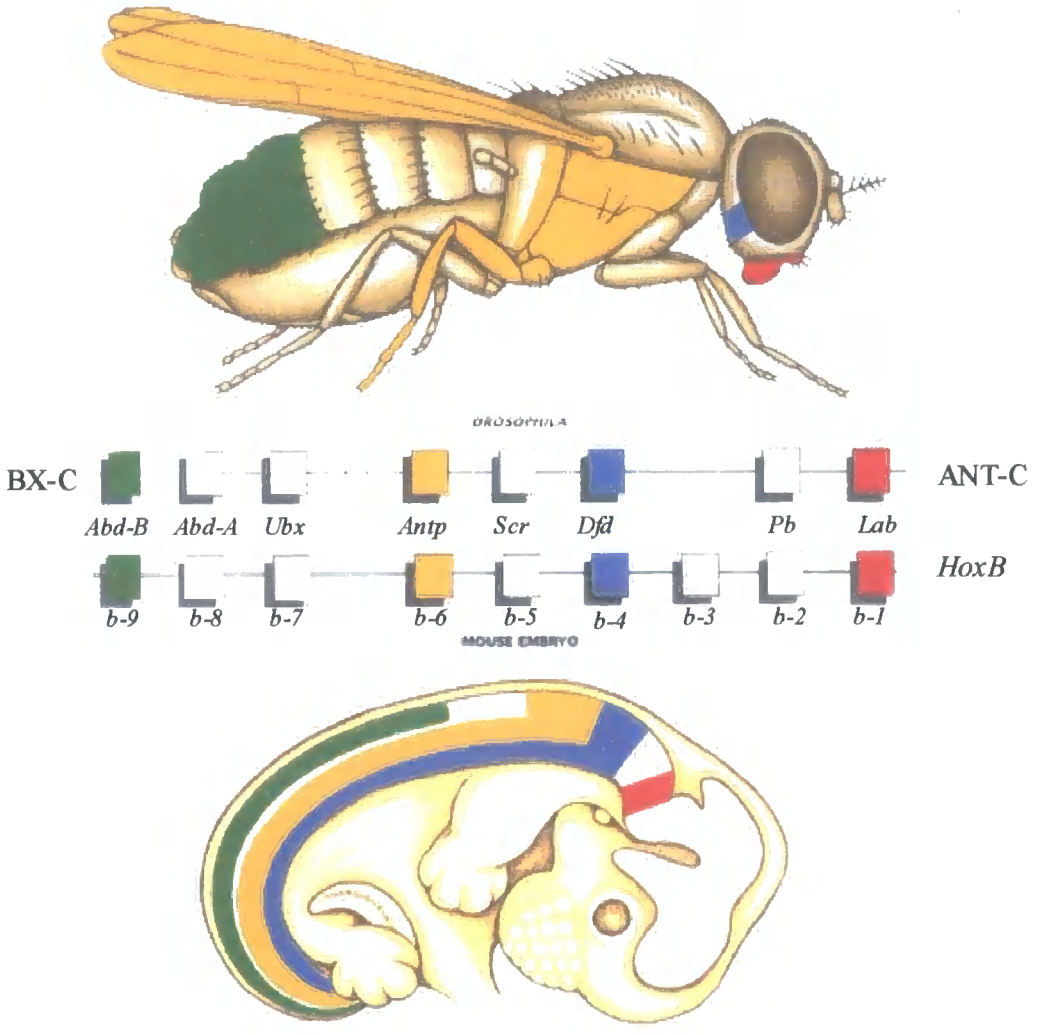


***Figure 3* - Co-linear expression of the homeodomain complexes in *Drosophila* and mouse** (adapted from De Robertis, 1990)

This figure highlights the co-linear expression pattern of the ANT-C cluster in *Drosophila* in relation to the *HoxB* cluster in the mouse embryo– The 3' located genes (*Lab- Dfd* and *Hoxb-1 –Hoxb-4*) are expressed in the anterior regions of the fly and embryo respectively.

1.2.1 The role of *Hox* genes in cell patterning

The chromosomal organisation of *Hox* loci is correlated to their axial expression pattern. The reasons to this colinearity at the chromosome level are unclear, but it is believed to involve the conservation of their mechanisms that regulate expression and function (Gaunt, 1989, Douboule and Dollé, 1989, McGinnis and Krumlauf 1992, Krumlauf 1994).

A number of studies have examined if precise *Hox* patterning is instructive or permissive for morphological patterning. There is a great deal of supporting evidence for instructive roles. Ectopic *Hox* expression or loss of function can lead to large-scale transformations as a result of altered gene expression in flies or mice (McGinnis and Krumlauf 1992)

Some evidence comes from loss of function studies in the mouse. *Hoxc-8* is normally expressed in the thoracic region. Following loss of function mutations there are anterior homeotic transformations in the vertebral column in the mouse (Le Moulleic *et al.*, 1992). When homeodomain proteins are ectopically expressed in the posterior of the embryo, anterior homeotic transformations can be found. For example, a mouse transgenic line was generated carrying 40 copies of the human Hox3.3 (Hoxc-6) gene. The resulting mice expressed large amounts of *Hox3.3* protein in posterior regions of the embryo where this homeodomain protein is normally not expressed. The transgene caused homeotic transformations of the skeleton, in particular the appearance of an extra pair of ribs in the lumbar region, transformation of the shape of posterior ribs into that of more anterior ones, and the joining of an additional pair of ribs to the sternum (Jegalian and De

Robertis, 1992). Ectopic expression of *Hoxc-8* also results in anterior transformations in thoracic segments T11, T12 and lumbar 1 (Pollock *et al.*, 1992).

Previous experiments in *Drosophila* have shown that detailed modulation of homeobox spatial and temporal patterns of expression within metamers can also play an instructive role (Mann 1994, Castelli-Gair *et al.*, 1994, Castelli-Gair and Akam, 1995). *Drosophila* has segmental fields within the embryo that contain groups of cells with the potential of developing into different organs.

Antp complex genes have been shown to be expressed in a specific manner within these segmental fields of the embryo, which include the region that develops Keilin's organs. There are also spatially separate groups of cells that have the potential to develop as spiracles, which map to specify Antp expression (Castelli-Gair and Akam, 1995).

However, neither gene is expressed outside these assigned domains in abdominal segments, due to the suppressing power of *Ultrabithorax (Ubx)* and other bithorax complex proteins. In the second and third abdominal segment, *Ubx* is activated in an intricate pattern of cells that includes the spiracle primordial. It is this specific expression of bithorax genes including *Ubx* that controls the specific morphology of each segment. In the leg primordia of the same segments, *Ubx* expression is absent during early stages of embryogenesis, which allows Keilin's organs to develop (Akam, 1997). This highlights the role of specificity of each *Hox/HOM-C* gene expression within a given metamar at precise time points in determining cell fate (reviewed Gellon and McGinnis 1998).

Similar experiments (Salser and Kenyon 1996) have revealed that upstream regulation of *Hox* genes is an important process in generating gene expression patterning within

functional domains, but why do different animals exhibit distinctive and diverse morphological features? What is controlling this variation in design? To answer these questions we need to comprehend what determines *Hox* gene patterning.

1.2.2 *Hox* gene patterning

In *Drosophila*, *HOM-C* gene patterning is established during the early blastoderm stage, and appears as 'stripes' of expression on the anterior-posterior axis (Akam *et al.*, 1987). At this stage of the embryo there is asymmetry of transcription factor distribution of both maternal and zygotic origin, produced by coordinate gap and pair-rule genes (Qian *et al.*, 1993). These gene products allow every nucleus to be exposed on the anterior-posterior axis to unique combinations of transcription factors. It is thought that alterations or loss of function of these proteins can result in constrictions, expansions or deletions of *Hox* expression domains (McGinnis and Krumaluf 1992).

Hox gene expression domains have been shown to be controlled by segmentation genes. These genes can be subdivided into (i) gap genes that are required in regions that are several segments wide to act in a repressive manner to 'mark out' the coarsest subdivisions in the embryo, (ii) pair-rule genes that affect alternate segments, (iii) segment polarity genes that are active in parts of every segment (Qian *et al.*, 1993). The repetitive pattern of similar segments is converted into a sequential pattern of individually different segments by action of the homeobox genes that gives each segment a unique identity.

It might be expected that given the apparent conservation of expression patterns and sequence amongst species (see figure 1.1a), that the regulatory networks upstream of *Hox*

are equally as conserved. It appears that there are many different mechanisms for establishing the initial boundaries of *Hox* gene expression in different animal phyla (vertebrates and flies), (Patel 1994, Sommer *et al.*, 1993, Wolff *et al.*, 1995, Brown and Denell 1996). These include trans-activating proteins, signalling molecules, cell silencing, auto activation and cross regulation.

1.3 *Hox* gene regulation:

1.3.1 Trans-activating proteins;

Transactivating genes induce expression of specific transcription factors. These factors are able to activate the transcription of the gene whose promoter or enhancer is bound. An example of such a transactivator is *Krox20.* The *Krox-20* gene encodes a zinc finger transcription factor that is expressed in regions of rhombomeres 3 and 5 (r3/r5) in the wild type murine hindbrain (Chavrier *et al.*, 1988, Wilkinson *et al.*, 1989).

Evidence that *Krox20* can act as a transactivator of *Hox* genes has been shown from a transgenic model. Cis-acting regulatory regions were characterised by flanking the *Hoxb-2* gene *in vitro* and in transgenic mice. The *Krox20* response element was identified acting as an enhancer capable of imposing spatially restricted domains of expression in rhombomere 3 and 5 or in response to the ectopic expression of *Krox20* (Sham *et al.*, 1993). This leads to the suggestion that *Krox20* is a direct upstream regulator of *Hoxb-2.* Supporting evidence also comes from the analysis of mouse mutants carrying null alleles of the *Krox20.* In the homozygous mutants, r3 and r5 begin to be established, but there is a loss of *Hoxb-2* expression, specifically in r3/5, that confirms *Hoxb-2* as an *in vivo* target of *Krox20.* In later stages there is a loss of r3/5, further suggesting that the *Hox* genes,

controlled via *Krox-20*, are required to maintain normal rhombomere patterning and growth gene (Schneider-Maunoury *et al.*, 1993, Swiatek and Gridley 1993).

1.3.2 Signalling molecules;

There are many cell-cell signalling molecules that have been identified as regulators of *Hox* gene expression in vertebrates.

Embryo exposure to retinoic acid (RA) can induce homeotic transformation (Boncinelli *et al.*, 1991, Marshall *et al.*, 1992, Krumlauf 1994). Exposure to RA also induces ectopic expression of *Hox* genes and alters spatial aspects of *Hox* gene expression patterns (Kessel and Gruss, 1991, Marshall *et al.*, 1992). The role of retinoids in regulating these genes during normal development is unclear. Marshall (Marshall *et al.*, 1994) identified two enhancers, 3' of the mouse *Hoxb-1* gene, which together reconstruct the early endogenous expression pattern and mediate the early ectopic response to retinoic acid. The enhancer that controls the retinoic acid response, and regulates expression predominantly in neuroectoderm, contains a retinoic acid response element (RARE). Point mutations in the RARE abolish expression in neuroectoderm. Therefore this RARE is not only involved in the ectopic expression to RA, but is also essential for establishing aspects of the early *Hoxb-1* expression pattern.

This ectopic expression in this most 3' gene of the *Hox B* cluster, expressed in the anterior part of the embryo reflects a colinear sensitivity in the level and time of response of *Hox* genes to RA that has been shown in both cells lines and embryos (Simeone *et al.*, 1990, 1991, Papalopulu *et al.*, 1991, Dekker *et al.*, 1993).

It had been assumed that RA works on Hox genes to modify their transcription, via indirect pathways that feed into *Hox* gene regulators searching for RA response elements (RARE) within the *Hox* cluster. It has been shown that ectopic exposure of mouse embryos to RA in late primitive streak stages can induce both transformation of r2 into an r4 identity and rapid changes in *Hoxb-1* expression in both rhombomeres (Marshall *et al.*, 1992). However, developmental phenotypes and changes in *Hoxb-1* expression can also be generated by the ectopic expression of *Hoxa-1* in transgenic mice (Zhang *et al.*, 1994). This suggests that RA may exert its effect by activating the *Hoxa-1* gene which then directly or indirectly altering *Hoxb-1* expression.

There are many other signalling molecules, like RA, that could potentially interact and regulate *Hox* genes.

These include *Bone Morphogenic Proteins* (*BMP*) 2, 4 and 7, which in response to *Shh* regulate the transcription of *Hox* in the growth stage of skeletal and mesenchyme tissue development (Iimura *et al.*, 1994, Roberts *et al.*, 1995, Watanabe *et al.*, 1998).

FGF8 can also regulate *Hox* gene expression. Using heterospecific grafting strategies and ectopic FGF8 expression, *Hox* gene expression can be induced in murine rhombomere 1. Loss-of-function of FGF8 function suggests that this gene is responsible for defining anterior limits of *Hox* genes in the developing brain (*Hoxa-4, a-3, a-1, b-1, b-2, a-2)* and therefore specifies the extent of rhombomere 1 boundary (Irving and Mason 2000).

As expression of *Hox* genes outside their designated expression domain can result in cell transformation, it is of equal importance to keep these genes inactive where their products are not morphologically wanted. This is achieved by repression – by which the enhancers

can be silenced or blocked to prevent active transcription (Bieberich *et al.*, 1990, Müller and Bienz 1991, Püschel *et al.*, 1991)

1.3.3 *Hox* gene silencing and repression;

In *Drosophila Hox* gene silencing is achieved in two steps (Garcia-Bellido and Capdevila, 1978). Initiation is the first step, and this provides the spatial specificity: gap gene products that are localised in the early embryo repress homeotic genes in sets of primordia (White and Lehman 1986, Harding and Levine 1988, Irish *et al.*, 1989, Qian *et al.*, 1991, Zhang *et al.*, 1991, Shimell *et al.*, 1994), The second step, maintenance, keeps the homeotic genes silenced even though the gap proteins have gone and the cell is dividing.

This silencing depends on a set of more than ten genes (Lewis 1978, Struhl 1981, Jürgens 1985), called the *Poly-comb group* (*Pc-G*), which includes the *Polycomb (Pc)* gene itself. The *Pc-G* maintain cell determination and function, and are uniformly distributed in the embryo (Paro and Hogness 1991, Franke *et al.*, 1992, Martin and Adler, 1993). Their importance can be demonstrated in loss of function studies. In *Pc* mutants, posterior *Hox* genes such as *Abd-A* and *Adb-B* are expressed in more anterior regions of the embryo, resulting in partial transformation in posterior abdominal identity (Lewis 1978, Struhl and Akam 1985, Wedeen *et al.*, 1986).

The *trithorax* group *(trx)* mutations are characteristic by their ability to suppress *Pc* group phenotypes (Shearn 1989, Kennison 1993). Loss of function does not abolish homeotic gene expression but affects the amount of expression, ranging from severe to mild (Breen and Harte 1993, Tamkun *et al.*, 1992).

In a manner similar to *Hox*, the sequence and genomic organisation of *Pc* and *Trx* are largely conserved amongst species (Simon 1995, Muller 1995), and provide a means of maintaining homeotic gene expression during stages of embryonic patterning (Struhl *et al.*, 1981, Jürgens *et al.*, 1985, Paro and Hogness, 1991, Franke *et al.*, 1992, Martin and Adler, 1993).

Bmi-1 a murine homologue (posterior sex comb Psc), a member of the polycomb group of genes provides evidence that it acts as a regulator of Hox gene expression in mice. Homologous recombination studies of Bmi-*1* repression in embryonic stem cells (van der Lugt *et al.*, 1994, Alkema *et al.*, 1995), provide evidence that it acts as a regulator of *Hox* gene expression in mice, and M33 a mouse homologue of polycomb (Pearce *et al.*, 1992, Paro and Hogness, 1991), substitutes the function of the Pc protein in transgenic flies, therefore further suggesting that the Pc genes play similar, conserved roles in mice and flies (Müller *et al.*, 1995).

It has been highlighted that *Hox* gene domain expression patterns along the anterior-posterior axis can be activated by regulator gap and pair-rule genes, trans-activating proteins, and retinoic acid as well as other signalling molecules, and the pattern of expression is maintained, at least in part the repression of polycomb and *trithorax* genes. However, some *Hox* genes in *Drosophila* and mice also rely on auto-activation circuits for the maintenance of expression during development.

1.3.4 Auto-regulation;

Comparisons of *Hoxb-1* regulatory regions from different vertebrates identified three related sequence motifs critical for rhombomere 4 (r4) expression in the hindbrain. Functional analysis in transgenic mice and *Drosophila* embryos demonstrated that the conserved elements are involved in a positive autoregulatory loop dependent on *labial (lab)* family groups. Binding of *Hoxb-1* to these elements *in vitro* requires cofactors, and the motifs closely resemble the consensus binding site for pbx-1, a homologue of the *Drosophila* extradenticle *(Exd)* homeodomain protein (van Dijk and Murre 1994). *In vitro, exd/pbx* serve as a *Hoxb-1* cofactor in cooperative binding and in *Drosophila* expression mediated by the r4 enhancer is dependent on both *lab* and *Exd (*Popperl *et al.*, 1995). This provides supporting *in vivo* and *in vitro* evidence that r4 expression involves direct autoregulation dependent on cooperative interactions of *Hoxb-1* with *exd/pbx* proteins as cofactors (Regulski *et al.*, 1991, Chouinard and Kaufman 1991, Chan *et al.*, 1996, Gould *et al.*, 1997).

Embryos that lack *Exd* are unable to maintain *Dfd* or *lab* transcription, although the overall transcriptional regulation of many other *Hox* genes is unaffected (Chan *et al.*, 1996, Pinsonneault *et al.*, 1997).

This is supporting evidence of direct auto-activation/regulation – the *Hox* genes are regulated by their products acting through their own control regions (McCormick *et al.*, 1990, Jiang *et al.*, 1991, Regulski *et al.*, 1991, Pöpperl and Featherstone 1992, Schier and Gehring 1992).

1.3.5 Cross-regulation;

Cross-regulatory circuits among *Hox* genes can play an important role in determining transcriptional patterns. The expression patterns of most *Hox* genes involve large, overlapping domain areas along the axis that extend from the anterior boundary back towards the caudal end of the embryo. In *Drosophila,* where *Hox* gene expression overlaps occur, there is often down-regulation of anterior genes through negative cross-regulation by loci expressed in a more posterior fashion (Hafen *et al.*, 1984, Struhl and White 1985, Carroll *et al.*, 1986, Wirz *et al.*, 1986, Bermingham *et al.*, 1990, Appel and Sakonju 1993, Gould *et al.*, 1997). It is not clear that such a system operates in vertebrates. Indirect evidence may be seen in the vertebrate hindbrain, where there is specific *Hox* gene patterning in each rhombomere (Lumsden and Krumlauf 1996). Each rhombomere appears to express a specific *Hox* pattern, but there is a high degree of spatial overlap, suggesting that there maybe regulatory elements shared between *Hox* genes (Simeone *et al.*, 1988, Krumlauf 1994, van der Hoeven *et al.*, 1996). Direct evidence is suggested in the case of *Hoxb-3*. In the mouse hindbrain *Hoxb-3*' and *Hoxb-4* share an expression domain caudal to the boundary between rhombomeres 6 and 7. Transgenic analysis reveals that an enhancer (CR3) is shared between both genes and specifies this domain to overlap (Gould *et al.*, 1997).

Homeodomain containing *Hox* genes have been strongly implicated in determining cell patterning, proliferation and migration during several developmental processes (reviewed by Gehring *et al.*, 1987, Levine and Hoey 1988, Akam 1989). Aberrant expression of

these transcription factors in transgenic mice result in morphogenic transformations, indicating their importance during development (Balling *et al.*, 1989, Wolgemuth *et al.*, 1989, Chisaka and Capecchi 1991, Fromental-Ramain *et al.*, 1996, reviewed by Krumlauf 1994.) *Hox* genes are best represented by their important roles in cell proliferation and normal differentiation control. One adult system that produces highly differentiated cellular progeny in a tightly regulated manner is the haematopoietic system, and this has become one focus of attention for workers investigating *Hox* gene function in adult tissues.

1.4 Haematopoiesis

Haematopoiesis is a series of complex pathways that allows the production of all blood cell types (lymphoid, myeloid, erythroid) from a single, multipotent stem cell, the haematopoietic stem cell (HSC)(Graham and Pragnell, 1992).

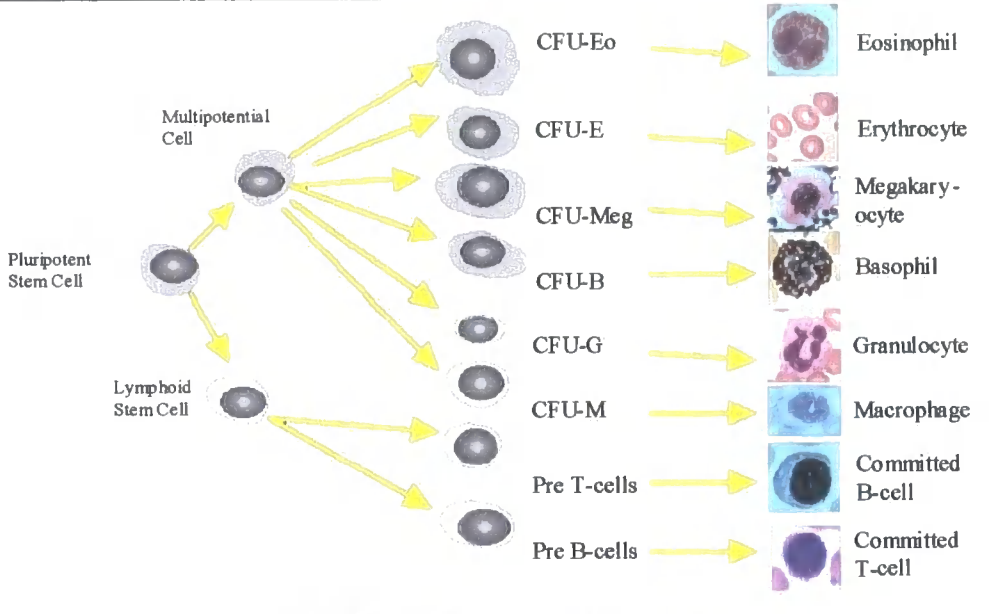


***Figure 4* - An outline of the haematopoietic pathway.**

This figure highlights the production of eight different blood lineages from one pluripotent stem cell type.

At present, the molecular controls involved in this differentiation pathway are incompletely understood, therefore it has become increasingly important to identify candidate genes that regulate 'normal' haematopoiesis.

Figures 5-11 illustrate the events involved in the development of the haematopoietic system in the mouse embryo. It is to be noted that these illustrations are not quantitative, but merely an indicator of such events taking place.

1.4.1 Primitive haematopoiesis

Haematopoiesis can be first detected as blood islands within the yolk sac of the mouse embryo at E7 (Russell and Bernstein 1968), illustrated in figure 5. The blood islands contain terminally differentiated blood cells of erythroid and monomyeloid lineages. At E8, primitive progenitors which can give rise to lymphoid lineages can also be seen (Moore and Metcalfe, 1970, Johnson and Barker, 1985, Wong *et al.*, 1986, Gordon *et al.*, 1992, reviewed Keller *et al.*, 1999). The blood cells begin to circulate between the extraembryonic yolk sac and the embryo at this stage, seen in Figure 1.4.6 (Medvinsky *et al.*, 1996). At this time, the yolk sac does not contain cells capable of repopulating the entire adult haematopoietic system (or long term repopulating (LTR) cells, (reviewed Dzierzak and Medvinsky, 1995, Cumano *et al.*, 1996, Medvinsky and Dzierzak, 1996, Dzierzak *et al.*, 1997), and are therefore termed primitive, but such progenitors that are found are able to self-renew and generate committed progenitors which lead to mature haematopoietic cells after E7-14 (Dzierzak and Medvinsky, 1995).

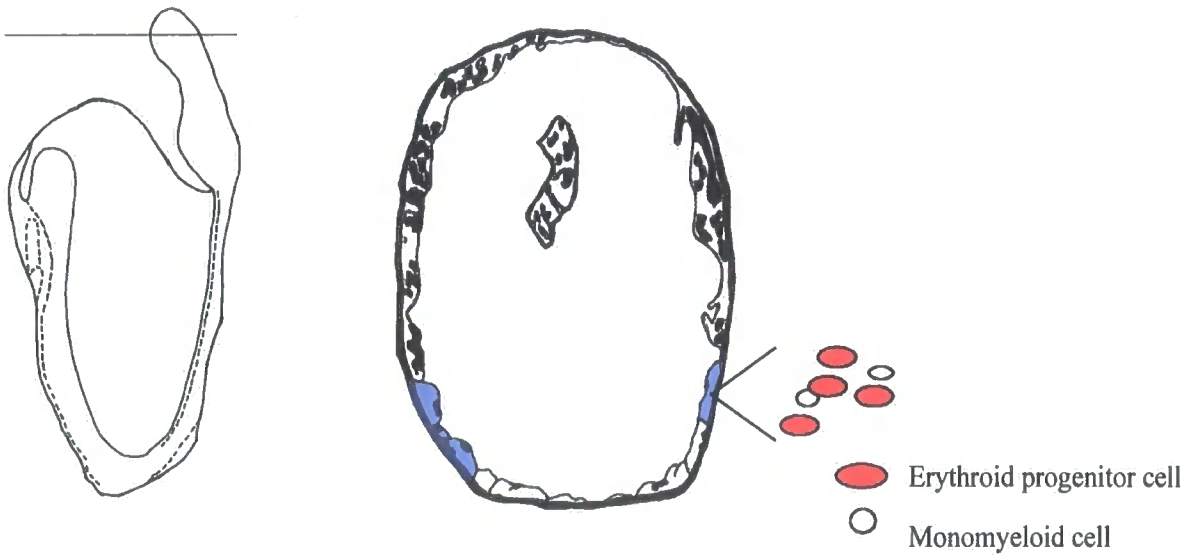


Figure 5a (left) **The E7.5 mouse embryo**

Figure 5b (right)**Haematopoiesis in the E7.5 mouse embryo** – transverse section shown in 5a

Initial haematopoietic events in the mouse begin in the blood island yolk sacs (highlighted in blue), where primarily erythroid lineage cells, and fewer monomyeloid cells are produced. Stem cells in the yolk sac are thought to be incapable of Long Term Reconstitution (LTR) (Cumano et. al. 1996, Medvinsky & Dzierzak *et al* 1996, reviewed Dzierzak et. al. 1998 and Medvinsky et. al. 1993).

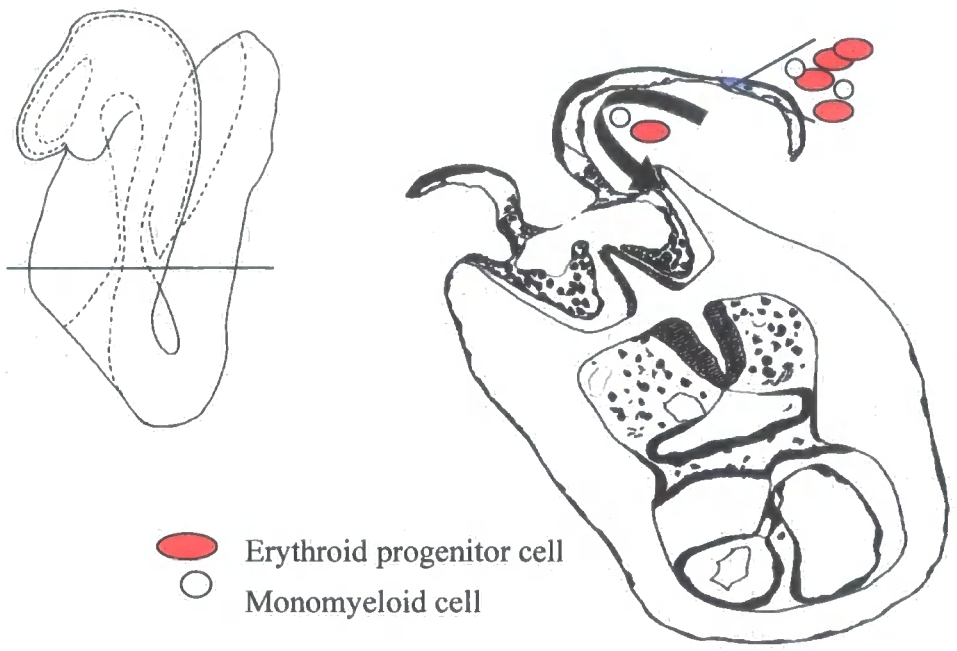


Figure 6a (left) **The E8.5 mouse embryo**

Figure 6b (right) **Haematopoiesis in the E8.5 mouse embryo**

Transverse section through *Figure 1.4.5.*

At this stage, blood cells begin to circulate between extraembryonic yolk sac (highlighted in blue), and the embryo, indicated by the arrow (Gordon *et al.*, 1991, Dzierzak *et al.*, 1995, Medvinsky et. al. 1996).

1.4.2 Definitive haematopoiesis

The aorta-gonad-mesonephros (AGM) has been identified as the first intraembryonic site of definitive, or adult, haematopoietic activity. This mesoderm derived region containing the dorsal aorta, genital ridge/gonads and pro/mesonephros has been shown to harbour adult-type multipotent haematopoietic progenitors and LTR-HSCs at E9-10 (Medvinsky,

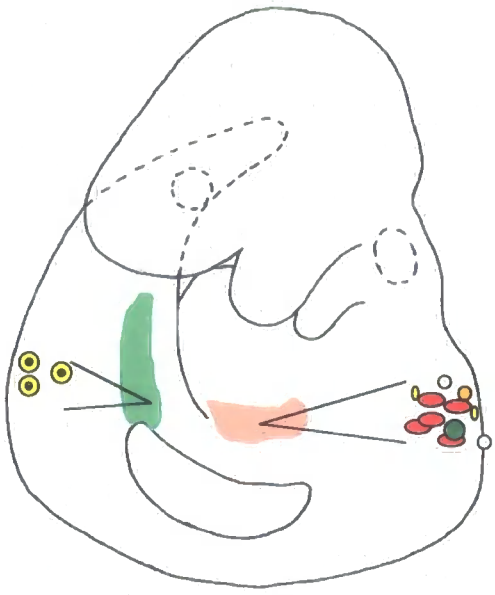
1993, Müller *et al.*, 1994). At these times, illustrated in figures 7-8, the AGM produces LTR-HSCs abundantly, and is the most important organ for the generation of the adult haematopoietic system as shown by functional haematopoietic assays (Tavassoli *et al.*, 1991, Medvinsky, 1993).

By E9.5 the foetal liver is another principal haematopoietic organ producing primitive erythroid cells, myeloid and B-cell lymphoid precursors (figure 7). From E9 upwards the foetal liver generates haematopoietic progenitors and mature blood cells, and by E11 contains definitive LTR haematopoietic stem cells that have migrated from the AGM. At E12 the yolk sac contains cells of the same nature that have migrated from other sites (Chang *et al.*, 1992, Medvinsky, 1993, Müller *et al.*, 1994, and reviewed Dzierzak *et al.*, 1998).

There is only one functional definition of LTR-HSC, but in order to isolate this comparatively rare population of cells, the surface antigen phenotype has been closely studied, and an indicative phenotype characterised for adult bone-marrow HSC. These include *c-kit*$^+$ and CD34$^+$, but negative for mature lineage markers such as CD4, CD8, B220 and *Gr-1* (Sanchez *et al.*, 1996). Evidence from Morrison (Morrison *et al.*, 1995), suggests that the adult bone-marrow HSC can be very highly enriched, if not a pure population of LTR-HSC. In a similar manner, the HSCs found in AGM and foetal liver have been studied.

1.4.3 The site of long term reconstituting (LTR) HSC production

The presence of definitive HSCs in the AGM with characteristic surface antigens suggests that definitive HSCs are produced solely in the AGM and then migrate to the



Erythroid progenitor cell
Monomyeloid cell
Committed B cell precursor cell
Granulocytic cell
Macrophage cell
Definitive HSC

Figure 7 - **Haematopoiesis in the E9.5 mouse embryo**

The foetal liver (area shown in pink) generates the primitive progenitors.

The Aorta-Gonad-Mesonephros (AGM), highlighted in green, harbours HSCs capable of long term reconstitution (Medvinsky *et al.*, 1993, Müller *et al.*, 1994).

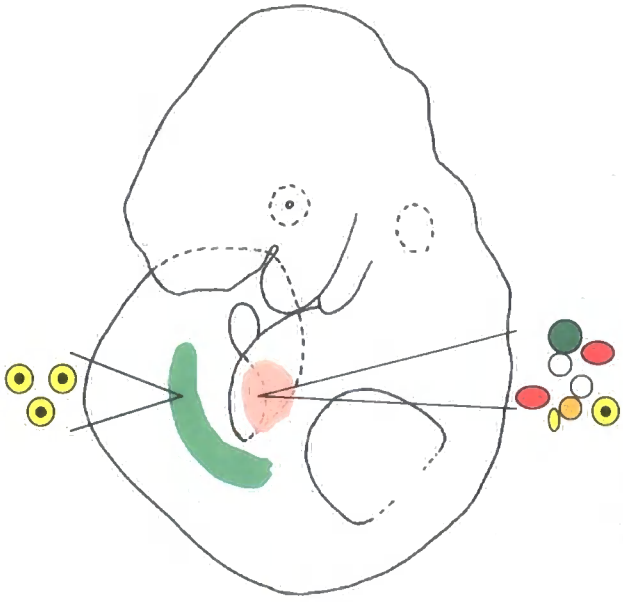


Figure 8 - **Haematopoiesis in the E10.5 mouse embryo**

The foetal liver (area shown in pink) continues to produce haematopoietic progenitor cells.

The intraembryonic (AGM) region, highlighted in green, is found to contain LTR haematopoietic stem cells (Dieterlan-Lievre *et al.*, 1992, Medvinsky *et al.*, 1996, Müller at al., 1997, Dzierzak *et al.*, 1998).

These organ culture experiments have shown that while yolk sac stem cells initiate primitive haematopoiesis as early as E7, they are independent from intraembryonic definitive LTR stem cells found in the AGM at E10. The migration of AGM stem cells to the yolk sac occurs around E11 and the liver by E12 (figure 9).

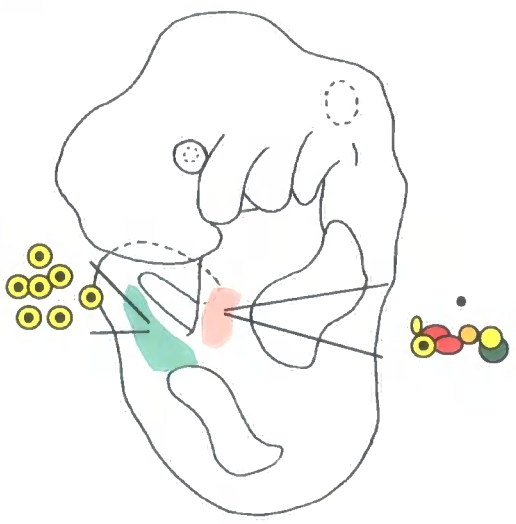


Figure 9 - **Haematopoiesis in the E11.5 mouse embryo.**
LTR HSCs are more abundant in the AGM, highlighted in green, compared to the foetal liver (pink) and the yolk sac surrounding the embryo (not shown).
These sites now contain definitive LTR HSCs that have migrated from the only site of production, the AGM (Dieterlan-Lieve and LeDourain 1993, Medvinsky *et al.*, 1993, Müller *et al.*, 1994, Dzierzak *et al.*, 1998). See 1.11 for cell key.

1.4.4 The transition to adult haematopoiesis

The foetal liver is the principal site of mid- and late- gestation haematopoiesis in mammals, beginning at E10 in mice. The adult haematopoietic system begins in the foetal liver and includes the emergence of a large and expanding pool of multipotent progenitors (Ikuta *et al.*, 1990). From E16 the foetal bone marrow takes over as the principal haematopoietic organ and produces adult type erythrocytes, macrophages, T-cells and conventional B cells (reviewed Bonifer *et al.*, 1998). This site of multi lineage cell production remains throughout adult life.

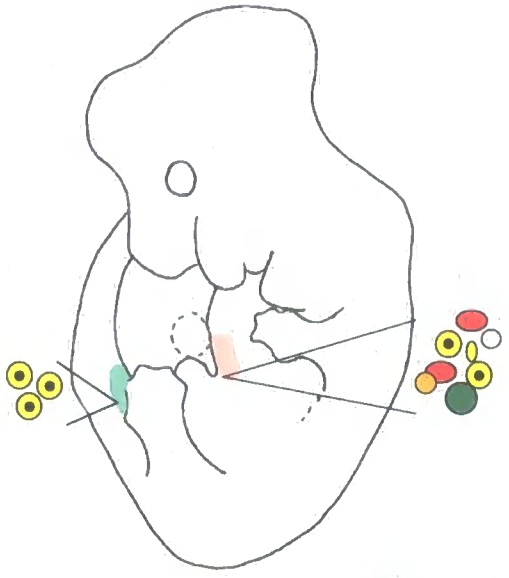


Figure 10 - **Haematopoiesis in the E12.5 mouse embryo.**

Definitive haematopoiesis increases in the foetal liver (pink), whilst activity in the AGM (green) declines.

The extraembryonic yolk sac now begins to degenerate, as the intraembryonic sites take over production (Medvinsky and Dzierzak 1996).

Erythroid progenitor cell
Monomyeloid cell
Committed B cell precursor cell
Granulocytic cell
Macrophage cell
Haematopoietic Stem Cell

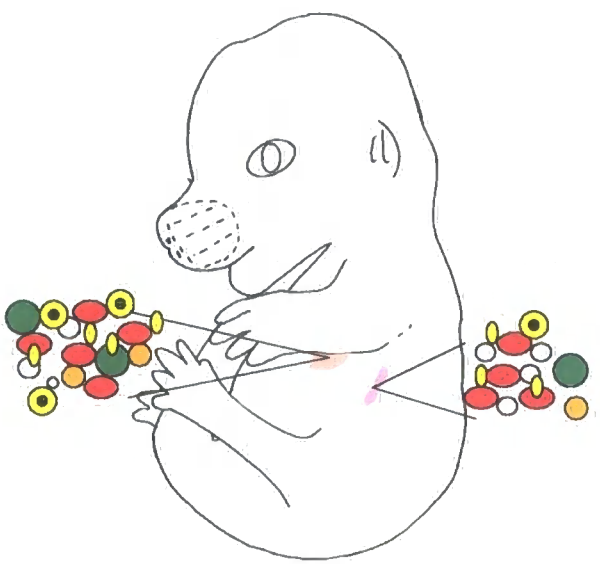


Figure 11 - **Haematopoiesis in the E14.5 mouse embryo.**

Definitive haematopoietic events have shifted to the foetal spleen (purple), whilst production continues in the foetal liver (pink).

Haematopoietic activity will move to the bone marrow, and contain precursor cells needed for adult life (reviewed Medvinsky and Dzierzak 1996).

1.4.5 Why regulate haematopoiesis?

The haematopoietic system of human adults produces approximately one trillion blood cells each day (Emerson, 1991). This continual process involves the sequential commitment of multipotential haematopoietic cells to gradually more-restricted progenitor cells, and finally to functionally eight distinct cells of mature blood shown in figure 1.4.13. The molecular mechanisms that determine these lineages remain central to the field of haematopoiesis.

Lineage-specific transcription factors have essential roles in lineage decisions (reviewed Shivdasani and Orkin, 1996), and include *GATA-1-FOG-1* interactions in erythroid and megakaryocyte differentiation (Pevny *et al.*, 1991, Fujiwara *et al.*, 1996, Shivdasani *et al.*, 1997, Crispino *et al.*, 1999, Vyas *et al.*, 1999, Nichols *et al.*, 2000), *PU-1* in the development of monocytic, granulocytic and lymphoid lineages (Scott *et al.*, 1994, Hromas *et al.*, 1993, McKercher *et al.*, 1996). *PU-1* interacts with *GATA-1* to down regulate *PU-1* expression in erythroid cells, (Moreau-Gachelin *et al.*, 1996). Enforced expression of *GATA-1* in myelo-monocytic cells perturbs their differentiation (Kulessa *et al.*, 1992) highlighting *GATA-1* as a regulator in this lineage pathway. *Scl* is essential for all ontogenetic stages of haematopoiesis. Following targeted mutagenesis of this gene, all haematopoietic lineages are eliminated (Robb *et al.*, 1995, Porcher *et al.*, 1996).

Ikaros is essential for lymphoid development. Targeted mutagenesis leads to the complete absence of B-cell and abnormal T-cell development (Wang *et al.*, 2996).

It is important that each lineage can be derived continually, and more importantly that on demand (following damage), the molecular mechanisms are able to activate the

production pathway of a particular lineage. However these pathway mechanisms are incompletely understood and require further study.

1.4.6 *Hox* genes as potential molecular markers of haematopoiesis

It would seem logical to study developmental genes in such a system that is initiated during embryogenesis and continued on into the adult.

A distinguishing hallmark of *Hox* complexes is the correlation between physical order of genes along the chromosome and their expression pattern along the anteroposterior axis of the embryo (Lewis 1978). This mechanism for representing and possibly predicting the regulation of regional identity, may suggest that *Hox* genes contribute to the control of this developmental pathway.

1.5 *Hox* gene expression in differentiating blood cells

Evidence from embryonic *Hox* gene expression studies has shown temporal patterning. There is increasing evidence of *Hox* gene transcripts present in a variety of primitive and definitive blood cell progenitors. Genetic manipulation by gene overexpression into primary cell lines, has revealed several *Hox* genes as key regulators in the production of blood cell lineages.

Figure 12 below highlights the expression patterns of specific *Hox* genes as potential regulators of lineage specific pathways, and the methods used for analysis.

***Hox* Gene**	**Lineage pathway involvement**	**Study**	**Reference**
HOX A cluster	$CD34^+$ (primitive)	RT-PCR analysis	Vieille-Grosjean *et al.*, 1992
HOXA5	Myeloid	RT-PCR analysis	Fuller *et al.*, 1999
		anti-sense oligo	Crooks *et al.*, 1999
HOXA9/a-9	Myeloid	Targeted disruption	Lawrence *et al.*, 1997
	T-cell	Targeted disruption	Izon *et al.*, 1998
	B-cell	Overexpression	Thorsteinsdottir *et al.*, 2002
HOXA10	Myelomonocytic	mRNA analysis	Lowney *et al.*, 1991
	Myeloid	Overexpression	Thorsteinsdottir *et al.*, 1997
		Transgenic mice	Bjornsson *et al.*, 2001
		Overexpression	Buske *et al.*, 2001
HOXB3	Lymphoid	Overexpression	Sauvageau *et al.*, 1995, 1997
HOXB4	Erythroid	Anti-sense oligo	Shen *et al.*, 1989
		Overexpression	Helgason *et al.*, 1996
	Myeloid	Overexpression	Helgason *et al.*, 1996
HOXB5	Erythroid	Library screening	Shen *et al.*, 1989
HOXB6	Erthroid	Anti-sense oligo	Shen *et al.*, 1989,
		Targeted disruption	Kappen *et al.*, 2000
	Granulocytic	RT-PCR	Giampaolo *et al.*, 1994
	Normal bone marrow	Library screening	Shen *et al.*, 1989
HOXB7	Erythroid	RT-PCR	Sauvageau *et al.*, 1994
	Granulocyte	Targeted disruption	Yaron *et al.*, 2001
	Myelomonocytic	Anti-sense oligo	Lill *et al.*, 1995
	Myeloid	RT-PCR	Sauvageau *et al.*, 1994

Hox Gene	Lineage pathway involvement	Study	Reference
HOXB8	Myeloid	Overexpression Library screening	Blatt *et al.*, 1988, 1992 Kongsuwan *et al.*, 1988
HOXC4	Lymphoid	Rnase protection assay	Meazza *et al.*, 1995
HOXC5	Lymphoid	RT-PCR RISH	Bijl *et al.*, 1996
HOXC6	Erythroid	Anti-sense oligo	Takeshita *et al.*, 1993

Figure 12 - *Hox* gene expression patterns in haematopoietic cell lineages.

In summary, much of the work on *Hox* gene expression in normal haematopoietic cells has been carried out on human and mouse transformed cell lines (Kongsuwan *et al.*,1988, Shen *et al.*, 1989, 1990, Lowney *et al.*, 1991, Magli *et al.*, 1991, Mathews *et al.*, 1991, Vieille-Grosjean *et al.*, 1992), primitive haematopoietic cells ($CD34^+$) (Sauvageau *et al.*, 1994), and undifferentiated bone marrow cells (Sauvageau *et al.*, 1994). The expression of *HOXA* cluster is associated with the myelomonocytic lineage (Lowney *et al.*, 1991, Vieille-Grosjean *et al.*, 1995), expression of *HOXB* genes to cells differentiating with erythroid potential (Shen *et al.*, 1989, 1990, Mathews *et al.*, 1991, Sauvageau *et al.*, 1994, 1995), and *HOXC* genes are largely expressed in cells of the lymphoid system (Takeshita *et al.*, 1993, Meazza *et al.*, 1995, Bijl *et al.*, 1996).

Information on the expression of *Hox* regulator genes, such as *polycombs* is limited, but it is known that the expression pattern of *Pc* genes does change during the differentiation of human bone marrow cells *in vitro* (Lessard *et al.*, 1998).

These findings of *Hox* gene expression during normal lineage development and differentiation suggest several potential roles for these homeotic genes as potential regulators of embryonic and or adult haematopoiesis.

The precise function of these genes awaits closer analysis. One approach is to examine their expression in pathological conditions, particularly ones in which normal commitment and differentiation is subverted; such an example can be found in neoplasia.

1.6 *Hox* genes and cancer

Disturbances in normal adult *Hox* gene expression can be found in carcinomas (Cillo *et al.*, 1992). In primary kidney tumours *HOX* genes can be turned off (*HOXB5, B9*) or on (*HOXC11*) or can display different-sized transcripts (*HOXD4*) if compared to the expression in normal kidneys (Cillo *et al.*, 1995). This association between altered *HOX* gene expression and kidney cancer, has subsequently been proven for other *HOX* and homeobox genes.

Dysregulated expression of *HOXA9, HOXB7,* and *HOXD11* has been detected in metastatic liver lesions that have originated from colorectal tumours, suggesting a possible involvement of *HOX* genes in colon cancer (De Vita *et al.*, 1993).

There is an association between the progression of small cell lung cancer and the inactivation of several *HOX* genes that are expressed in the normal lung (Tiberio *et al.*, 1994).

In mammary glands, *Hoxc-6* transcripts, active during puberty and maturity, become silent during pregnancy (Friedmann *et al.*, 1994), transcripts are also undetectable in mammary adenocarcinomas . In contrast, *Hoxa-1*, silent in normal mammary gland tissue, becomes active in the presence of breast cancers in both mice and humans. Furthermore, in mammary epithelial cells, *Hoxa-1* and *Hoxb-7* are regulated by extracellular matrix-dependent signals (Srebrow *et al.*, 1998). The transduction of *HOXB7* has been shown to induce bFGF expression and alter growth characteristics of breast cancer cells (Carè *et al.*, 1998).

There is also evidence that the expression of several *HOX* genes expression is altered in other forms of cancer including: cancer of the prostate (Robbins *et al.*, 1996, Sciavolino *et al.*, 1998); corectal cancer (Vider *et al.*, 1997); papillomas (Chang *et al.*, 1998); melanomas (Carè *et al.*, 1996); and neuroblastomas (Osborne *et al.*, 1998).

However, the strongest data for a role of *Hox* in neoplasia is in the onset of leukaemia.

1.6.1 *Hox* genes in leukaemia

The potential regulatory roles of *Hox* genes in the normal development and commitment of haematopoiesis have been shown (table 1.1e). It can be suggested from these previous studies that *Hox* genes play a regulatory role during leukemogenesis (Shen *et al.*, 1989, Borrow *et al.*, 1996, Nakamura *et al.*, 1996, 1996a, Knoepfler *et al.*, 1997, Lawrence *et al.*, 1997, Thorsteinsdottir *et al.*, 1997).

The transduction of normal bone marrow with a retroviral-*Hoxb-8* construct together with the activation of the IL-3 gene was able to induce myeloid leukaemia in mice (Perkins *et al.*, 1990). Also in mice, the overexpression of *Hoxa-10* induces myeloid leukaemia after

long latency periods in mice (Thorsteinsdottir *et al.*, 1997). Cooperation between *HOXB3, HOXB4* and *Pbx1* homeoproteins are likely to play an important part in blood cell proliferation and transformation (Krosl *et al.*, 1998).

Retroviruses can be used to induce myeloid leukaemia in BXH-2 mice by insertional mutation of cellular proto-oncogenes or tumour suppressor genes. Potential disease genes can thus be identified by proviral tagging through the identification of common viral integration sites. Using this technique, Nakamura (Nakamura *et al.*, 1996a), identified that proviral activation of *Hoxa-7,*and *Hoxa-9,* associated independently correlated with proviral activation of *Meis1* (a Pbx related homeobox gene), resulting in the onset of acute myeloid leukaemia, suggesting that a *Pbx-1* related gene cooperates with these two *Hox* genes in leukaemia formation.

Homeodomains of paralogue groups 1 to 10 are stabilised in their binding activity by PBX1, whereas *AbdB*-like *Hox* proteins require interaction with *meis1* in order to bind to their target (Shen *et al.*, 1997).

Hoxa-9 overexpression in mouse bone marrow cells is closely associated with the development of acute myeloid leukaemia. Thorsteinsdottir (Thorsteinsdottir *et al.*, 2002) used two different mouse models (transplantation chimeras of overexpressing bone marrow cells and transgenics) to study the effects of *Hoxa-9* overexpression on haematopoietic cells prior to the occurrence of leukaemic transformation. They demonstrated that contrary to a previous loss of function study (Izon *et al.*, 1998), both the HSC and myeloid progenitor cell pools are significantly increased in the *Hoxa-9* transplantation chimeras. In both models there was a reduction in B-cell progenitors, suggesting a further role for this *Hox* gene in lymphoid cell development.

Evidence for a role for *Hox* genes in leukaemia comes from spontaneous human leukaemia. In human acute myeloid leukaemia (AML), the translocation t(7;11)(p15;p15) fuses the nucleoporin gene NUP98 in frame with the *HOXA9* gene (Borrow *et al.*, 1996, Nakamura *et al.*, 1996). Chimeric fusion proteins resulting from the transcription activator domain of one protein and the DNA binding domain of the other protein display increased oncogenic properties when compared to the components. Fusion between PBX1 and E2A proteins alters the sequence-specific binding to the *HOX* genes directing the homeoprotein on a different target and inducing leukemogenesis (Knoepfler *et al.*, 1977). There are several invaluable studies investigating the role of *Hoxa-9* in leukaemia however, the function of *Hoxa-9* in normal haematopoiesis remains to be elucidated.

1.7 Aims

Hox genes are invaluable and essential for cell identity in regulating several embryonic developmental processes in a broad variety of species.

There is already evidence for several *Hox* genes playing key roles during definitive haematopoiesis and in particular *Hoxa-9* in haematopoietic disease, which lead this study to focus on the understanding of the function of *Hoxa-9* during normal stages of blood cell development, especially in the embryo. To investigate the role of *Hoxa-9* during embryonic mouse development, it was intended to use conventional wholemount and *in situ* hybridisation techniques on a series of mouse embryos from E7-E14. This allowed the anatomical study to screen the different events seen by the expression pattern of *Hoxa-9* during the course of essential haematopoiesis in the embryonic mouse (E7-E14),

as well as allowing the study of other potential roles for *Hoxa-9* during embryonic development. To extend this expression study further, an embryonic stem cell model was used to detect the expression patterns of *Hoxa-9* during the early differentiation and development of ES cell derived cell lineages. In order to investigate a potential function for *Hoxa-9* during this developmental process, genomic manipulation techniques were applied to this conventional differentiation model to study the effects on lineage commitment following the overexpression of *Hoxa-9*.

Chapter Two – Wholemount and *in situ* hybridisation

2.1 Introduction

Hox genes have been shown to be crucial in several developmental processes during embryogenesis. Several of these findings were identified using *in situ* and wholemount hybridisation techniques, with further functional studies using knock out and overexpression procedures.

Previous overexpression and targeted mutation studies have shown that *Hoxa-9* like several other *Hox* genes (figure 12) play a regulatory role in myeloid, and lymphoid cell development (Lawrence *et al.*, 1997, Izon *et al.*, 1998, Thorsteinsdottir *et al.*, 2002). This gene is also associated with the onset of acute myeloid leukaemia in mice (Nakamura *et al.*, 1996, Thorsteinsdottir *et al.*, 2002), and humans (Nakamura *et al.*, 1996b, Borrow *et al.*, 1996, Dash *et al.*, 2002).

It followed therefore to use conventional *in situ* and wholemount hybridisation techniques to study the expression pattern of *Hoxa-9* in normal embryonic development of the mouse of the core time course of embryonic haematopoiesis (E7-E14).

Previous *Hoxa-9 in situ* hybridisation studies generated invaluable data on the expression pattern during embryogenesis. However, these limited studies did not address the patterning at haematopoietic sites. Therefore due to the potential regulatory roles of *Hoxa-9* during haematopoiesis and the correlation with AML onset, it would seem logical to use these methods that have been used in previous *Hoxa-9* gene studies to further investigate the normal molecular control of *Hoxa-9* during haematopoiesis in the mouse embryo.

2.1.1 The application of *in situ* hybridisation

The detection of specific nucleic acid sequences in cells, tissues, or whole organisms by *in situ* hybridisation has important applications in many areas of biology. There is an abundance of cloned genes with potential roles in embryonic tissues. Analysis of the spatial and temporal regulation of the expression of these genes by *in situ* hybridisation to mRNA is a crucial step towards understanding gene function and elucidating the biology system at the molecular level.

The ease and speed with which patterns of gene expression can be visualised by wholemount and *in situ* hybridisation makes it an attractive technique for screening large numbers of candidate clones for region- or tissue-specific expression. This method also enables the detection of significant changes in gene expression that may occur only in small sub-populations of cells.

2.1.2 Detection of *Hox* genes during development using wholemount and *in situ* hybridisation methods:

There is increasing use of *in situ* and wholemount hybridisation to detect expression and identify functions of *Hox* genes in several areas of developmental biology. The most explored developmental areas using these techniques have largely contributed to understanding the regulation of the neural crest, hindbrain, axial skeleton, limbs and gut, all of which will be discussed in this chapter.

2.1.2.1 Expression in neural tube and crest development;

In situ and wholemount hybridisation techniques with *Hox* genes in the chick have identified expression boundaries and regulatory mechanisms in neural crest cells.

In the chick, the expression pattern of *Hoxa-2* was analysed during early development in the rhomboencephalic neural tube and derived neural crest using wholemount and *in situ* hybridisation techniques.

The experiments using a digoxigenin-labelled riboprobe in stage 9 and 10 chick embryos, showed that *Hoxa-2* has a rostral limit of expression in the rhombencephalic neural tube corresponding to the boundary between rhombomeres r1 and 2. This *in situ* data along with corresponding grafting experiments showed the specificity of *Hoxa-2* expression in neural crest-derived rhombomere patterning (Prince and Lumsden, 1994).

A similar experiment using *in situ* and wholemount hybridisation demonstrated *Hoxa-3* expression throughout chick development, in the neural plate and later in the neural tube, corresponding to the boundary between rhombomeres 4 and 5. This method followed *Hoxa-3* expression in caudally migrating neural crest cells from their rhombomeres of origin to determine neural crest cell regulation (Saldivar *et al.*, 1996).

The maintenance and regulation of vertebrae hindbrain segmentation into rhombomeres in not fully understood. Using wholemount *in situ* hybridisation following screening from a subtracted embryonic chick hindbrain cDNA library, a large proportion of genes, including *Hoxd-3,* with restricted expression patterns and previously unknown functions in the embryonic brain were identified (Christiansen *et al.*, 2001). Further regulatory mechanisms in hindbrain development have been identified using these techniques.

Hoxb-2 and *Hoxa-2* gene expression in rhombomeres 3 and 5, have been shown to regulate patterning of the vertebrae hindbrain via *Krox20* (Nonchev *et al.*, 1996).

2.1.2.2 Cranio-facial development;

In situ hybridisation was used to establish temporal and spatial expression patterns of *Hox-7,* in the developing embryonic cranium and nervous system of the mouse between E9.5 and E15.5. It was suggested from the resulting expression patterns that *Hox-7* might be a good candidate as one of the genes involved in the formation and development of the outer ear, and the differentiation of the cell types of the anterior pituitary (MacKenzie *et al.*, 1991).

By using specific probes against the message of eight *HOX* genes, *HOXB1, A2, B2, A3, B3, D3, B4* and *C4* were found to be expressed in the human embryonic hindbrain and branchial arches at 4 weeks of development. The combinatorial patterns of expression of genes representing the first three paralogous groups parallel the patterns described for their homologues in various animal models, demonstrating a high degree of conservation of the 'brachial Hox code' (Vieille-Grosjean *et al.*, 1997) – the developmental strategy whereby positional specification made axially within the neural tube is transmitted to the periphery via migrating neural crest cells (Hunt *et al.*, 1991).

2.1.2.3 Axial skeletal and limb development;

Mapping the altered patterns of gene expression in mutant embryos can yield valuable information about the mutant phenotype and elucidate the genetic interactions that are involved.

Mice were generated with a targeted mutation of *Hoxb-9* (Chen and Capecchi, 1997),. Homologous mice showed defects in the development of the first and second ribs, and a significant role was suggested in the role for *Hoxb-9* in specifying thoracic skeletal elements. It has been reported that in some cases a mutation in one *Hox* gene can affect the expression of a neighbouring *Hox* gene (Suemori *et al.*, 1995, Borrow and Capecchi, 1996). To determine if the *Hoxb-9* mutation affected *Hoxb-8* or *Hoxb-7* expression, wholemount *in situ* hybridisation was carried out on E10.5 and E12.5 embryos of all three *Hoxb-9* genotypes. It appeared from using this technique that the absence of functional *Hoxb-9* did not affect the expression of neighbouring genes.

In limb development, several *Hox* genes (*Hox-1.8 (a-10), 1.9(a-11), 1.10(a-13)* have been shown to be expressed in a temporal and spatial manner using *in situ* hybridisation (Haack and Gruss, 1993).

2.1.2.4 Gut development;

To elucidate the anteroposterior patterning of the digestive tract, expression patterns of *Hox* genes belonging to paralogue groups 6, 7, 8, and 9 were examined using wholemount *in situ* hybridisation. The expression patterns of these genes showed co-linearity along the wall of the digestive tract, with later expression in individual gut subdomains, suggesting a function for *Hox* genes in specifying mesenchymal differentiation of the gut (Sekimoto *et al.*, 1998). Roberts (Roberts *et al.*, 1995) found a similar expression pattern in the developing gut of the chick using wholemount *in situ* hybridisation.

2.1.2.5 Other developmental processes

In situ and wholemount studies using *Hox* genes have also contributed to a lesser degree in other areas of development including tooth initiation and shape – *Hoxc-8* was detected during the initiation and development of the molar and incisor teeth in the neural crest-derived mesenchyme tissue beneath the site of future tooth formation in the embryonic mouse (MacKenzie *et al.*, 1992). *Hox 2.2 (Hoxb-6),* has been suggested to play a role in epidermal development following detected transcripts in 17-day foetal skin using *in situ* hybridisation (Mathews *et al.*, 1993). An association between *HOXB6* and erythropoietin (*EPO*) expression in the E8.5 mouse embryo was found using wholemount *in situ* hybridisation. It was shown that the sequential transfer of erythropoiesis in different organs during development was followed by a similar transfer of *HoxB6* and *EPO* gene expression (Zimmermann and Rich, 1997).

The importance of wholemount and *in situ* hybridisation applications to *Hox* genes during development have been shown to contribute largely to understanding functional roles for these genes during the maturity of several processes.

2.1.3 Aims

The significant association of *Hoxa-9* in the onset of leukaemia has previously been highlighted, and therefore understanding the normal biology of *Hoxa-9* during haematopoiesis becomes significant for further investigation.

Previous developmental studies of *Hox* genes have applied conventional wholemount and *in situ* hybridisation techniques to detect and highlight expression patterns in the embryo,

which can be further analysed to identify potential functional roles. Subsequent *Hoxa-9* expression studies did not report findings in the haematopoietic system of the embryo.

To further the current study, the established hybridisation techniques described above, were used to analyse the *Hoxa-9* expression pattern in the mouse embryo throughout the crucial developmental stages of embryonic haematopoiesis from initiation in the yolk sac (E7.5) to definitive stages (E12), in an attempt to further understand the normal functions of this gene during blood cell production and differentiation.

2.2 Materials and Methods

All chemicals were supplied by Sigma, (Poole, Dorset, England) unless otherwise specified.

All procedures were carried out at room temperature unless otherwise stated.

Dilutions were carried out using DEPC treated water (A.4), unless otherwise stated.

2.2.1 Polymerase Chain Reaction (PCR)

The PCR was set up by mixing together 0.5 µg cDNA with 5 µl 5x PCR NH_4 reaction buffer (Bioline, London, England), 1.5 mM $MgCl_2$ (Bioline), 10 mM dNTPs (Promega, Southampton, Hampshire, England), 0.1 ng of both forward and reverse PCR primers (Invitrogen) and 1 unit of Taq polymerase (Bioline).

The PCR reactions were run using Omn-E thermal cycler (Hybaid, Ashford, Middlesex, England) using the following programme:

94°C for four minutes

94°C for one minute
x°C (hybridisation temperature) for one minute } x35 cycles
72°C for one minute

72°C for ten minutes

2.2.2 Gel electrophoresis

PCR products were run on 2% agarose gels, made by dissolving 2% (w/v) agarose powder (Bioline) in 1xTAE buffer (see A.18). Once cool (50°C) 0.5 µg/ml ethidium

bromide was added and poured into a pre-cast gel tray containing combs and left to solidify at room temperature. Once cool, the combs were removed and the gel placed into a gel tank submerged in 1xTAE buffer. 15 µl of PCR product was mixed with 2 µl loading stain (Promega) and loaded into individual wells. Once all samples had been loaded 5 units of a DNA ladder (Promega) was added and the gel run at 150 volts for forty minutes. The DNA on the gel was then visualised using Bio-Rad 'Gel Doc' Ultra Violet illuminator at 366nm, and the results recorded.

2.2.3 Collection of mouse embryos

Mouse embryos from Balb/C mice were collected between E9 and E12 days of gestation. Their developmental stage was confirmed according to Kaufmann's stages of development (Kaufman, 1992). Males were introduced to a cage with females for twelve hours overnight and dated matings were identified by the presence of vaginal mucous plug, and considered to be embryonic day 0.5. Pregnant females were sacrificed by a schedule I procedure of cervical dislocation. The abdominal skin was cleaned with 70% v/v ethanol, the uterus removed and dissected in order to remove the embryos. Up to nine embryos were collected at each time point, and for every wholemount and *in situ* hybridisation in chapter two, three whole embryos were used for independent experimental procedures. Whole mouse embryos for wholemount *in situ* hybridisation were a kind gift from Dr. Heiko Peters at The International Centre for Life at the University of Newcastle, and used as described in Peters *et al.*, 1995, 1999.

All embryos were fixed overnight in 4% paraformaldehyde (w/v) (PFA) in PBS at 4°C. Following a brief rinse in Diethyl Pyrocarbonate (DEPC) treated PBS (see appendix A.4.

A.15), embryos were dehydrated through an ethanol series starting at 50% (v/v), 70% (v/v), 85% (v/v), 95% (v/v) and finally 100% ethanol, each for 30 minutes at room temperature, transferred to Histoclear and stored in a pre-baked glass jar at 4°C.

2.2.4 Wax embedding and sectioning

Histoclear was removed from the embryo tissue and replaced with wax, by immersion into molten paraffin wax that had been pre-warmed in a 60°C oven, for three, two-hour incubations. The embryos were left in the final wax change overnight, at 60°C.

Paraffin wax was then poured into sterile casts (BDH), and the tissue placed centrally, approximately 0.5 cm below the surface, and the wax block submerged immediately into cold water, to solidify.

The paraffin wax blocks containing fixed embryos were oriented into the required plane to enable transverse or sagittal sectioning and mounted on a Leitz 152 microtome stage.

The microtome, bench area and cutting accessories were cleaned thoroughly with 0.1% (v/v) DEPC in 96% (v/v) ethanol to ensure as far as possible that they were RNAse-free.

7μm thick sections were cut using a standard Leitz 152 microtome and allowed to dry overnight on pre polysine-treated slides.

Embedded embryos were either used immediately or stored in sealed plastic bags at 4°C until needed.

2.2.5 Wholemount and *in situ* hybridisation riboprobes

Anti-sense and sense, single stranded RNA probes were synthesised for wholemount and *in situ* hybridisation from cDNA clones contained in vectors. The *Hoxa-9* sense and anti-

sense were generated by inserting the full coding sequence (813bp) into a CT-TOPO-GFP vector in both a sense and anti-sense orientation, therefore using only one promoter site. However the other riboprobes (*Hoxc-9, vMLC*) were synthesised in a Bluescript vector, therefore being inserted in a sense orientation and using the two RNA polymerase promoter sites to generate both sense and anti-sense probes. The cDNA clones used for this purpose are described below:

(i) The murine ***Hoxa-9 cDNA clone*** was generated by PCR and cloned using the CT-TOPO-GFP cloning kit (Invitrogen, Paisley Scotland, UK).

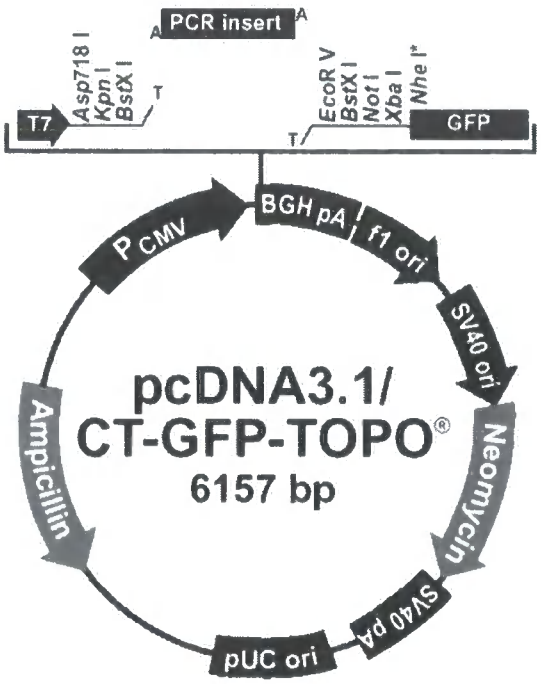


Figure 13 - The CT-TOPO-GFP-*a9* vector

2.2.6 CT-TOPO-GFP cloning

This vector, used to generate the *Hoxa-9* clone required that the PCR product that was to be inserted into the CT-TOPO-GFP directly carried a Kozak sequence at the 5' terminus. Therefore, PCR primers were designed to add the 'ACC' codon, before the 'ATG' start codon in the insert DNA, shown in sequence appendix, B.1.

CT-TOPO-GFP acquired the insert by incubating up to 5 µg of Kozak sequence containing PCR product with 1µg of the CT-TOPO-GFP vector for five minutes at room temperature. After five minutes, 1µl of 'stop' solution (see A.21) was added to complete the reaction.

2 µl of the CT-TOPO-GFP-DNA mix was added to one shot TOP10 chemically competent *E.coli* (Invitrogen, #C4040-10) competent cells respectively and stored on ice for thirty minutes. 500 µl of provided SOC medium was added and incubated on a platform shaker for thirty minutes at 37°C. Following incubation, 50-100 µl of mix was plated out onto LB agar plates (see appendix A.9) containing 50 µg/ml ampicillin for vector ampicillin resistance, and incubated overnight at 37°C.

ii) Murine Hoxc-9 cDNA clone provided from IMAGE clone consortium (#101188). The cDNA clone has been inserted into pBluescript KS+ (Stratagene).

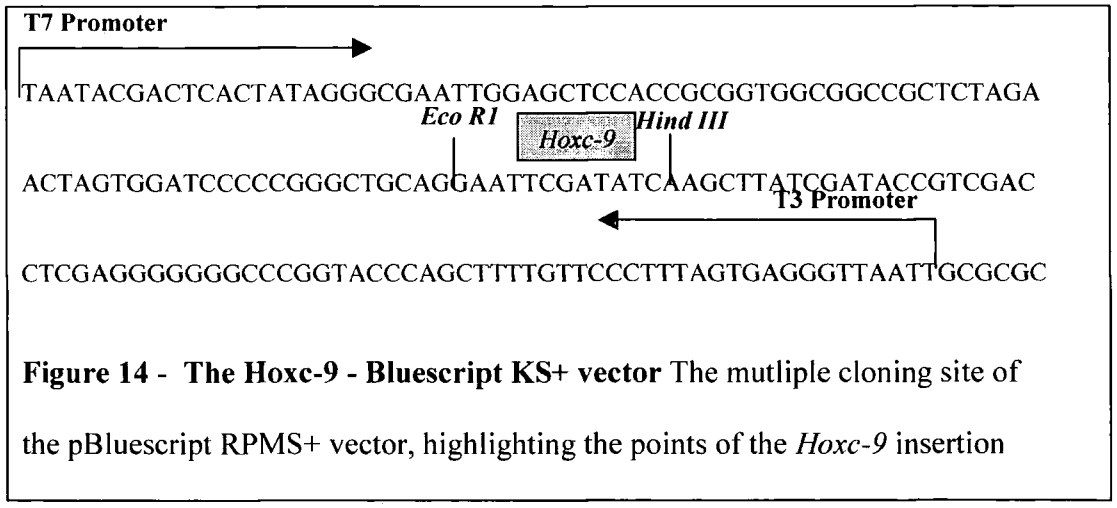


Figure 14 - The Hoxc-9 - Bluescript KS+ vector The mutliple cloning site of the pBluescript RPMS+ vector, highlighting the points of the *Hoxc-9* insertion

The *Hoxc-9* DNA insert contained the majority of the *Hoxc-9* full coding sequence (719bp) shown in B.6, and was again sequenced in both directions to ensure there were no errors, and confirm orientation.

iii) Ventricular Myosin Light Chain2 (vMLC2) was a kind gift from Professor David Wilson (University of Southampton).

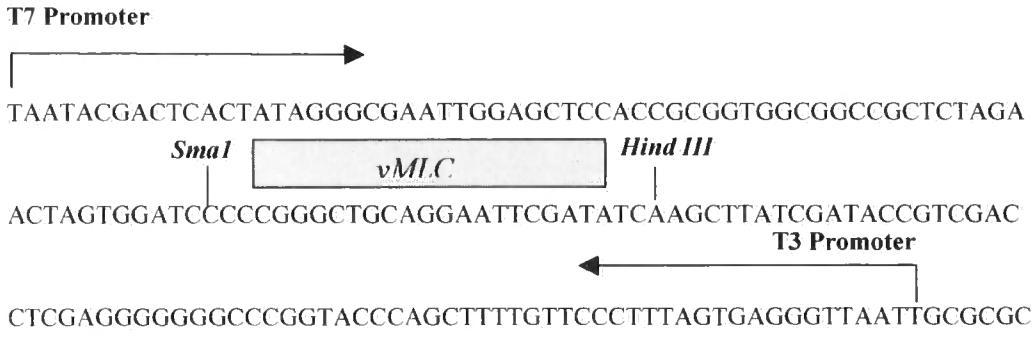


Figure 15 - The *vMLC2* - Bluescript vector – The multiple cloning site of the pBluescript KS+ vector, highlighting the points of the *vMLC* insertion

The *vMLC2* coding sequence was ligated into Bluescript KS+ (Stratagene), and sequenced as shown in B.4.

iv) Pax-1 was a kind gift from Dr. Heiko Peters, at The Centre of Life in Newcastle-Upon-Tyne as reported in Peters *et al.*, 1995, 1997.

In order to control for non-specific staining of a riboprobe, throughout this study, a negative control sense strand riboprobe (inverse vector insertion of the gene), that would

not recognise the single strand RNA in the mouse embryo sample, was always hybridised under the same conditions as the corresponding anti sense strand.

2.2.7 Riboprobe production

Prior to digoxigenin labelling each plasmid had to be linearised by mixing 4 units of a sequence specific enzyme that cuts only at the multiple cloning site, to 1 μg of plasmid DNA, and 5x enzyme buffer in a 50 μl total volume mix. This mix was left at 37°C for three hours and confirmed by gel electrophoresis. The single stranded RNA probes incorporated digoxigenin (DIG)-UTP and were produced by *in vitro* transcription with T7 or T3 RNA polymerase (shown in figure 16). The residues are incorporated at approximately every 20-25th nucleotide of the transcript (Roche, Lewes, East Sussex, England).

Figure 16 - The enzymes used for plasmid linearisation and transcription

Riboprobe	**Linearisation enzyme**	**Polymerase used for transcription**
CT-TOPO-GFP-*Hoxa-9s*	*Pvu1*	*T3*
CT-TOPO-GFP-*Hoxa-9a*	*Pvu1*	*T3*
Bluescript-*Hoxc-9s*	*EcoR1*	*T7*
Bluescript-*Hoxc-9a*	*HindIII*	*T3*
Bluescript – *vMLC2s*	*Sma1*	*T7*
Bluescript – *vMLC2a*	*HindIII*	*T3*

s = sense strand a = anti-sense strand

1 μg of linearised plasmid was added to a DIG labelling mix following the manufacturers instructions. Briefly, the linearised plasmid was mixed with 2 μl 10x DIG RNA labelling mix, 2 μl 10x transcription buffer, 1unit of respective polymerase (figure 16). The mix was left for two hours at 37°C and treated with 1unit DNAse 1 (Promega) at 37°C for fifteen minutes. A 5 μl sample of the reaction was run on a 1% agarose gel to confirm labelling. The labelled probe was stored under 100μl 100% ethanol at –20°C. When needed, the probe could be diluted to a concentration of 0.1 ng/μl using DEPC water.

2.2.8 Testing probe labelling – Dot/Southern blot analysis

Dot blot analysis was carried out in order to test whether the riboprobe had been labelled efficiently.

Riboprobe vector, as described using the CT-TOPO-GFP-*Hoxa-9* was linearised as described and a range of concentrations from 100 pg of linearised vector to 1 pg, and 1 μl of the vector was dotted onto a positively charged nylon membrane and allowed to air-dry.

The blot was then denatured by immersion in a denaturing buffer (see appendix A.2) for 10 minutes, followed by a neutralisation step for a further ten minutes (see appendix A.12). After air-drying the blot, the DNA was fixed by being exposed to a transilluminator ultra violet light (366nm) for three minutes. Pre-hybridisation of the blot was carried out by immersion in hybridisation buffer (Clontech, Basingstoke, Hampshire, England) for thirty minutes at 37°C. 100ng of labelled probe was added to 5ml of fresh hybridisation buffer, and the filter was incubated in a hybridisation oven, under constant agitation for one hour at 37°C.

Stringency washes were carried out at room temperature initially in wash solution 1 (see appendix A.22) twice for five minutes each wash, and then twice in wash solution 2 (see appendix A.23) at the hybridisation temperature for five minutes.

After being rinsed in maleic acid buffer (see appendix A.11), non-specific binding was blocked by incubating for thirty minutes at room temperature in 1x blocking solution (see appendix A.1). Anti-DIG alkaline phosphatase antibody (Roche, Lewes, East Sussex, England) was added at a dilution of 1:5000 in 1x blocking solution and the blot was incubated at room temperature for a further 30 minutes.

A further two fifteen minute washes with maleic acid buffer at room temperature followed, and then a brief rinse in detection buffer (see appendix A.3). A dilute colour solution of 0.4mg NBT (nitro blue tetrazolium) and 0.175 mg BCIP (5-bromo-4-chloro-inodyl-phosphate) (both from a 50 mg/ml stock in 70% and 100% dimethylformamide respectively) was added to the detection buffer and then incubated with the blot in the dark, until development was sufficient.

The blot was either stored in 50 ml Tris-EDTA buffer (TE buffer) (see appendix A.20), until being dried at room temperature on filter paper, or on occasions the blot was re-probed by submersion in 100% dimethylformamide for twenty minutes at room temperature to strip the DIG colour stain from the blot, and then rinsed briefly in TE buffer, before being re-submerged in hybridisation buffer.

2.2.9 *In situ* hybridisation

All glassware was baked overnight to ensure minimal RNAse activity.

All the solutions were made up fresh and used on the day, except where directed.

Three independent sets of wholemount and *in situ* hybridisation results were generated for each set of results shown in figures 18-32.

To remove the paraffin wax, the sections prepared as described in 2.2.4 were placed in Histoclear for ten minutes at room temperature, followed by seven, five minute rehydration step series through methanol starting with two immersions in 100% methanol followed by 90% (v/v), 70% (v/v), 50% (v/v) methanol and finally into PBS twice. Sections were bleached in a final concentration of 6% (v/v) hydrogen peroxide in PBT (PBS + 10% (v/v) tween 20) for one hour at room temperature, followed by three PBT washes.

In order to increase the accessibility of target RNA, the sections were incubated in 10mg/ml proteinase K in PBT for fifteen minutes at room temperature.

Sections were washed for five minutes in PBT and post fixed in 0.2% (v/v) glutaraldehyde in 4% (w/v) paraformaldehyde (in PBS) for twenty minutes at room temperature, and then rinsed in two, five minute washes of PBS. The sections were then dehydrated for five minutes each in 25% (v/v) methanol, 50% (v/v) methanol, 75% (v/v) methanol, and finally immersed into 100% methanol, and then allowed to air dry.

A specific concentration (see figure 17) of labelled probe as determined by dot blot analysis, was diluted in hybridisation buffer to make a final volume of 100 µl (see appendix A.6). For each slide 100 µl of diluted probe was placed in a 'sureseal' cover slip (BDH) and incubated overnight in a moist chamber at the indicated hybridisation temperature.

The sections, were then given two washes of thirty minutes in '*in situ*' solution 1 (see appendix A.7), twice, for thirty minutes at the indicated washing temperature, followed

by three thirty-minute washes in 'in-situ' solution 2 (see appendix A.8) at the same temperature. Sections were then washed twice for ten minutes in TBST (see appendix A.19), and a further wash in TBST with the addition of 30mM levamisole for thirty minutes to minimise any background alkaline phosphatase activity.

Meanwhile 6mg embryo or heart powder, produced as described in 2.2.11, was heated to 65-70°C for one hour in 1ml TBST to deactivate any residual complement activity. Anti DIG was diluted 1:5000 in 5% (v/v) lamb serum (in PBS) and added to the embryo or heart powder, and agitated for one hour at 4°C. The reaction mix was centrifuged at 4000*g* (Sigma 2-15) for two minutes at room temperature. 1ml of the resulting supernatant was added to a further 1ml of lamb serum. This mixture was further added to 98 ml TBST and the sections were left overnight at 4°C.

Vector	Hybridisation Temperature (°C)	Stringency Wash Temperature (°C)	Amount of labelled probe added for hybridisation
Hoxa-9	55	50	300 ng
Hoxc-9	63	60	500 ng
VMLC2	65	60	300 ng
Pax-1	70	70	100 ng

Figure 17 - to show the hybridisation and stringency wash temperatures for the wholemount and *in situ* hybridisation sense and anti sense riboprobes.

Sections were washed three times for twenty minutes in PBT, followed by three thirty minute washes in NTMT (see appendix A.14) containing 2 mM levamisole.

Sections were then placed in a moist chamber in the dark, and a colour substrate of 0.4 mg NBT and 0.175 mg BCIP were added to 1ml NTMT, until the desired colour intensity had developed.

The sections were then viewed using a bright field microscope, mounted, and photographs taken using bright field microscopy and the 'SPOT' image capture unit, and 'SPOT RT software version 3.0, Diagnostic Instruments Incorporated'.

2.2.10 Wholemount *in situ* hybridisation of mouse embryos

Embryos were collected as in 2.2.3 and were prepared for wholemount *in situ* hybridisation by puncturing the amnion (E8) or myencephalon (E9 and older) using a sterile needle. This procedure has been previously shown to improve probe penetration and reduce non-specific stain trapping (reviewed Wilkinson 1992). Subsequent procedures were carried out with embryos resting in small sieves (Costar netwell inserts). Washes and hybridisation steps were carried out in twelve well plates (IWAKI, Paisley, Scotland). Embryos were fixed overnight at 4°C, in 4% (w/v) PFA in DEPC treated PBS (see appendix A.4, A.15). Following fixing, the embryos were washed in PBT (see appendix A.16) at 4°C for five minutes, and dehydrated through a methanol series in 1xPBT starting with 25% (v/v), 50% (v/v), 75% (v/v), followed by two 100% methanol immersions. Each dehydration step was carried out for five minutes each at room temperature. If necessary, the embryos could be stored in the final methanol step at –20°C for up to four weeks.

The embryos were rehydrated at room temperature for five minutes each down a methanol series starting with 75% (v/v) in PBS, 50% (v/v), 25% (v/v) into a final PBS and then rinsed in PBT for five minutes. They were then bleached in 6% (v/v) hydrogen peroxide in 1xPBT for one hour at room temperature, followed by three, five minute washes in 1x PBT. To increase RNA accessibility, embryos were incubated in proteinase K (10μg/ml) in PBT for fifteen minutes at room temperature, and then rinsed in 2mg/ml glycine in PBT, followed by a PBT wash for five minutes.

Embryos were re-fixed in 2% (v/v) glutaraldehyde in 4% (w/v) paraformaldehyde in PBT for twenty minutes at room temperature, and rinsed in 1xPBT.

Labelled riboprobe was added at the desired concentration (see figure 17), to the wholemount hybridisation solution (see appendix A.24), and the embryos left to hybridise overnight. Evaporation was suppressed by an overlay of mineral oil being added on top of the embryos during hybridisation.

After hybridisation embryos were washed twice at 70°C for thirty minutes in a pre warmed, wholemount post hybridisation washing solution 1 (see appendix A.25), followed by three, five minute washes at room temperature, in wholemount post wash solution 2, (see appendix A.26), diluted at 50:50 in the previous wholemount post wash solution 1. 100μg/ml RNAseA was then added to the final post wash solution 2 and the embryos were incubated for a further thirty minutes, at 37°C.

This procedure was followed by a rinse in wholemount post wash solution 3 (see appendix A.27) for five minutes, and a further thirty minutes in fresh wash solution 3 at 65°C.

Embryos were washed three times for five minutes each in TBST (see appendix A.19) A pre-blocking stage followed, in which the embryos were incubated for 150 minutes at room temperature in 10% (v/v) sheep serum in 1xTBST.

Meanwhile 3 mg of embryo powder (see method *2.22*) was incubated for thirty minutes at 70°C in 0.5ml TBST. The solution was cooled on ice and Anti DIG alkaline phosphatase antibody added to a dilution of 1:5000. The solution was agitated on a shaker platform for one hour at 4°C, and then centrifuged at 3000*g* (Sigma 2-15) for two minutes at room temperature. The supernatant was collected and added to a 10% sheep serum solution in TBST, and the embryos were incubated overnight at 4°C in this antibody solution.

After the overnight incubation, the embryos were then subjected to a wash series in TBST at room temperature: three washes of five minutes each, followed by five washes of sixty minutes each. The tissue was then given three washes of ten minutes each in NTMT (see appendix A.14).

Embryos were then placed in a freshly made colour substrate of 0.4 mg NBT and 0.175 mg BCIP in 1ml TBST, and kept in the dark until the colour had developed. Once this was reached the embryos were washed in NTMT for ten minutes and post-fixed in 4% (w/v) paraformaldehyde in PBS and 0.1% (v/v) glutaraldehyde for one hour at room temperature.

The embryos were then taken through a glycerol series (50%, 70%, 80% and 100%) (v/v with PBS) for five minutes for bright field microscopy and photography using the SPOT image capture unit, and the 'SPOT RT software version 3, Diagnostic Instruments, Incorporated'.

2.2.11 Preparation of embryo and heart powder

To reduce non-specific binding of Anti-DIG alkaline phosphatase, it was pre-absorbed with powder prepared from the target tissue : adult heart or embryo.

To prepare the powder, either ten E13 embryos were collected in PBS as described in previous sections (2.2.3), or three hearts from adult mice previously perfused with 4% (w/v) PFA (courtesy of Dr. Chris Thompson) were used.

All glassware and instruments were baked overnight to ensure minimal RNAse activity.

The tissues were snap frozen by immersion in liquid nitrogen ground down by pestle and mortar. Nitrogen was allowed to evaporate and the resulting ground material suspended in 20 ml ice cold acetone, and left on ice for thirty minutes.

The samples were then centrifuged at 3000*g* (Sigma 2-15) for ten minutes at room temperature, and the supernatant removed.

The pellet was resuspended in 1ml cold acetone and re-centrifuged as before for 2 minutes. The supernatant was again removed and the pellet collected and left to air-dry under sterile conditions.

Once dry, the pellet was loosened, and could be transferred into a sterile container, and stored at 4°C until needed.

2.2.12 Dot blot analysis

The dot blot analysis to test the specificity of the riboprobes was carried out by dotting 100 pg of linearised vector (both *Hoxc-9* and *Hoxa-9*) onto positively charged nylon filter

paper and allowing to air dry before being fixed by exposure to a transilluminator ultra violet light (366nm) for three minutes. The blot was further treated as in 2.2.8.

2.3 Results

In order to better understand the function of *Hoxa-9* in normal development, the expression pattern of this gene in the early mouse embryo was assessed using wholemount and conventional *in situ* hybridisation. A wholemount approach was used in those developmental stages where both size was small enough for effective probe penetration, and where numbers of specimens were not limiting. Conventional *in situ* hybridisation was used for all other stages. The first test in a complex procedure, such as *in situ* hybridisation, is to confirm that all steps are functioning. To address this, wholemount *in situ* hybridisation was used to assess the expression of *Pax-1,* a gene whose expression profile has previously been well characterised was used.

2.3.1 Wholemount *in situ* hybridisation using *Pax-1*

Pax-1 riboprobe was hybridised to whole E10.5 mouse embryos (figure 18). In three independent experiments, expression was found in the segment boundaries of the developing sclerotomes and mesenchyme tissue of the developing brachial arch, a result in clear agreement with previously published reports (Peters *et al.*, 1995, 1999). This data suggested that the technique was functioning correctly. The expression of *Hoxa-9* could now be examined.

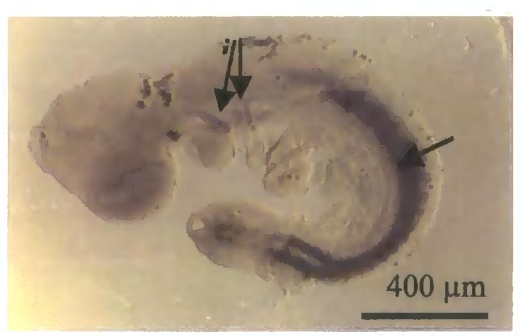


Figure 18 – **A *In situ* hybridisation of *Pax-1*** on E10 mouse embryo (*i*) indicates developing sclerotomes, (*ii*) indictaes the brachial arches.

2.3.2 Wholemount *in situ* hybridisation control riboprobes

In order to be confident that results seen by *in situ* hybridisation for *Hoxa-9* were transcript-specific, and not due to non-specific binding of probes, groups of embryos from each stage under examination were subjected to two parallel, but discrete hybridisations. A control hybridisation, using a 'sense' probe which would not hybridise to the target transcript and therefore acts as a control for non-specific binding, and a hybridisation with 'anti-sense' probe, complementary to the mRNA transcript under study. Comparison between this pair allows interpretation of specific hybridisation, and thus for every embryonic stage, results are shown for both specific (anti-sense) and control (sense) hybridisation. In each case at least three embryos were used for both sense and anti-sense hybridisations.

The *Hox* gene family are highly conserved, and share substantial sequence similarity (see figure 1). The risk therefore is that riboprobes derived from one family member, such as *Hoxa-9,* could hybridise to another family member, particularly one that shares the highest homology (a paralogue), such as *Hoxc-9. Hoxa-9* and *Hoxc-9* share more than 98% homology, and show some regions that are close to 100% homology (figure 19). If it were possible to use *Hoxa-9* riboprobe hybridisation conditions that discriminated between *Hoxa-9* and *Hoxc-9* targets then this would give greater confidence in the specific nature of hybridisations seen using *in situ* techniques.

```
IMAGE     588  gccaactggatccacgcncgttncacaaggaagaagcgctgcccctacaccaagtaccag 647
               ||||||||| | || || || | ||| |||||||||| ||||| ||||| ||  |||||
Hoxa-9    1855 gccaactggctacatgctcgctccactcggaagaagcgatgcccttacacaaaacaccag 1914

IMAGE     648  acgctggaactggagaaggagtttctctttcatatgtatttaaccagggacccgtcgtac 707
               |||||||||||||||||||||||||| ||| | |||||  | ||  |||||||    ||||
Hoxa-9    1915 acgctggaactggagaaggagtttctgtttaacatgtacctcacacgggaccgcaggtac 1974

IMAGE     708  gaggtggcccgttgtctcaatctcactgagcggcaggtcaaaatctgggttcagacccga 767
               ||||||||||||    ||||| ||||| ||  ||||||||||| |||||| | |||| |||
Hoxa-9    1975 gaggtggcccggctgctcaacctcaccgaaaggcaggtcaagatctggttccagaaccgc 2034

IMAGE     768  gggatga 774
               |||||||
Hoxa-9    2035 aggatga 2041
```

Figure 19 – **HGMP sequence pile up** to show the sequence between *Hoxa-9* (GI#3080547), and *Hoxc-9* (GI#4059801), the sequence prior to that shown shares 100% homology. The numbers correspond to the base pair number in the sequence used by GENBANK.

Dot blot analysis was carried out to further confirm the specificity of the *Hoxa-9* riboprobe (figure 20). In this experiment 100 pg of respective *Hoxa-9* and *Hoxc-9* plasmids were hybridised independently with both *Hoxa-9* and *Hoxc-9* anti sense and sense riboprobes under identical hybridisation conditions as used for previous experiments. It appeared that the *Hoxa-9* riboprobe only recognised the *Hoxa-9* targets (both dsDNA sense and anti sense), whilst *Hoxc-9* failed to recognise *Hoxa-9,* and was only detected when bound to *Hoxc-9* targets. This series of experiments confirmed the riboprobe specificity, and showed that cross hybridisation was not an issue in this study.

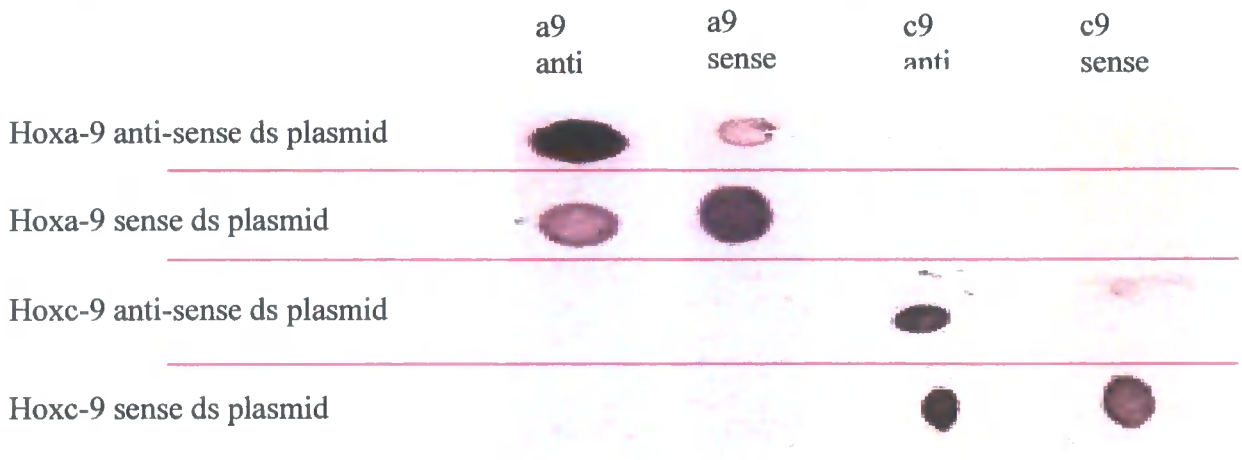


Figure 20 - **Dot blot analysis of riboprobe specificity**

2.3.3 E7.5 Wholemount *in situ* hybridisation

As haematopoiesis can first be detected around E8 the earliest embryo studied was E7.5. The results from the control *Hoxa-9* sense and anti-sense riboprobe are shown in figure 21.

The control hybridisation shows no detectable staining. In contrast the respective wholemount *in situ* hybridisation using *Hoxa-9* anti-sense shows a widespread staining pattern in all three germ layers.

2.3.4 E9 *In situ* hybridisation

The widespread expression pattern seen at E7.5 is also shown in the developing E9 mouse (figure 22). There was no obvious tissue patterning, but this hybridisation was specific shown by the sense strand hybridisation that showed undetectable levels of background staining.

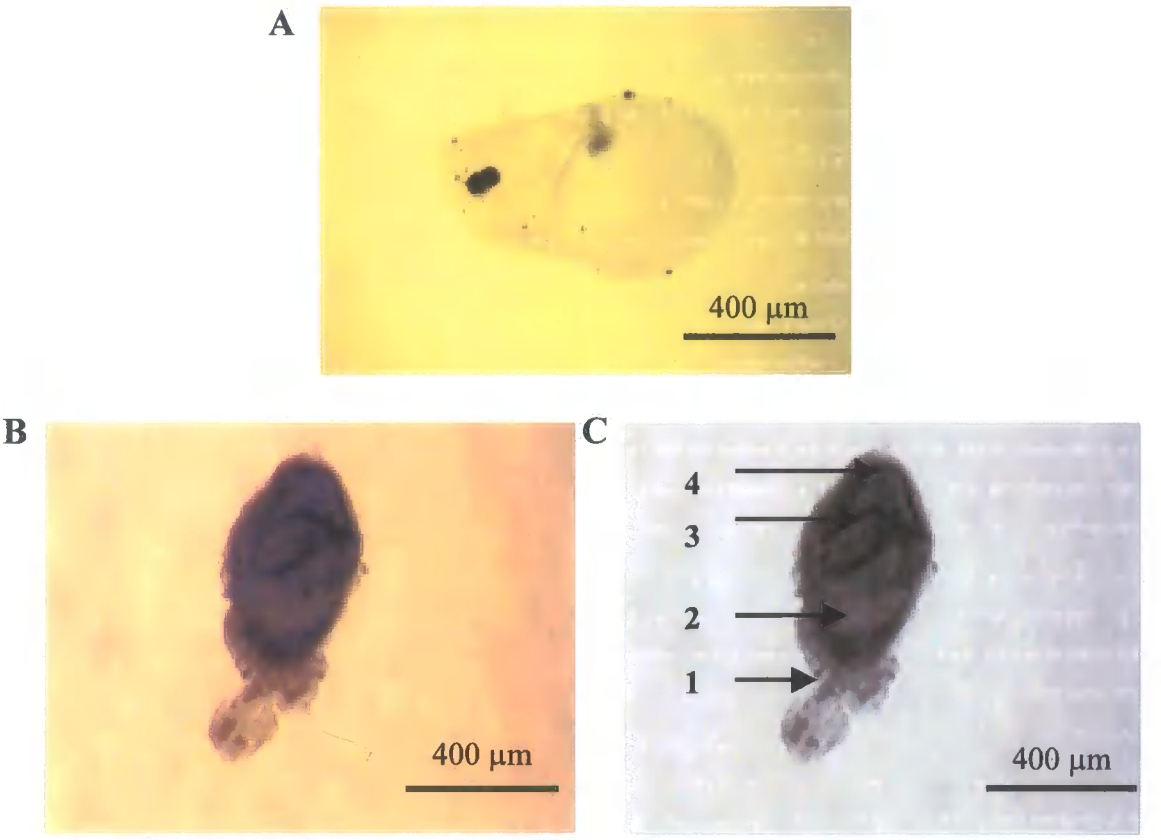


Figure 21 - **Wholemount *in situ* hybridisation of *Hoxa-9* at E7.5**
A - Wholemount *in situ* hybridisation of sense *Hoxa-9*
B - Wholemount *in situ* hybridisation of anti sense *Hoxa-9* embryos
C – Illustration of the extraembryonic tissue (1), ectoderm tissue(2), exocoelomic tissue (3), and amniotic tissue (4) shown in **B**

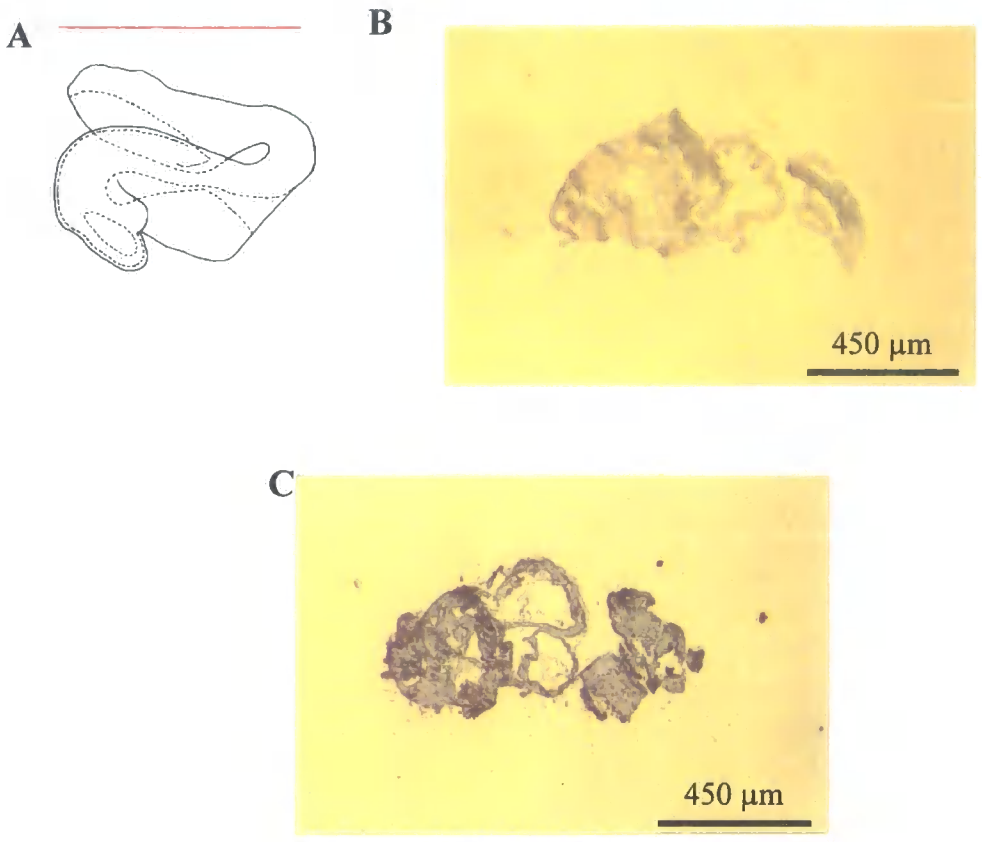


Figure 22 - ***In situ*** **hybridisation of** ***Hoxa-9*** **at E9**
A – Sagittal section taken through the centre of the E9 mouse embryo
B - *In situ* hybridisation of sense *Hoxa-9*
C - *In situ* hybridisation of anti sense *Hoxa-9*

2.3.5 E9.5 Wholemount *in situ* hybridisation

Control sense riboprobe hybridisation with whole E9.5 mouse embryos showed some non-specific staining in the head region (figure 23), a result consistent with stain trapping following insufficient cerebral puncture (Wilkinson, 1992). In contrast, anti-sense hybridisation showed clear and reproducible staining in the heart atrium (*i*), the first brachial arch (ii), and the gut region *(iii)* (figure 23). Close examination of the cardiac region suggested that this expression did not extend to the ventricle or surrounding cavities. Expression of *Hoxa-9* was also found in vertebrae and the developing gut at this stage of development.

2.3.6 E10.5 Wholemount *in situ* hybridisation

E10.5 embryos showed a specific hybridisation pattern that was very similar to that seen at E9.5 (figure 24). Expression was observed in the cardiac atrium, the gut and vertebrae. However, In contrast to E9.5, expression was also observed in the outflow tract. Expression of *Hoxa-9* was not observed in structures adjacent to these tissues.

2.3.7 E11 *In situ* hybridisation

Control hybridisation of E11 embryos with sense *Hoxa-9* riboprobe demonstrated negligible non-specific staining. However, in marked contrast with hybridisations in embryos at E9-10.5, apparently widespread hybridisation with anti-sense probe was seen (figure 25).

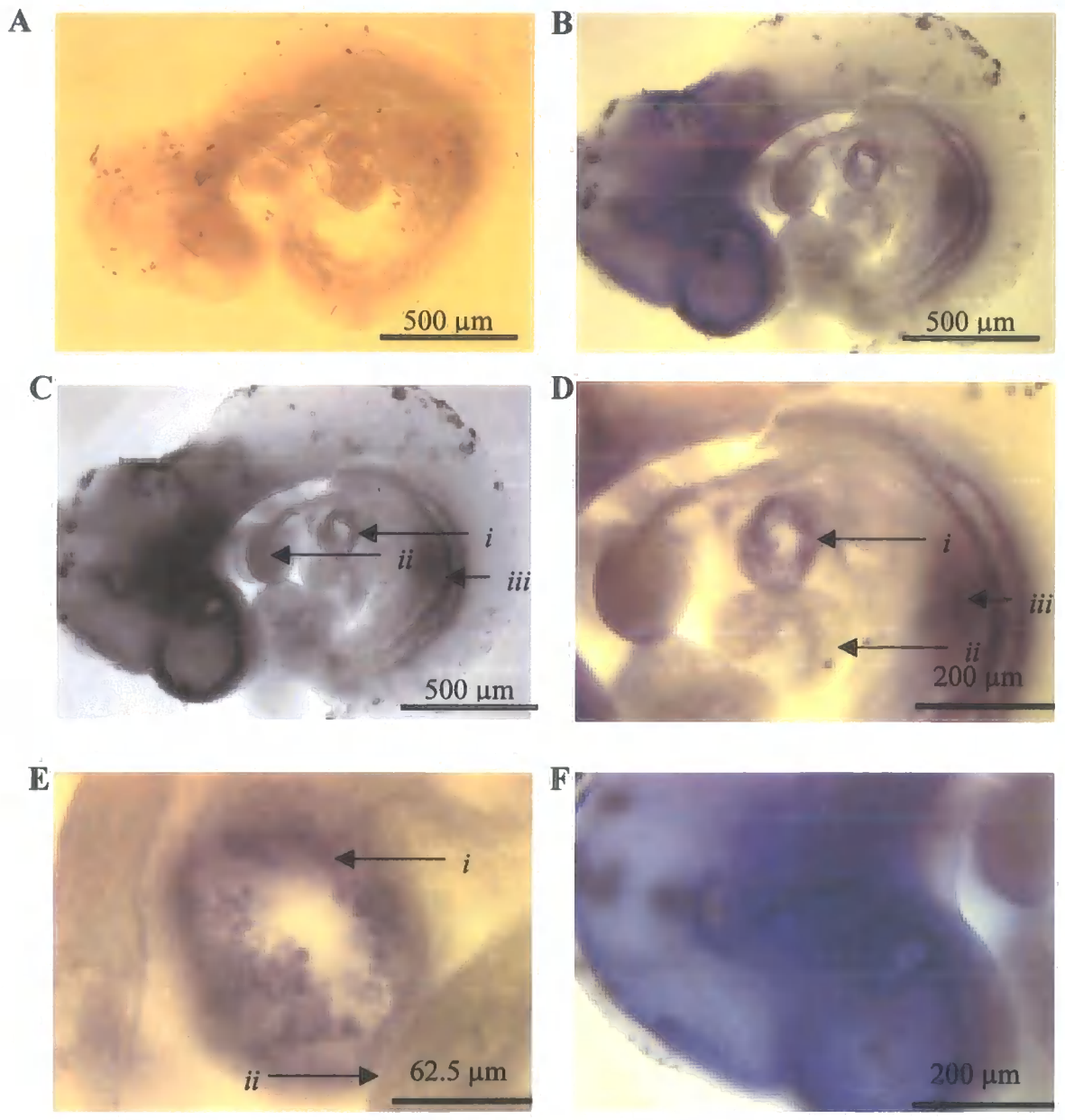


***Figure 23* - Wholemount *in situ* hybridisation of *Hoxa-9* at E9.5**
A - Wholemount *in situ* hybridisation of sense *Hoxa-9*
B - Wholemount *in situ* hybridisation of anti-sense *Hoxa-9*
C, D – Illustration of the heart atrium *(i),* the first brachial arch (ii), and the gut region *(iii)* shown expressing in **B**
E - Wholemount *in situ* hybridisation of anti-sense *Hoxa-9* showing expression in the atrial (*i*) region and undetectable levels in the ventricle (*ii*)
F - Wholemount *in situ* hybridisation of anti-sense *Hoxa-9* showing stain trapping in the head region

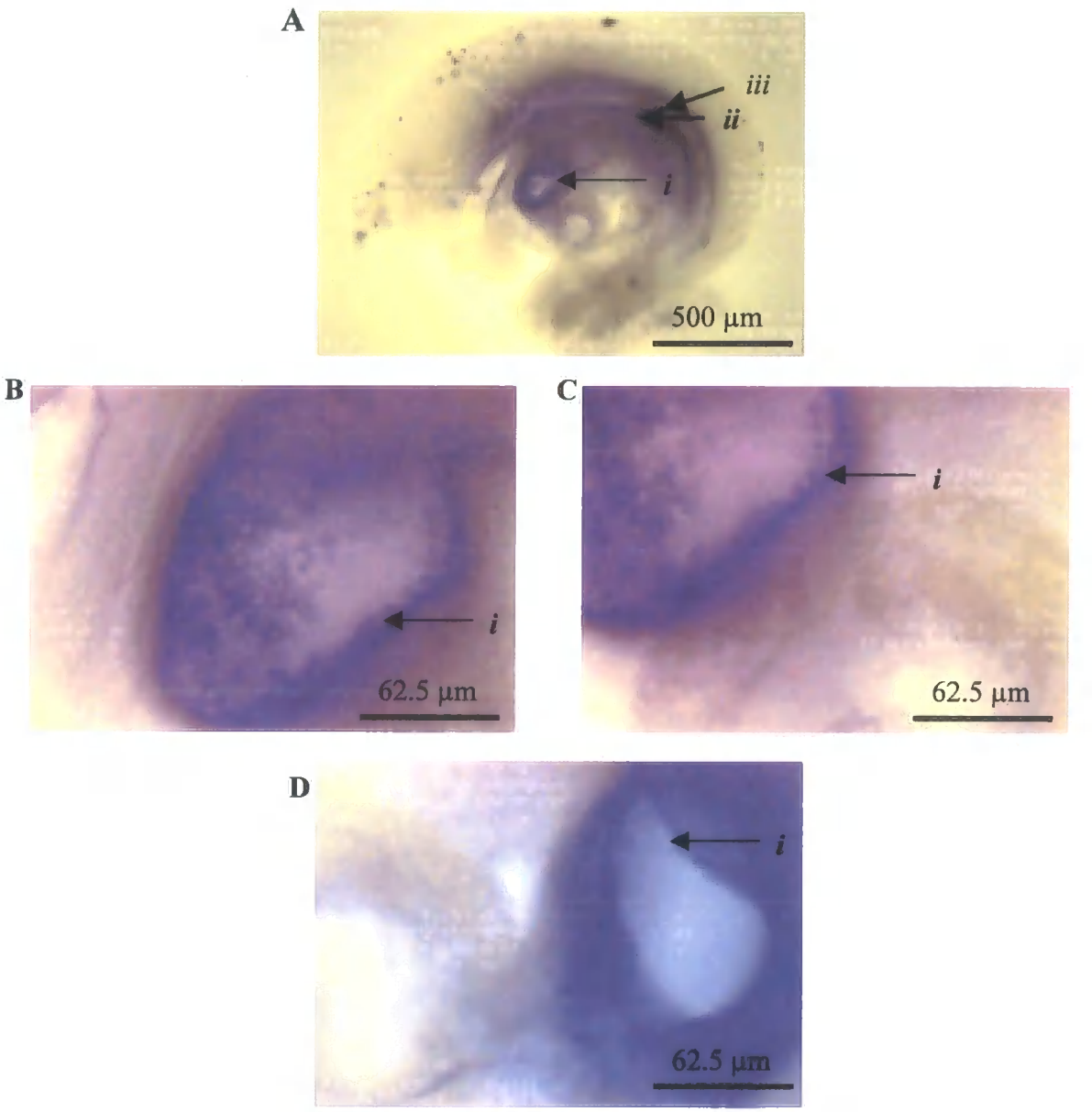


Figure 24 - **Wholemount *in situ* hybridisation of anti-sense *Hoxa-9* at E10.5**
A – *Hoxa-9* expression seen in the anatomical areas of the heart atrium (*i*), the gut region (*ii*), and the notochord (*iii*).
B, C, D - *Hoxa-9* expression detected in the atrial region (*i*)

Close examination of the staining pattern did however identify some distinction in the staining of different tissues. In particular, a consistent, intense *Hoxa-9* expression pattern at the liver/heart boundary (atrial septum). However, in marked contrast to hybridisation with embryos staged only 0.5 days earlier (figure24), there appeared to be little difference in staining between the cardiac atrial, ventricular and vascular tissues. Although this pattern was reproducible (as with all hybridisation experiments described here, three embryos were independently examined), this remarkable change in apparent expression profile demanded the study of additional controls.

To confirm that *in situ* hybridisation could discriminate expression of the cardiac genes in these embryos, riboprobes specific for *vMLC* were produced and used in this technique. The cardiac-specific expression of this gene has been previously well characterised.

2.3.7.1 Detection of cardiac expression of genes at E11

To examine the RNA quality of E11 embryos, riboprobes specific for known ventricular marker gene (*vMLC*) were hybridised with E11 mouse embryos under conditions identical to those used for *Hoxa-9* hybridisations described above (figure 26). Control sense strand *vMLC* riboprobe produced a detectable background staining. However it was possible to distinguish this from the cardiac specific hybridisation seen with anti-sense riboprobe hybridisation (figure 26). This suggested that at least ventricular RNA in these embryos was of sufficient quality to detect specific levels of gene expression seen with *vMLC*. However, the high level of background staining seen with anti-sense probes for this cardiac-specific gene in these embryo stages suggests that the widespread staining seen with the *Hoxa-9* anti-sense riboprobe is not necessarily due to specific transcript

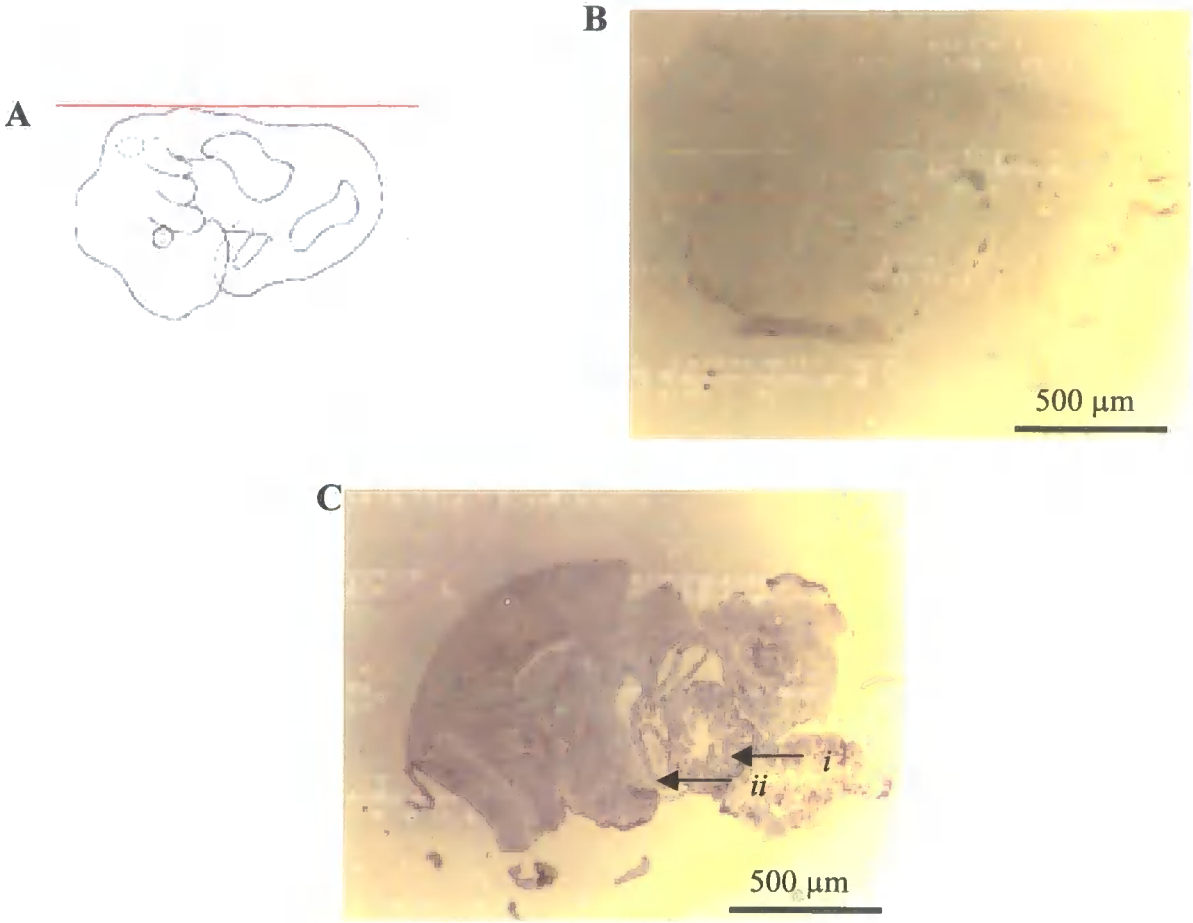


***Figure 25 - In situ* hybridisation of *Hoxa-9* at E11**
A - Sagittal section taken through the centre of the E11 mouse embryo
B - *In situ* hybridisation of sense *Hoxa-9*
C - *In situ* hybridisation of anti sense *Hoxa-9*. Illustrated are the developing liver region (*i*) and the gut (*ii*)

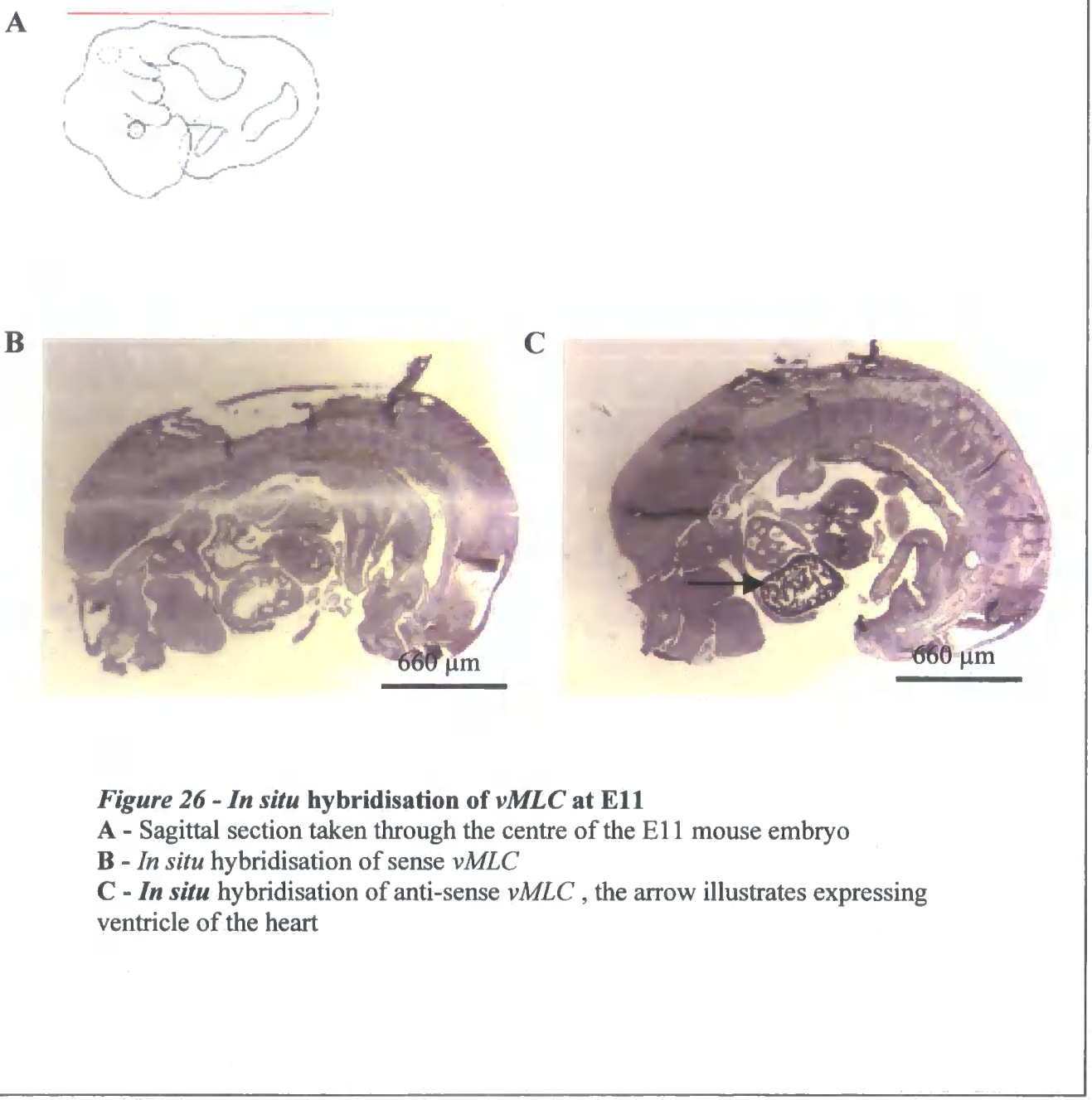


***Figure 26 - In situ* hybridisation of *vMLC* at E11**
A - Sagittal section taken through the centre of the E11 mouse embryo
B - *In situ* hybridisation of sense *vMLC*
C - ***In situ*** hybridisation of anti-sense *vMLC* , the arrow illustrates expressing ventricle of the heart

hybridisation but may reflect other, non-specific effects, such as may be caused by preparation of embryos at this time point.

2.3.8 E12 *In situ* hybridisation

The areas that had previously been expressing high levels of *Hoxa-9* at E9.5 and E10.5 include the gut and atrial region. At E12, there appeared to be a high level of expression not only in the heart atrium as at E9.5-10.5, but also in the developing ventricle (figure 27).

Non-specific binding, also seen in the sense strand hybridisations include tissue dense and ductal areas in which the stain is trapped in the deep cavities, potentially due to insufficient washing (Wilkinson, 1992).

2.3.9 E13 *In situ* hybridisation

At E13, specific *Hoxa-9* expression became undetectable. The sections hybridised with the sense strand *Hoxa-9* were clean and very similar in staining intensity to anti-sense hybridisations (figure 28).

As these undetectable levels by the anti sense strand have not been seen previous in this study, the RNA quality of E13 sections was tested as before.

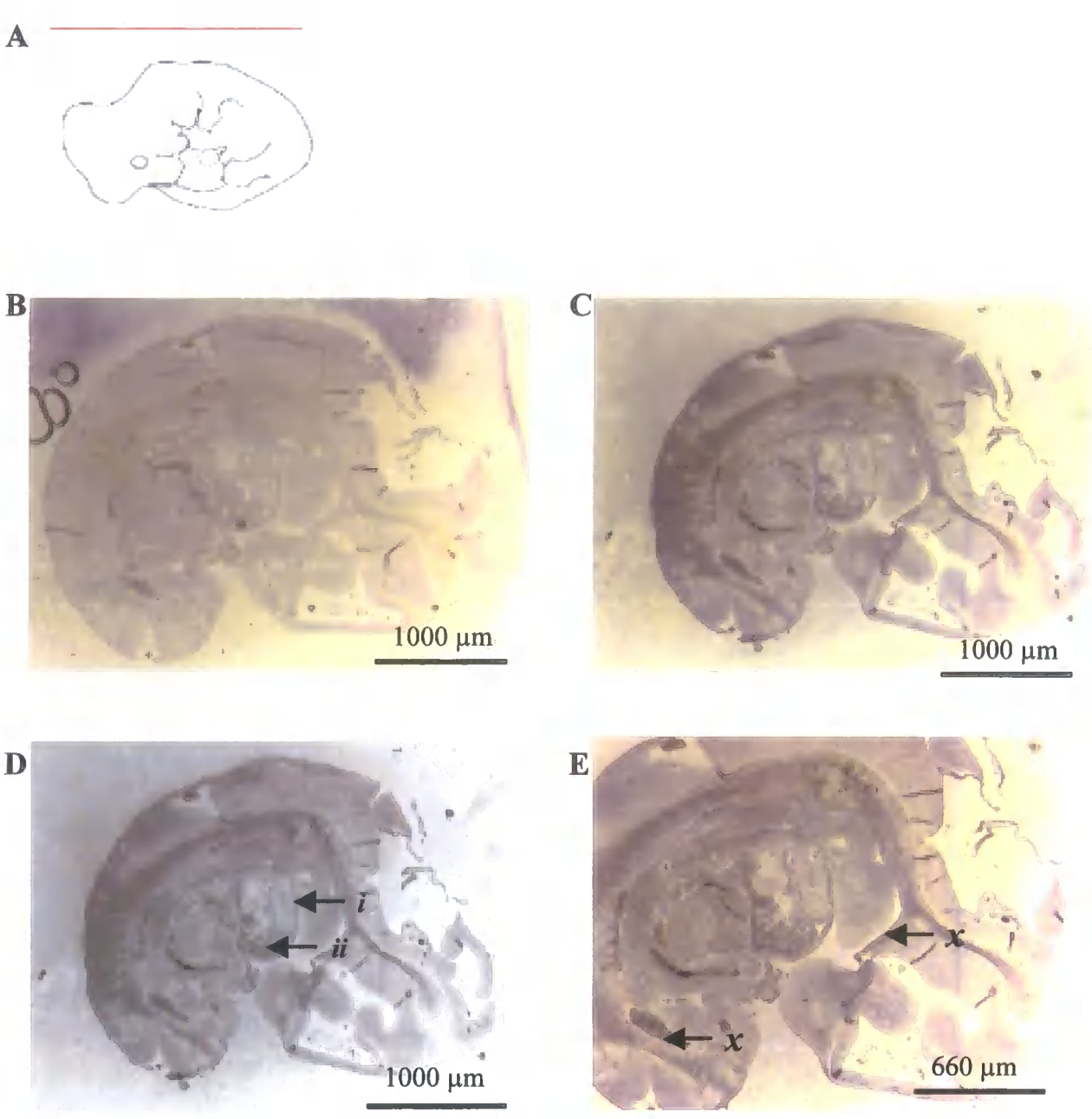


***Figure 27 - In situ* hybridisation of *Hoxa-9* at E12**
A - Sagittal section taken through the centre of the E12 mouse embryo
B - ***In situ*** hybridisation of sense *Hoxa-9*
C, D - ***In situ*** hybridisation of anti-sense ***Hoxa-9,*** **D** highlights the heart atrium *(i),* the heart ventricle (*ii*) expressing in **C**
E - ***In situ*** hybridisation of anti-sense ***Hoxa-9, x*** illustrates some ductal areas of non-specific binding

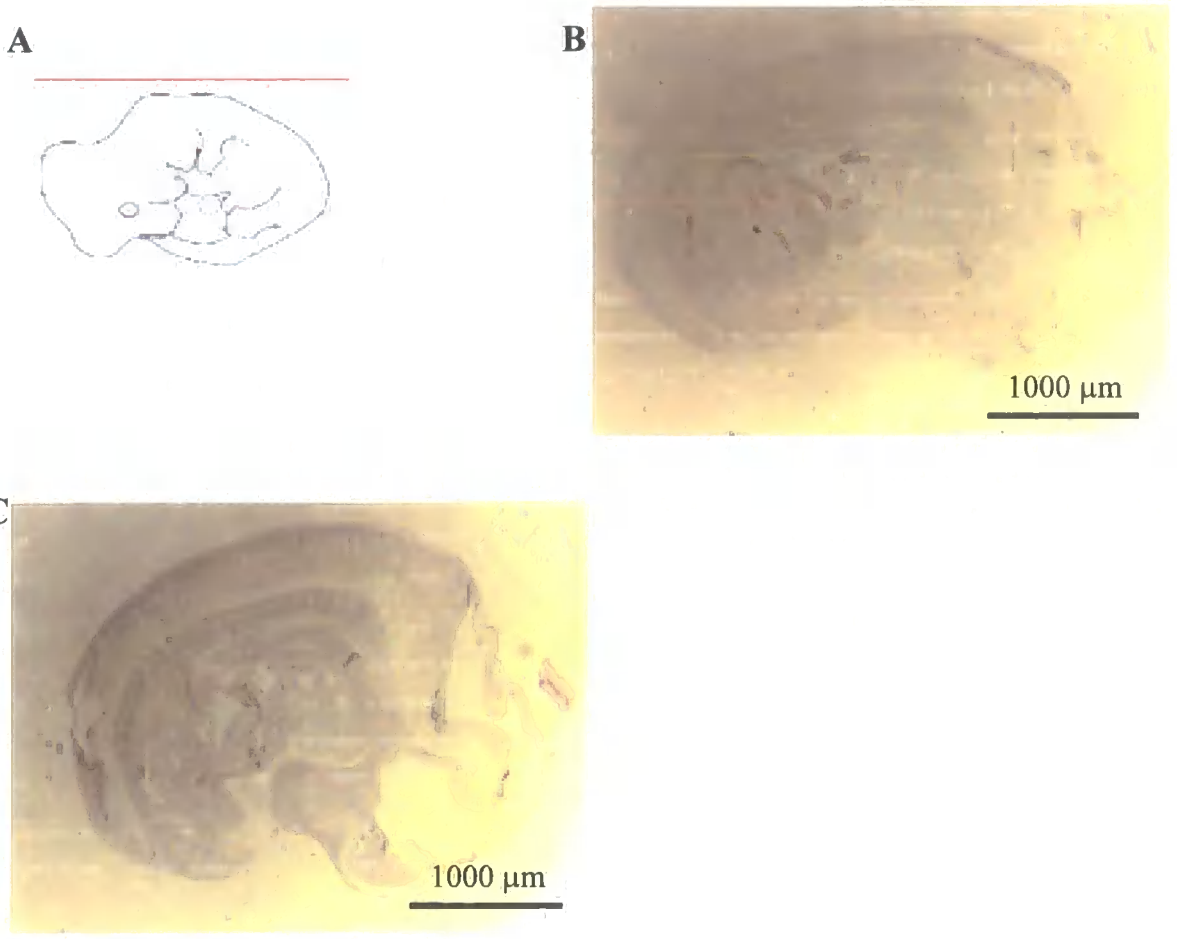


***Figure 28 - In situ* hybridisation of *Hoxa-9* at E13**
A - Sagittal section taken through the centre of the E13 mouse embryo
B - ***In situ*** hybridisation of sense *Hoxa-9*
C - ***In situ*** hybridisation of anti-sense *Hoxa-9*

2.3.9.1 Detection of cardiac expression of genes at E13

Compared with the sense strand control, the anti sense hybridisation with *vMLC* showed intense specific staining of the ventricle (figure 29). This suggests that the RNA quality of embryo sections at this stage was at least good enough to detect expression levels seen with this gene.

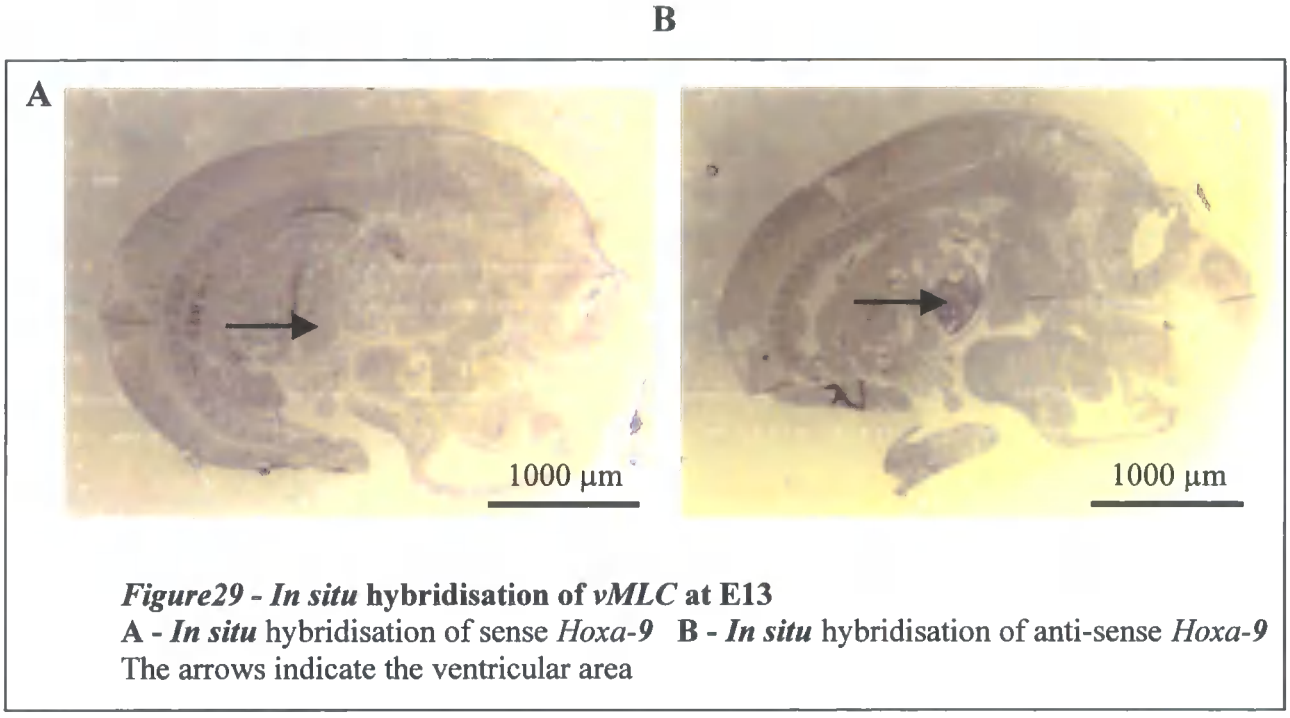


***Figure29 - In situ* hybridisation of *vMLC* at E13**
A - *In situ* hybridisation of sense *Hoxa-9* **B - *In situ*** hybridisation of anti-sense *Hoxa-9*
The arrows indicate the ventricular area

2.3.10 E13 *In situ* hybridisation using *Hoxc-9* riboprobe

The undetectable staining using the *Hoxc-9* sense strand (figure 30), can be seen in clear comparison with the widespread expression patterns produced from hybridising with the *Hoxc-9* anti sense strand, and shows a different pattern from hybridistaion using *Hoxa-9* anti-sense at this time point (figure 28), demonstrating further the specificity of the *Hoxa-9* riboprobe.

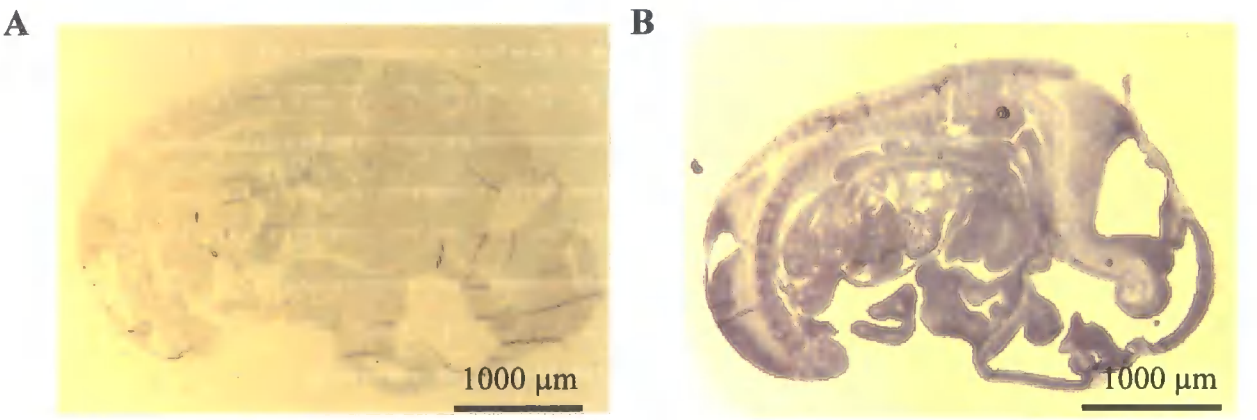


***Figure 30 - In situ* hybridisation of *Hoxc-9* at E13**
A - *In situ* hybridisation of sense ***Hoxc-9*** **B - *In situ*** hybridisation of anti-sense ***Hoxc-9***

2.3.11 *In situ* hybridisation of the adult murine heart

By E13, the major morphological changes associated with heart development in the mouse are largely complete. However gene expression in the fully formed adult heart needs to be examined. Hybridisation with *Hoxa-9* anti-sense riboprobe was unable to demonstrate detectable staining in the adult heart; indeed this result was indistinguishable from the sense control (figure 31). Although a reproducible result, one possibility is that RNA quality in this tissue was insufficient for successful hybridisation. In order to test this, adult heart sections were tested for hybridisation with sense and anti-sense riboprobes for *vMLC*.

The ventricle of the adult heart stained very quickly with undetectable levels in the adjacent atrium as expected (figure 32), suggesting at least that RNA quality was sufficient for the detection of cardiac genes expressed at least at the level of this gene.

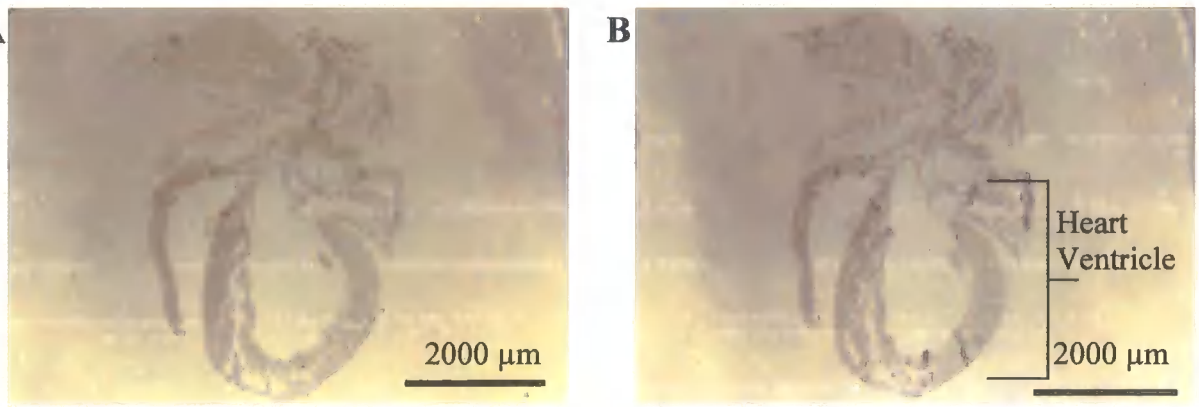


Figure 31 **- Sagittal section *in situ* hybridisation of *Hoxa-9* in the murine adult heart**
A - *In situ* hybridisation of sense *Hoxa-9* **B -** *In situ* hybridisation of anti-sense *Hoxa-9*

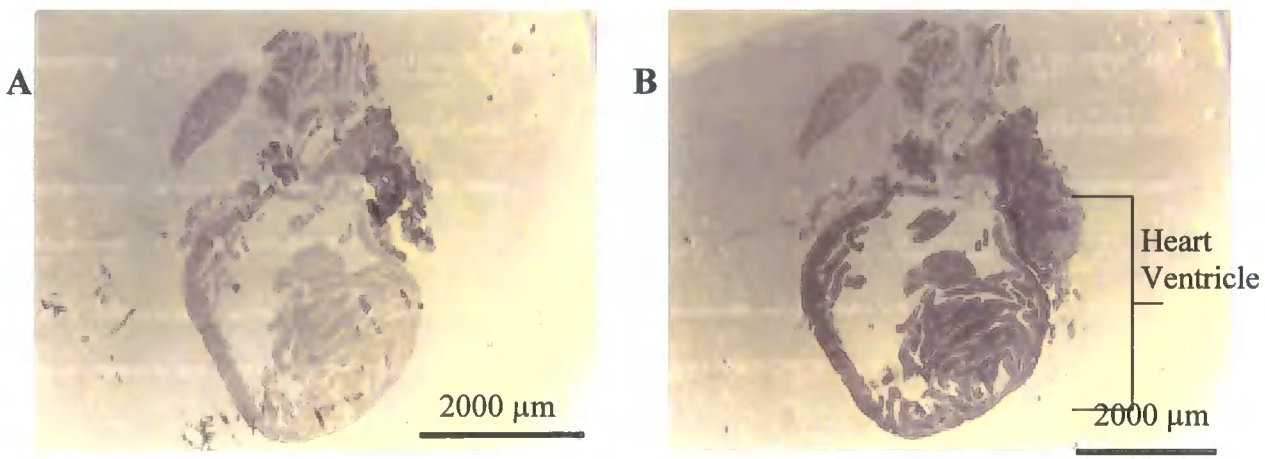


Figure 32 - Sagittal **section *in situ* hybridisation of *vMLC* in the murine adult heart**
A - *In situ* hybridisation of sense *Hoxa-9* **B -** *In situ* hybridisation of anti-sense *Hoxa-9*
The heart ventricle is shown to be expressing

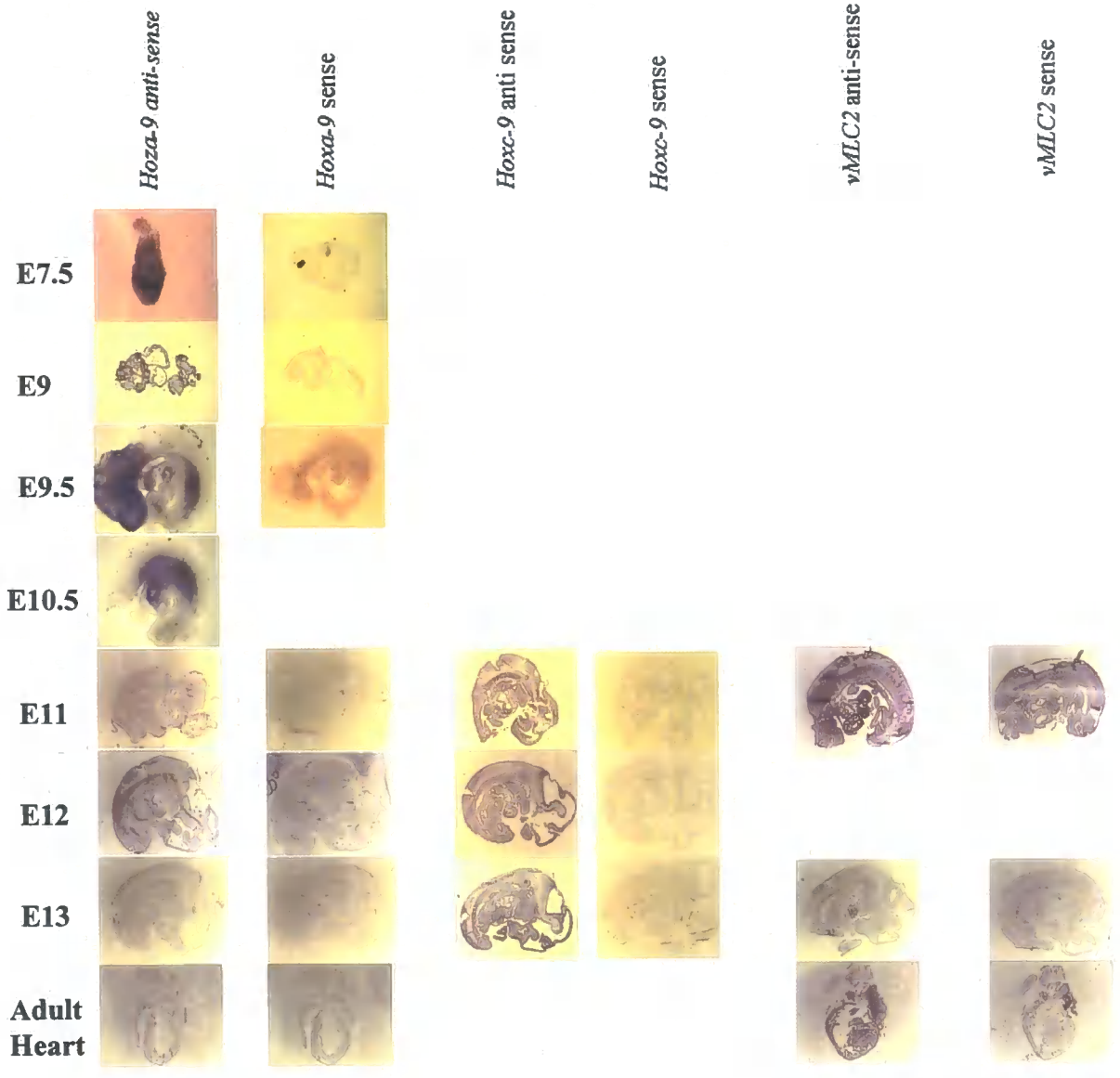


Figure 33 – **A summary of the wholemount and *in situ* hybridisation study**

2.4 Discussion

It has been intended in this study to combine the successful techniques of *in situ* and wholemount hybridisation as seen in previous work, to analyse the expression pattern of *Hoxa-9* during embryonic mouse development to further investigate the role of *Hoxa-9* during normal developmental processes, including haematopoiesis. A previously unreported *Hoxa-9* expression pattern was identified from this study using such techniques in the developing heart of the mouse embryo. In this discussion, previous *Hoxa-9* expression studies will be analysed, along with how the findings in this study may potentially implicate embryonic heart development.

2.4.1 The expression pattern of *Hoxa-9* in the developing mouse embryo

The mouse *Hoxa-9* gene is the most 3' located gene amongst the whole set of *Hox a* genes phylogenetically related to Drosophila Abdominal B (Adb-B) homeotic gene (Rubin *et al.*, 1987), which control the development of mid to posterior parasegments in the insect.

Previous work using *in situ* hybridisation and other techniques has revealed that during the midgestational mouse embryo, the gene is expressed along the primary body axis, including the spinal cord and prevertebral column, and limb buds (Duboule and Dollè, 1989, Chisaka & Capecchi 1991, James and Kazenwadel, 1991, Fromental-Ramain *et al.*, 1996, Sekimoto *et al.*, 1998, Shin *et al.*, 1998). The *in situ* hybridisation studies used in previous studies supported expression patterns shown by this study at E9.5 and E10.5 (James and Kazenwadel, 1991, Sekimoto *et al.*, 1998, Shin *et al.*, 1998), however, this study also identified additional expression in the developing heart and vasculature.

2.4.1.1 *Hoxa-9* expression in the E7.5 mouse embryo;

Previous *in situ* hybridisation studies have shown high expression levels of *Hoxa-9* in all three germ layers of the murine embryo (ectoderm, endoderm and mesoderm) at E7.5, as well as in extraembryonic tissues including the amnion and ectoplacental cone, shown in figure 35 (Shin *et al.*, 1998).

Using similar wholemount *in situ* hybridisation in this study, supporting evidence has been shown that *Hoxa-9* has a widespread expression pattern in the mouse embryo at E7.5 (Shin *et al.*, 1998). As previously mentioned, it is at this time point when haematopoiesis is initiated in the blood islands of the yolk sac (Moore and Metcalfe, 1972), but on a full 360° examination of wholemount embryos, there did not appear to be intense levels of expression at the cellular level in this extraembryonic tissue.

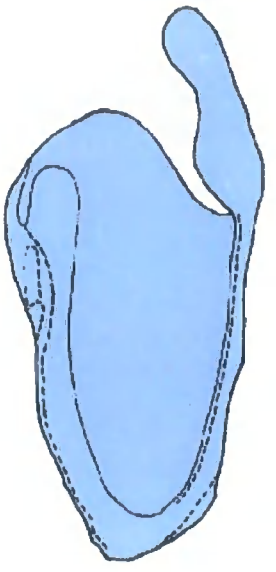


Figure 34 ***Hoxa-9* expression at E7.5 in the mouse embryo**

Hoxa-9 is highly expressed in all three germ layers, Including extra embryonic tissue in the mouse embryo (Shin *et. al. 1998).*

The presence of *Hoxa-9* transcripts at E7.5, although widespread, suggested a potential role during early development of the mouse. It therefore became important to study any

changes in expression pattern during the next stage of development (E8.5), at a time point when primitive erythroid progenitor cells begin to circulate from the yolk sac and the rest of the embryo (see figure 6).

2.4.1.2 *Hoxa-9* expression in the E9 mouse embryo;

This widespread patterning continued in E9 embryos in this study, with similar findings to that from published reports demonstrated in figure 35 (Shin *et al.*, 1998).

Gene knockout data by Fromental-Ramain suggested that at this stage in the developing embryo, expression was seen to the somites and developing limbs (Fromental-Ramain *et al.*, 1996). To some extent, this current study has shown expression in those areas, but there was also a high degree of expression outside these areas. These differences in data could be due to the different approaches and techniques used in the two studies, and the report regarding *Hoxa-9* knockouts was incomplete (Fromental-Ramain *et al.*, 1996).

Although there was no distinct *Hoxa-9* expression seen in embryonic sites of haematopoiesis, there was some positive expression identified during critical stages of early development. These widespread expression findings of *Hoxa-9* in this study largely support that of other reports and therefore suggested that expression should be further studied.

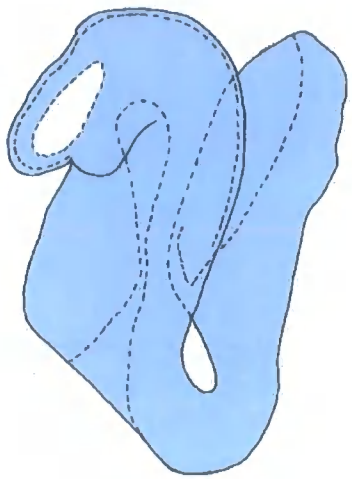


Figure 35 ***Hoxa-9*** **expression at E9 in the mouse embryo**

Hoxa-9 expression continues to be widespread within embryonic endoderm, ectoderm and mesoderm at E8.5 as well as in extra- embryonic tissues including the amnion and ectoplacental cone (Shin *et. al.* 1998).

2.4.1.3 E9.5 wholemount *in situ* hybridisation in the mouse embryo

Previous *Hoxa-9 in situ* hybridisation studies showed expression in the developing gut at E9.5 (James and Kazenwadel, 1991, Sekimoto *et al.*, 1998) (figure 35) and data presented here supports this finding.

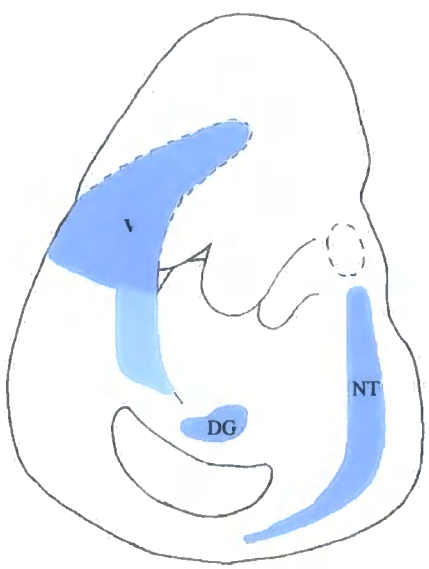


Figure 36 ***Hoxa-9*** **expression at E9.5 in the mouse embryo**

Hoxa-9 expression is found in the thoracic vertebrate (v) (Sekimoto *et. al.* 1998).

Expression is also found in the visceral mesoderm in the developing gut (DG), (James and Kazenwadel, 1991 and Sekimoto *et. al.* 1998)

and the neural tube (NT) (Shin *et. al.* 1998).

However, in addition to these regions, there was specific *Hoxa-9* expression detected in the developing wall of the heart atria and the vasculature. This expression pattern was restricted to the atria in contrast to the adjacent ventricle that showed undetectable levels. There are considerable differences between the expression pattern seen in E9 using *in situ* hybridisation, and E9.5 using wholemount hybridisation. However, this difference was noted in two other independent experiments. As only *in situ* hybridisation was carried out at E9, detecting expression in the posterior region of the developing heart vessel prior to this looped state of the heart at E9.5, was not carried out.

It is at E9.5 that the foetal liver and aorta-gonad-mesonephros (AGM) regions became primary sites for producing haematopoietic precursors including committed B-cells, monomyelocytic cells and erythroid progenitors. (Medvinsky *et al.*, 1993, Müller *et al.*, 1994). However, at the level of analysis adopted in this study, expression in these areas was not detected.

2.4.1.4 Stain trapping in the whole mouse embryo

Stain trapping results from insufficient puncturing of the embryo prior to the wholemount procedure, and is characterised by a concentration of stain colour in cavity areas (Wilkinson, 1992). There was evidence in this study of stain trapping in the head of the E9.5 embryo, as the cavities of the developing eye and ear were also heavily stained like the surrounding head region.

When these wholemount embryo was sectioned coronally, the staining diffused out from the head region but remained in the positively expressing areas – the gut, heart atrium and

vertebrae (data not shown), eliminating the speculation of stain trapping occurring in the heart.

2.4.1.5 E10.5 wholemount *in situ* hybridisation in the mouse embryo

This high levels of expression in the atrium of the developing heart continued into E10.5, and became more obvious in the outflow vasculature. There were no published *in situ* or wholemount studies to compare these findings with, and it therefore seemed important to continue our investigation further in development.

At this time, the gut also continued to express *Hoxa-9* and although difficult without coronal sectioning of this embryo, there was possible expression in the developing notochord lying below the neural tube.

At E10.5, the continued expression of *Hoxa-9* in the developing heart suggested that the patterning should be studied further in E11 mouse embryos.

2.4.1.6 E11 *In situ* hybridisation in the mouse embryos

Due to the larger size of E11 embryos, probe penetration becomes more difficult (Wilkinson, 1992) and therefore for E11 mouse embryos onwards, the technique was switched to using conventional *in situ* hybridisation techniques in sectioned material using the same *Hoxa-9* riboprobe.

The staining pattern once again became widespread at E11, but with consistent expression in the boundary between the liver and the heart (atrial-septum) of the embryo, also seen in duplicate E11 embryos (data not shown).

It could be suggested that this E11 stage could be an 'intermediate' between the high levels of specific expression in the developing heart (gut, and vertebrae) at E9.5 and E10.5, and the more specific patterning seen at E12.

2.4.1.6.1 Testing RNA quality in the E11 mouse embryo

To eliminate the poor quality of RNA causing non-specific binding in the E11 mouse embryo, the embryo was hybridised with a known ventricular marker - ventricular myosin light chain (*vMLC*) under identical conditions. The clear ventricular expression suggested sufficient RNA quality for the *Hoxa-9* riboprobe to bind to in the E11 *in situ* sections.

The expression found in this *in situ* and wholemount hybridisation study of *Hoxa-9* in the developing mouse embryo showed initial wide spread expression at E7.5 with specific distinct patterning in the heart atrium wall at E9.5 and E10.5, followed by widespread patterning at E11. The reasons for this change in patterning are unclear, and require further investigation by studying the expression at E12.

2.4.1.7 E12 *in situ* hybridisation in the mouse embryo

The *in situ* hybridisation with the *Hoxa-9* riboprobe on E12 mouse embryos highlighted several areas of interest. Firstly the similar areas of staining that were seen in both the sense strand control and the anti-sense strand were eliminated. These were tissue dense ductal areas and cavities including the peritoneal cavity, which trap the stain following

insufficient washing (Wilkinson, 1992) and were discarded as positive *Hoxa-9* expressing areas.

It has been demonstrated that in E12 embryos hybridised with anti sense *Hoxa-9* there was specific expression in the atrium of the heart as seen before in days E9.5 and 10.5, this expression pattern had continued into the heart ventricle by E12, with an intermediate widespread expression at E11.

Previous *in situ* and wholemount hybridisation studies on *Hoxa-9* did not report this same expression pattern in the developing embryonic heart (figure 37), and this encouraged this study to look further into this atrial expression during later stages of development.

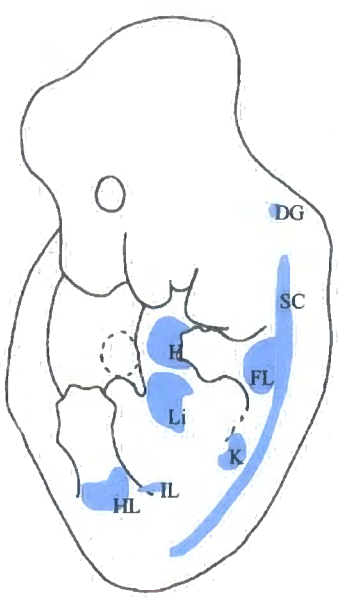


Figure 37 ***Hoxa-9* expression at E12 in the mouse embryo**

Hoxa-9 expression can be found on the rostral border Between the ileum and the caecum (IL) (Sekimoto *et. al.* 1998).
Expression can also be seen in the spinal cord (SC), the heart (H), Liver (Li), kidney (K), forelimb (FL), and hindlimb bud (HL), and the dorsal root ganglia (DG) (Shin *et. al.* 1998)

2.4.1.8 E13 *in situ* hybridisation in the mouse embryo

By E13 the structural development of the heart is almost complete. It could be suggested that the undetectable levels of *Hoxa-9* seen by *in situ* hybridisation in this thesis at E13 are due to this gene no longer being required during cardiogenesis.

By E14 haematopoiesis has shifted to the foetal spleen, but continues in the liver, in preparation for the bone marrow taking over as the primary site of production for adulthood. Again, using the methods adopted to this study, expression levels at these anatomical sites were undetectable. *Hoxa-9* expression was also undetectable in the adult murine heart, suggesting a more embryonic role for *Hoxa-9.* However there is evidence from other studies for *Hoxa-9* in adult cell tissues (Fromental-Ramain *et al.*, 1996, Taylor *et al.*, 1997, Chen and Capechhi, 1999)..

In order to understand the apparent spatial and temporal expression of *Hoxa-9* in the developing heart, it is important to recognise how the heart is forming during these developmental stages.

2.4.2 Formation of the developing heart

The heart is the first organ to form in vertebrates, and it arises through a series of complex interactions involving cells from several embryonic origins. Figures 38-44 illustrate cellular events involved in the formation of the multi-chambered heart.

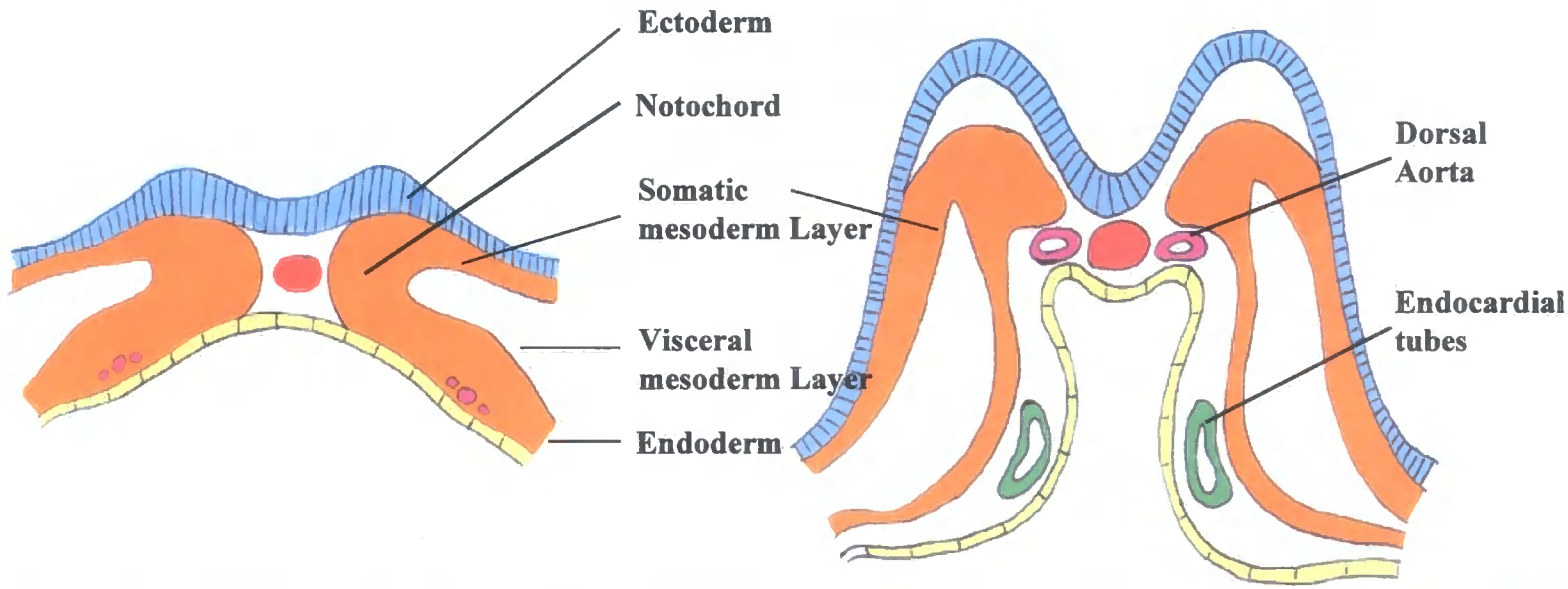


Figure 38 - **Heart formation in the E7.5mouse embryo**
Cardiac precursors form the parallel cardiac primordia in response to endoderm signals, commit to mesoderm (Schulthesis et al., 1995, 1997, Lough et al., 1996, Ladd et al., 1998, Schwartz and Olson 1999).

Figure 39 - **Heart formation in the E8.5mouse embryo**
At E8 the primordia begins to fuse at the midline, in preparation of forming the cardiac tube. Endocardial cells proliferate across ventral midline into intraembryonic coelom and extend into left-right horns surrounded by ectoderm tissue.

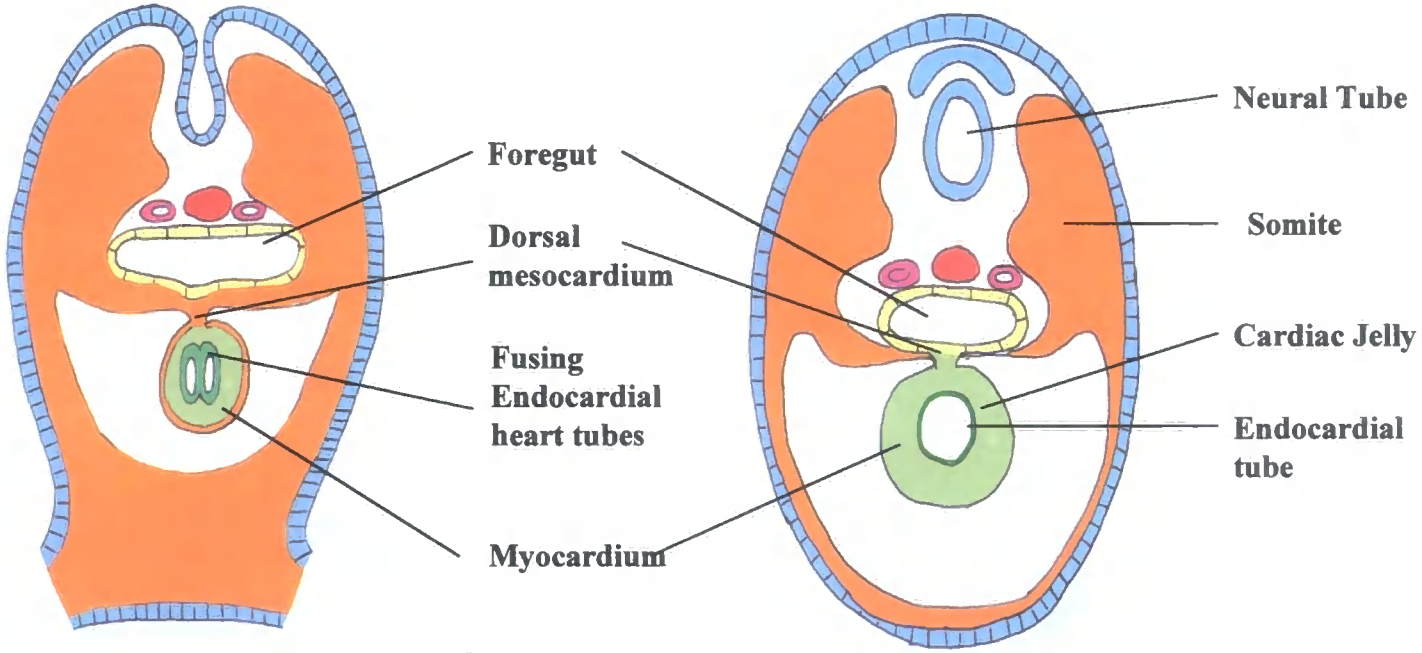


Figure 40 - **Heart formation in the E8.5 mouse embryo**

The cardiac primordia continues to fuse, and
The heart tubes begin to form following fusion of the endocardial cells.

Figure 41 - **Heart formation in the E9 mouse embryo**

The primordia has fused to form the cardiac tube, which contains an outer myocardium, and inner endocardium (cardiac jelly). The neural tube too has formed following fusion.
It is this cardiac tube that separates and gives rise to the atrial and ventricular chambers.

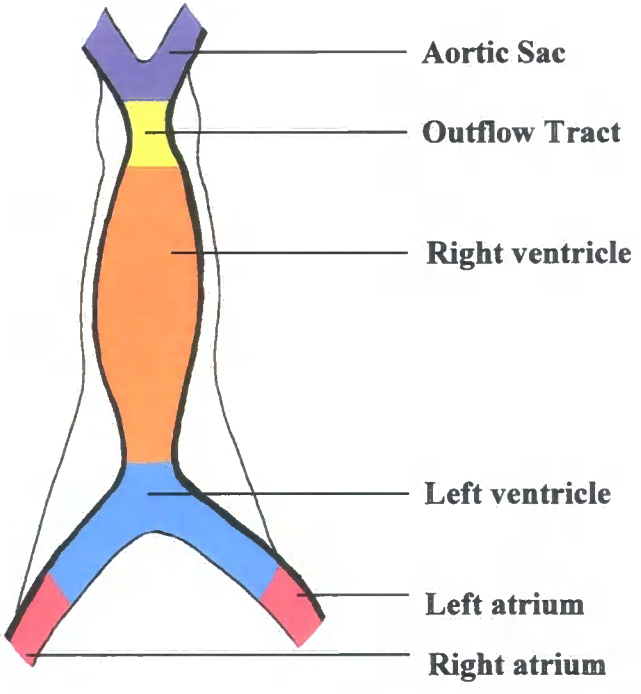


Figure 42 **Heart formation in the E9.5 mouse embryo**

A sagittal schematic view of the foetal cardiac heart tube at E9. The potential chambers of the heart have been identified

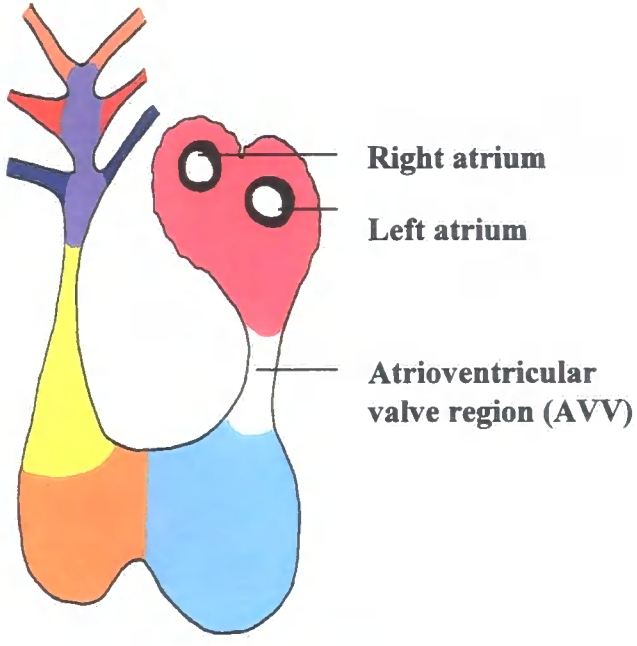


Figure 43 **Heart formation in the E10 mouse embryo**

The heart undergoes rightward looping, and the cardiac tube separates into the relevant chambers. Rhythmic contractions are initiated at this stage.

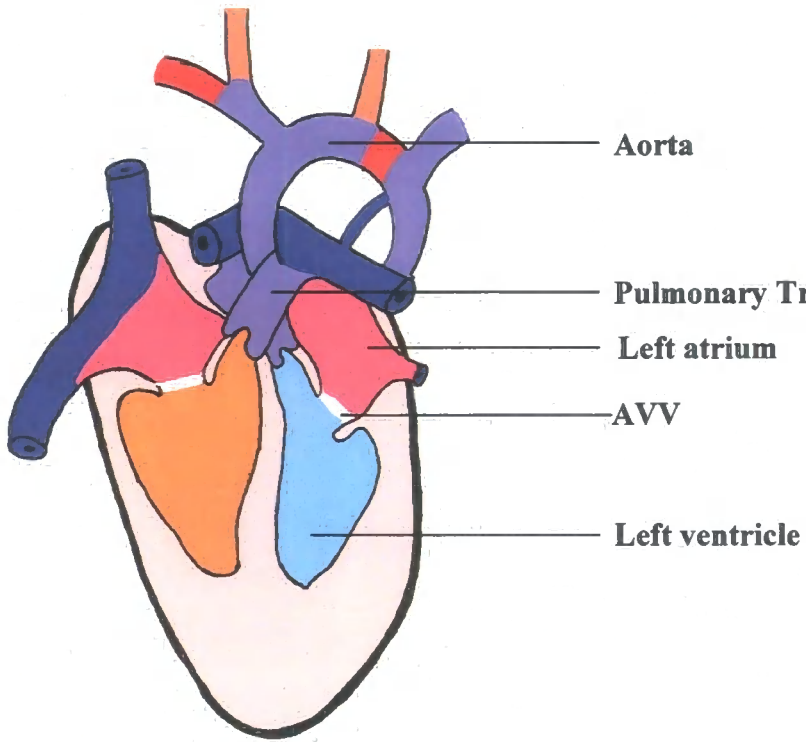


Figure 44 **Heart formation in the E12mouse embryo**

By E12, asymmetry is prominent, and the venous system is developed.
By 13.5 there is inter-atrial and inter-ventricle septation, and the cardiac valves can be recognised. At E14.5 the two septa allow oxygenated blood from the placenta to flow into the right atrium from the left umbilical vein (Olson and Srivastava 1996, Fishman and Chien 1997)

Data presented here suggests specific *Hoxa-9* expression shows a temporal and spatial pattern over critical time points of heart formation. Cardiac cell commitment and lineage differentiation begins during early gastrulation, in response to peptide growth factor signals from adjacent endoderm, which include BMP2 and 4 (Schulthesis *et al.* 1995, 1997), Fibroblast Growth Factor (Lough *et al.*, 1996) and *Activin* (Ladd *et al.*, 1998) (reviewed by Schwartz & Olson 1999). In response to growth factor stimulation, the paired regions of the anterior lateral mesoderm develop into parallel cardiac primordia, which in turn fuse, forming the heart tube in the ventral midline of the embryo.

Looping and chamber maturation follow around E10, when *Hoxa-9* expression is apparent, to form a fully functional multi chambered heart (Olson & Srivastava 1996, Fishman & Chien 1997). The fate of each cell type in becoming the chambers of the heart is highlighted in figures 41-43.

2.4.3 Gene expression in the developing heart

Embryonic patterning is a necessary first step for development of the primordial heart and throughout this orderly developmental system the expression of several other known homeobox genes and transcription factors have been demonstrated.

Figure 45 shows the temporal expression of genes that are known to regulate heart formation.

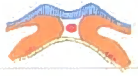
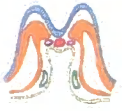
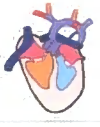
		Activin	BMP2,4	FGF	GATA-4	Gax	HAND	Id	MEF2	Mesp2	Msx1,2	Nkx2.5	Pax3	Pitx2	RxRα
E7.5		√	√	√	√	√		√	√	√					
E8					√	√			√			√			
E8.5					√	√						√			
E9					√	√						√			
E9					√	√						√			
E10		√				√	√				√	√	√	√	√
E12 – adult					√	√			√			√			

Figure 45 **The expression of lineage markers during heart formation**
(reviewed Olson and Srivastava, 1996)

The *Drosophila* homeobox gene, *tinman* (Azpiazu and Frasch 1993, Bodmer, 1993, reviewed in Harvey 1996), and its homologues in mouse (*Nkx2-5* (Lints *et al.*, 1993)), play vital roles in establishing normal cardiac development . The gene is expressed in primitive mesoderm tissue and its mutation leads to the absence of a heart in Drosophila, and severe, early cardiac defects in mouse (Barinaga 1994).

Vertebrae homeobox genes *Msx-1* and *Msx-2*, related to *Drosophila msh,* have shown expression restricted to a distinct subpopulation of myocardial cells. In later stages, of heart development there are cells that are found to coincide morphologically with the cardiac conduction system, suggesting a potential model for conduction tissue development (Chan-Thomas *et al.*, 1993).

Overexpression of the homeobox gene *Pitx2* in chick and xenopus (Campione *et al.*, 1999, Logan *et al.*, 1998, Piedra *et al.*, 1998, Ryan *et al.*, 1998) and knockout studies of the same gene in mice (Gage *et al.*, 1999, Kitamura *et al.*, 1999, Lu *et al.*, 1999), support the idea that this family of genes play an important role in asymmetric morphogenesis of multiple organs, including the heart.

There is also a clear evidence to support a role of transcription factors, other than homeobox genes, in cardiac cell specificity.

2.4.3.1 GATA gene expression in the developing heart

Certain members of the *GATA* family of transcription factors appear to participate in cardiac muscle differentiation. *GATA-4* is expressed in the precardiac mesoderm and

subsequently in the endocardial and myocardial layers of the heart tube in the developing heart (Heikinheimo *et al.*, 1994). Inhibiting *GATA-4* expression, using anti sense RNA, blocks the differentiation of cardiac myocytes in a pluripotent carcinoma cell line (Ip *et al.*, 1994, Grepin *et al.*, 1995). *GATA-5* and *GATA-6* bind the same DNA sequence as *GATA-4* and their expression patterns overlap that of *GATA-4* in the cardiogenic lineage, suggesting that they may perform similar functions (Laverriere *et al.*, 1994, Jiang *et al.*, 1996, reviewed Olson and Srivastava 1996).

2.4.3.2 basic helix-loop-helix gene expression in the developing heart

Two transcription factors *HAND1* and *HAND2* control aspects of cardiac left-right differentiation. The two are structurally related basic-Helix-Loop-Helix (bHLH) proteins, and their mRNAs are present bilaterally during early fusion stages of heart development (Srivastava *et al.*, 1995, Srivastava *et al.*, 1997).

Other bHLH genes expressed in early mouse cardiac cell development include Mesp2 (Saga *et al.*, 1997) and Id (Evans *et al.*, 1993).

2.4.3.3 Myosin enhancing binding-factor-2 expression in the developing heart

Myosin enhancing binding factor-2 (*Mef2*), like *Hoxa-9* is a transcription factor expressed in precursors of the cardiac, skeletal, and smooth muscle lineages, as well as in other cell types (Edmondson *et al.*, 1994, Chambers *et al.*, 1994, Wong *et al.*, 1994). Loss-of-function studies in *Drospohila* (D-Mef2) show a block in all muscle cell type differentiation in the embryo (Lilly *et al.*, 1995, Bour *et al.*, 1995, Ranganayakulu *et al.*, 1995). Phenotypic consequences of *Mef2* mutations in mouse are not yet known, but

evidence from D-Mef2 studies and expression patterns suggest a likely role in cardiomyocyte differentiation (reviewed Olson and Srivastava 1996).

Chisaka and Cappechi showed that the targeted disruption of Hoxa-3, which is expressed at E12.5, results in atrial hypertrophy, aortic and pulmonic valve defects and left ventricular hypertrophy as well as other non-cardiac defects (Chisaka and Capecchi,1991).

2.4.4 Heart chamber formation

Data in this chapter demonstrated expression initially in the left and right atrial wall of the developing heart between E9.5-10.5. During this stage, heart looping occurs which is the first insight into left/right symmetry. This stage is essential in distributing this organ system correctly in connecting the outflow and inflow vasculature systems. It is at E10 when the chambers of the heart form from specific sites within the tubular heart during and after looping (Christoffels *et al.*, 2000).

These chambers (atria and ventricles) differ in morphology, physiology and in the muscle contractile protein genes each expresses (Zadeh *et al.*, 1986, Evans *et al.*, 1988, Wang *et al.*, 1996).

2.4.4.1 Specific heart chamber gene expression

Many cardiac genes are broadly expressed in the early heart and become restricted to the atria or ventricles as development proceeds.

Chamber myocardium tissue forms locally from the embryonic myocardium of the tubular heart.

It has been proposed that diversification of atrial and ventricular cells may occur earlier than chamber formation around E10 (mouse), because an atrial-specific *MyHC* gene (myosin heavy chain), *AMHC1*, is first expressed in the posterior region of the fusing cardiac tube, and is a future atrial component (Yutzey *et al.*, 1994).

Another myosin heavy chain gene *MyHC 3*, has been identified as atrial specific during chamber development in the quail heart. Initially the gene is expressed throughout the tubular heart, but as the heart chamberises, expression is down regulated in the ventricles, but maintained in the atria (Schleinitz and Stockdale, unpublished data, Wang *et al.*, 1996).

In situ and wholemount data has shown *BMP-10* expression in the developing heart. Transcripts were first detected at E9.0 in the common ventricular chamber and by the beginning of E12.5 additional expression was found in the inner atrial wall (Neuhaus *et al.*, 1999). This temporal expression pattern demonstrated by *BMP-10* is very similar to that shown by *Hoxa-9* in this manuscript, but the spatial expression is in different chambers of the heart eg. *Hoxa-9* first shows expression in the atria of the heart and develops into the ventricle at E12.5

It could be suggested that these two gene expression patterns may share a degree of repressive or activating mechanisms. *Bone Morphogenic Proteins* (*BMP*) 2, 4 and 7 have been demonstrated previous as upstream signallers of *Hox* genes in response to *Shh* regulate during the growth stage of skeletal and mesenchyme tissue development (Iimura *et al.*, 1994, Roberts *et al.*, 1995, Watanabe *et al.*, 1998), and therefore there may be a relationship in the developing heart.

The atrial natriuretic factor (ANF) gene is specifically expressed in this developing chamber myocardium and is one of the first hallmarks of chamber formation. In the mature heart ANF gene expression is restricted to the atrial auricles, and during development its expression is specific for the formation of atrial and ventricular chambers (Christoffels *et al.*, 2000).

Signalling molecules in pre-atrial cells are not clearly understood, but studies have shown that retinoic acid (RA) synthesis and activity within the embryonic mouse heart support the importance of endogenous RA for cardiac anterior posterior patterning (Moss *et al.*, 1998). As the murine heart forms, the RA-synthesising enzyme RALDH2 is only present in pre-atrial regions. Transgenic mice carrying a reporter gene under the control of a RA-responsive promoter element demonstrate that retinoid signalling within the myocardium controls atrial differentiation (Moss *et al.*, 1998, reviewed Yelon and Stainier 1999). Posterior transformations of anterior digits, rhombomeres or vertebrae by RA are thought to be controlled by RA-mediated shifts in *Hox* gene expression (Langston and Gudas, 1994). It is yet not clear what roles *Hox* genes play in cardiac anterior posterior regions, but several *Hox* genes have been upregulated in RA-treated precardiac avian experiments, which include *Hoxd-3, Hoxa-4* and *Hoxd-4*, suggesting a role in heart patterning and the regulation of early lineage specification (Searcy & Yutzey, 1998). However, as yet a retinoic acid response element to regulate function has not been detected in *Hoxa-9* transcription.

Another homeodomain protein belonging to the Iroquois family, *Irx4*, directs ventricular-specific gene expression (Bao *et al.*, 1999) and the orphan nuclear receptor COUP-TF (chicken ovalbumin upstream promoter-transcription factor) II, is required for atrial, but

not ventricular development (Pereira *et al.*, 1999). Members of the HRT family of hairy-related bHLH proteins, are expressed in a spatial manner in the embryonic heart – HRT1 in the atria and HRT2 in the ventricles (Nakagawa *et al.*, 1999, Leimeister *et al.*, 1999, reviewed Yamagishi *et al.*, 2001).

These cardiac genes must demonstrate spatial and temporal expression patterns to ensure they regulate the correct cell type at the intended time point. *Hoxa-9* has shown both of these criteria, but its regulation and function in the atrium remains unclear.

2.4.5 Conclusions

In situ and wholemount hybridisation techniques have been used successfully to demonstrate the anatomical expression patterns of desired genes during stages of embryonic development. However these did not focus on the expression pattern at haematopoietic sites during the essential stages of blood cell development in the embryo (E7-E14). This study, using similar techniques on Balb/C mouse embryos showed initial widespread patterning in the early E7.5 embryo, with a previously unreported specific expression pattern in the developing atrium of the heart at E9.5-E10.5, as well as the previously known areas of the gut and vertebrae. *Hoxa-9* expression then appeared more widespread, but with specific expression seen in the heart atria and ventricle at E12, before becoming undetectable by E13. This patterning has not been seen in previous *Hoxa-9* reports, and could have important implications in future heart development studies.

This specific patterning seen in the heart, gut and vertebrae, does not correlate with the time course of the corresponding haematopoietic pathway in this mouse strain, although

there appeared to be a degree of positive staining in the liver at E11, when haematopoietic function was high in this organ. However, the undetectable levels of *Hoxa-9* in relation to haematopoietic organs in these *in situ* and wholemount hybridisation embryos does not mean that *Hoxa-9* is not involved in the haematopoietic pathway. It may be, that this conventional technique is not suitable for detecting low levels of expression at the cellular level, for example the foetal liver is a vital haematopoietic organ, but it is the blood islands within the liver that are important for producing haematopoietic progenitors. This study cannot exclude the possibility of expression at these sites, but the techniques adopted did not show detectable levels and therefore alternative gene expression models should be studied to investigate further *Hoxa-9* expression during haematopoiesis, but perhaps more importantly to further investigate the expression patterns identified in the heart in this study and a possible role for *Hoxa-9* during cardiogenesis.

Chapter Three – *Hoxa-9* expression during embryonic stem cell differentiation *in vitro*

3.1 Introduction

In situ and wholemount hybridisation analysis in the previous chapter identified an expression pattern for Hoxa-9 in the atrium of the developing heart, but levels at haematopoietic sites were undetectable. This does not mean however, that Hoxa-9 definitively is not involved in this process (Lawrence *et al.*, 1997, Izon *et al.*, 1998, Thorsteinsdottir *et al.*, 2002), it merely suggests that the methods used may not be sensitive enough to detect expression at those sites, and an alternative technique should be used to further investigate a potential functional role. One approach that has been previously used to investigate a functional role for developmentally regulated genes has been to exploit the biology of embryonic stem cells.

3.1.1 Embryonic stem cells

Early embryogenesis is characterised by the formation, expansion and differentiation of a population of pluripotent cells. At the end of this early developmental stage, cleavage divisions occur within the cells and the developmental potential is from the resulting inner cell mass (ICM) of nascent blastocysts.

In vivo, the ICM is a stem cell population from which develops all tissues of the embryo proper including germ cells (Hogan *et al.*, 1994). *In vitro* it is possible to avoid the loss of pluripotency, seen during gastrulation in vivo (Hogan *et al.*, 1994), and isolate immortal stem cell lines known as embryonic stem cells (ES cells) (Evans and Kaufman, 1981, Martin *et al.*, 1981, Brook and Gardner, 1997).

ES cells are derived from the epiblast of preimplanted blastocysts. The discovery that LIF can suppress this differentiation (Smith and Hooper, 1987, Smith *et al.*, 1988, Williams *et al.*, 1988) has allowed the *in vitro* culture of these cells as ES cells. Their continued pluripotency following *in vitro* culture can be demonstrated following injection into host blastocysts (reviewed Robertson *et al.*, 1986, Smith *et al.*, 1992); the ES cells can contribute to all tissues of the developing embryo. This functionality has made them a powerful tool in the study and analysis of the molecular mechanisms involved in *in vivo* development of different cell lineages. However, their potency, and their ability to spontaneously differentiate *in vitro* in the absence of LIF have also encouraged several groups to investigate the potential of ES cells as *in vitro* assays of cell differentiation.

3.1.2 ES cell differentiation *in vitro*

A number of techniques have been used to facilitate the differentiation of ES cells into different tissue types. One approach has been to form ES cells into aggregates prior to differentiation (figure 46). These differentiating structures are known as embryoid bodies and have been shown to generate a variety of tissues, including, muscle (Doetschman *et al.*, 1985, Rohwedel *et al.*, 1994), neuronal (Zhu *et al.*, 1996, Brüstle *et al.*, 1999), and endothelial (Wang *et al.*, 1992, Reynolds *et al.*, 1996, Weiss *et al.*, 1996). However, of particular relevance to studies of *Hoxa-9,* differentiation of ES cells *in vitro* has also been used to produce haematopoietic (Schmitt *et al.*, 1991, Keller *et al.*, 1993, McClanahan *et al.*, 1993) and cardiac tissue cell types (Robbins *et al.*, 1990, Miller-Hance *et al.*, 1993). The latter two will be reviewed here.

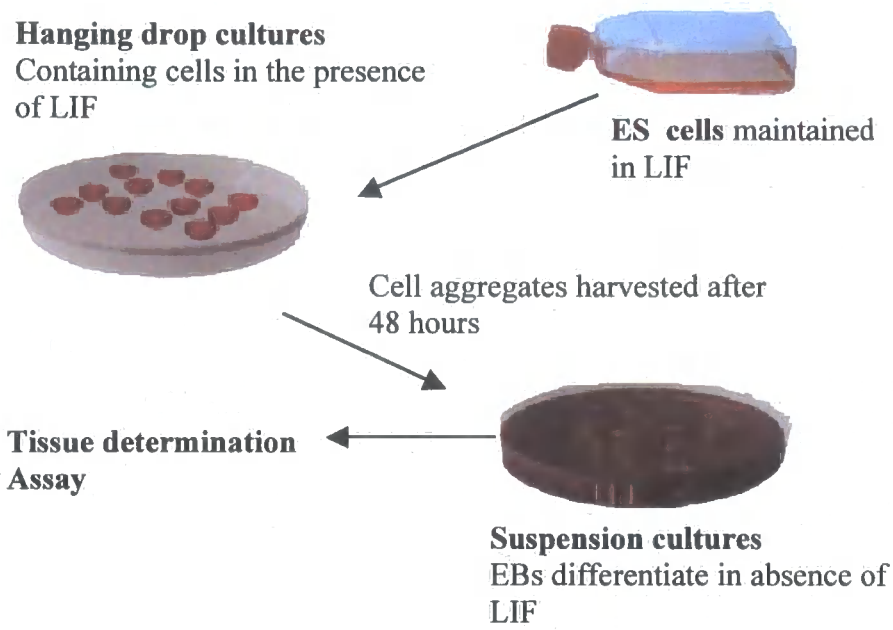


Figure 46 **The formation of embryoid bodies from undifferentiated ES cells.** Following the removal of LIF growth factors can be added in suspension cultures and the tissue phenotype analysed using a variety of assays.

3.1.3 Haematopoietic differentiation of ES cells *in vitro*

Haematopoietic development in EBs is efficient and highly reproducible. EBs have been shown to contain cells of erythroid, myeloid and to a lesser extent lymphoid progenitor cells. EBs themselves could provide an environment that supports haematopoiesis, as genes encoding various growth factors and their receptors are expressed early in EB development (Schmitt *et al.*, 1991, Keller *et al.*, 1993, McClanahan *et al.*, 1993). The earliest stages of haematopoietic development within EBs (defined as the onset of specific gene expression) follows an ordered sequence of events similar to those in the

developing embryo (Keller *et al.* 1993, Burket *et al.*, 1991, Schmitt *et al.*, 1991, McClanahan *et al.*, 1993). Haematopoiesis from ES cells, as in the mouse embryo, has been observed as islands of haemoglobinised erythroid cells or blood islands within the developing EBs (Doetschuman *et al.*, 1985).

Haematopoietic studies in ES cells have investigated and developed the understanding of the early foetal development and commitment of the multi lineages from the pluripotent HSC (Burket *et al.*, 1991, Wiles and Keller, 1991, Keller *et al.*, 1993, Bigas *et al.*, 1995). The understanding of lymphopoiesis, has been greatly enhanced using ES cell *in vitro* models to study the development, commitment and generation of lymphoid cells (Chen, 1992, Chen *et al.*, 1992, Gutierrez-Ramos and Palacios, 1992, Müller and Dzierzak, 1993, Nakano *et al.*, 1994, Nisitani *et al.*, 1994). There is evidence that long-term repopulating stem cells can be produced (Hole *et al.*, 1996). As an adjunct to these studies, morphogens (such as dimethyl sulphoxide (DMSO) and retinoic acid (RA)) have been used to modulate this haematopoietic commitment, and this has proved useful in characterising the expression of developmentally regulated genes in haematopoietic commitment (Lako *et al.*, 2000). The expression of *Hoxa-9* during haematopoietic differentiation of ES cells *in vitro* has however, not been described.

3.1.4 Cardiomyocyte differentiation of ES cells *in vitro*

Several groups have defined the culture conditions that allow ES cells to generate cardiomyocyte lineages (Wobus *et al.*, 1991, 1997 Drab *et al.*, 1997, Kolossov *et al.*, 1998, Müller *et al.*, 2000). ES cell-derived cardiomyogenesis has been unequivocally

shown that at the terminally differentiated stage, all different cardiac phenotypes – sinus nodal, pacemaker, atrial and ventricular-like cells can be detected (Wobus *et al.*, 1997). *In vitro* differentiation of ES cells into cardiac lineages has proven to be a useful screen to identify genes involved in cardiogenesis including *GATA-4* (Narita *et al.*, 1996), and *Hand-1* (Riley *et al.*, 2000). There is currently no published information on the expression of *Hoxa-9* during cardiac differentiation of ES cells *in vitro*.

3.1.5 Aims

The aim of this study was to examine the expression patterns of *Hoxa-9* during haematopoietic and cardiac differentiation of ES cells *in vitro*, and examine whether such expression correlates to the appearance of lineage specific molecular markers of differentiation.

3.2 Materials and methods

3.2.1 ES cell culture and embryoid body formation

The CGR8 ES cells were routinely passaged and maintained in an undifferentiated state as described by Smith (Smith *et al.*, 1991) in ES cell medium (GMEM; Gibco BRL, Paisley, Scotland, UK) with 7.5% $NaHCO_3$, 100x non-essential amino acids (Gibco BRL), 2 mM L-glutamine (Gibco BRL), and 1 mM pyruvate (Gibco BRL), 0.1 mM 2-mercaptoethanol and 10% foetal calf serum (Gibco BRL) with 66 µl LIF /10ml media (v/v) leukaemia inhibitory factor (LIF) which was produced as described (Smith, 1991).

In order to form embryoid bodies (EBs), ES cells were cultured in hanging drops (10 µl) at a concentration of 3×10^4 cells/ml in the presence of LIF for 48 hours in a humidified 5% CO_2 atmosphere at 37°C. ES cell aggregates were harvested into a petri dish (10^3 aggregates/10ml ES cell medium) and allowed to differentiate in the absence of LIF for up to 6 days, in figure 3.1.2. Medium was replaced every two days. Selected samples were treated with either 1% (v/v) dimethylsulfoxide (DMSO) or 10^{-8} M all - trans retinoic acid (RA) for the first 48 hours of differentiation (see figure 46).

The cardiomyocyte differentiation of ES cells was carried out essentially as described (Muller et.al. 2000). Briefly, the ES cell aggregates were produced in hanging drops, as described above, and after fourty-eight hours were collected by centrifugation at 800*g* (Sigma 2-15), for three minutes. The aggregated ES cells were then transferred into ungelatinised petri dishes (IWAKI, Paisley, Scotland) in CGR8 media containing LIF (described above). After 72 hours, the cells were passaged and transferred to 1% (v/v) gelatinised petri dishes in CGR8 media with LIF being removed and allowed to differentiate.

3.2.2 mRNA extraction

Each series of ES cell differentiation was carried out using three independent experiments. For each sample, approximately 100 EBs were collected for each time point, washed in PBS (see A.14) and spun at 4000*g* for three minutes (Sigma 2-15). The supernatant was removed and mRNA extracted by adding 1ml of RNAzol (Biogenesis, Poole, Dorset, England) to the pellet, followed by 100 µl chloroform and leaving on ice for five minutes. The samples were centrifuged for ten minutes at 4000*g* (Sigma 2-15), and the upper phase containing RNA transferred to a fresh tube.

RNA was precipitated with an equal volume of propan-2-ol and centrifuged at 4000*g* for ten minutes. The pellet was washed in fresh 70% (v/v) ethanol and allowed to air-dry before being resuspended in 20µl of TE buffer (see A.20).

3.2.3 The generation of cDNA

mRNA was reverse transcribed using the 1st strand cDNA synthesis kit from Roche (Lewes, East Sussex, England) following their instructions. In brief, 1 µg of mRNA was denatured by incubating at 90°C for ten minutes, followed by cooling on ice for five minutes. For each 1 µg of mRNA, 1unit of AMV-reverse transcriptase enzyme, 2 µl 5X AMV-RT buffer, 2 µg random primers, 10 mM dNTPs, and sterile water up to 10 µl total volume, were mixed and incubated at 37°C for one hour. Following incubation, the cDNA reaction was stored at –20°C. PCR was carried out as described in 2.2.1.

Figure 47 - **Sequences and annealing conditions for RT-PCR primers**

Gene/ Accession number	Product size(bp)	Anneal temp. °C (x)	5' RT-PCR primer	3' RT-PCR primer
Activin B_β	426	50°C	gtcaatttgacgtggtttcc	gcaagaatgtgctgatcaac
AFP	410	50 °C	gctcacaccaaagcgtcaac	cctgtgaactctggtatcag
B-Actin	934	63 °C	aggggccggactcatcgtactc	gtgacgaggcccagagcaagag
Brachyury	947	50 °C	tccaggtgctatatattgcc	tgctgcctgtgagtcataac
c-kit	765	55 °C	tgtctctccagtttccctgc	ttcagggactcatgggctca
Collagen	463	55 °C	caagcatagtggtccgagtc	aggcaggtcaagttctagcg
flk-1	398	60 °C	taggtgcctccccataccctgg	tggccggctctttcgcttactg
GATA-1	404	60 °C	ggaattcgggccccttggtgagg ccagagag	cggggtacctcacgctccagcca gattcgaccc
GATA-4 MMU85046	211	55 °C	ctgtcatctcactatgggca	tacgcggtgattatgtcccc
Hoxa-9 AB005457	724	59 °C	atcatggccaccaccggggc	cgtcttttgctcggtccttg
Mef2C MUSMEF2CA	269	60 °C	gtactctctgagtagcgcag	gtgtgttgtgggtatctcga
Mesp2 NM_008589	577	60 °C	gctcagatgcttggtcctag	caggtcttcctgccaaagtt
MLC NM_010861	188	65 °C	gaaggctgactatgtccgggaga tgc	tgtgggtcacctgaggctgtggtt cag

Gene/ Accession number	Base pair size (bp)	Anneal temp. °C (x)	5' RT-PCR primer	3' RT-PCR primer
Nkx-2.5 AF083133	248	55 °C	cagtcaaagacatcctgaac	caccagcacgggcaccgcga
Troponin T AB052890	578	57 °C	cagcagcgtattcgcaatga	ttcttcttccgggcctcatc

3.2.4 Colony forming unit-assay (CFU-A)

The *in vitro* colony forming unit-assay (CFU-A) of embryoid bodies was carried out essentially as described previously (Pragnell *et al.* 1988, Hole and Smith 1994). In brief, a feeder layer consisting of 0.6% agar in a modified Eagle's medium (α-MEM; Gibco BRL) with conditioned medium from two cell lines (AF1-19T; a source of granulocyte macrophage colony stimulating factor, GM-CSF; and L929 a source of CSF-1) was poured into 3 cm diameter tissue culture grade dishes (IWAKI)(1 ml per layer). Embryoid bodies (normally 50 per dish) were added to 0.3% agar in α-MEM and plated to form an upper layer. The dishes were incubated for 11 days at 37° C in a humidified atmosphere with 5% O_2/ 10% CO_2. The presence or absence of haematopoietic progeny in the differentiated EBs was determined by the formation of mixed colonies with diameter greater than 2 mm after 11 days of incubation. CFU-A assays were normally carried out in triplicate as an average percentage of the number of EBs showing proliferation and commitment.

3.3 Results

Two distinct ES cell differentiation strategies were used, optimised for either haematopoietic or cardiomyocyte differentiation. In each case these approaches were first studied for the appearance of functional cells and then for the appearance of molecular markers of lineage commitment. Having validated these systems and determined the time course of lineage commitment and differentiation, the expression of *Hoxa-9* was examined by RT-PCR and correlated with molecular and cellular events occurring during ES cell differentiation *in vitro*.

3.3.1 Haematopoietic differentiation: cellular assay

Haematopoietic commitment of the embryoid bodies was assessed using the Colony Forming Unit Assay (CFU-A) (Pragnell, 1988). This assay detects the presence of early haematopoietic progenitors (Pragnell, 1988) and is thus appropriate for the examination of the onset of haematopoiesis in ES cell differentiation *in vitro* (Hole *et al.*, 1996). When haematopoietic committed progeny are present within the EBs, they proliferate and can be visualised as a haematopoietic halo, as shown in figure 48 (Hole *et al.*, 1996). Figure 49 shows the percentage of EBs that demonstrated this haematopoietic halo in CFU-A at different time points. This graph shows the mean of the three independent experiments, and indicates that EBs differentiating in media alone (control) showed haematopoietic commitment first detected at day 4. The percentage of embryoid bodies containing detectable haematopoietic progenitors increased to a maximum of 40% after 6 days. The presence of DMSO or RA dramatically changed this differentiation profile. The percentage of EBs that demonstrate haematopoietic commitment was dramatically

increased following treatment with 1% DMSO for 48 hours, resulting in 80% of EBs showing detectable CFU-A activity after 6 days. In contrast, the presence of low concentrations of RA early in ES cell differentiation appear to suppress haematopoietic commitment. These results are in full agreement with previous work (Lako *et al.*, 2000).

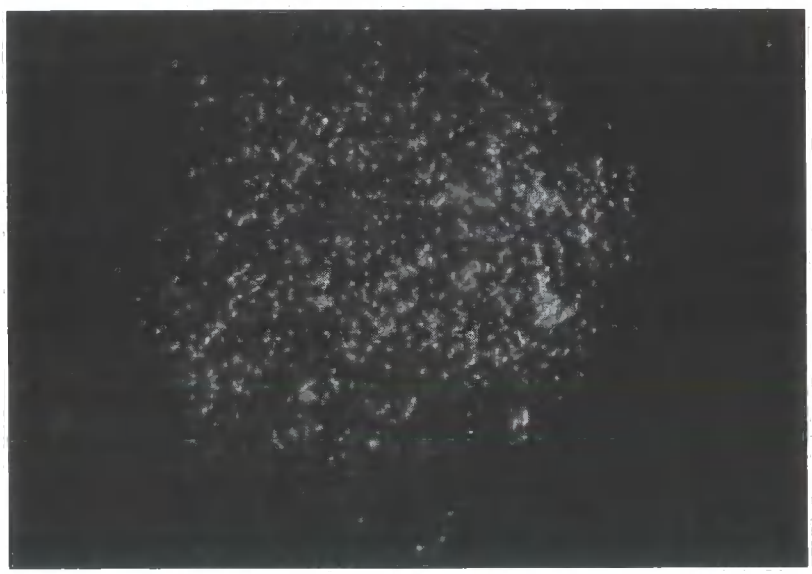


Figure 48 – **Colony forming unit assay** showing the haematopoietic halo of proliferative haematopoietic cells

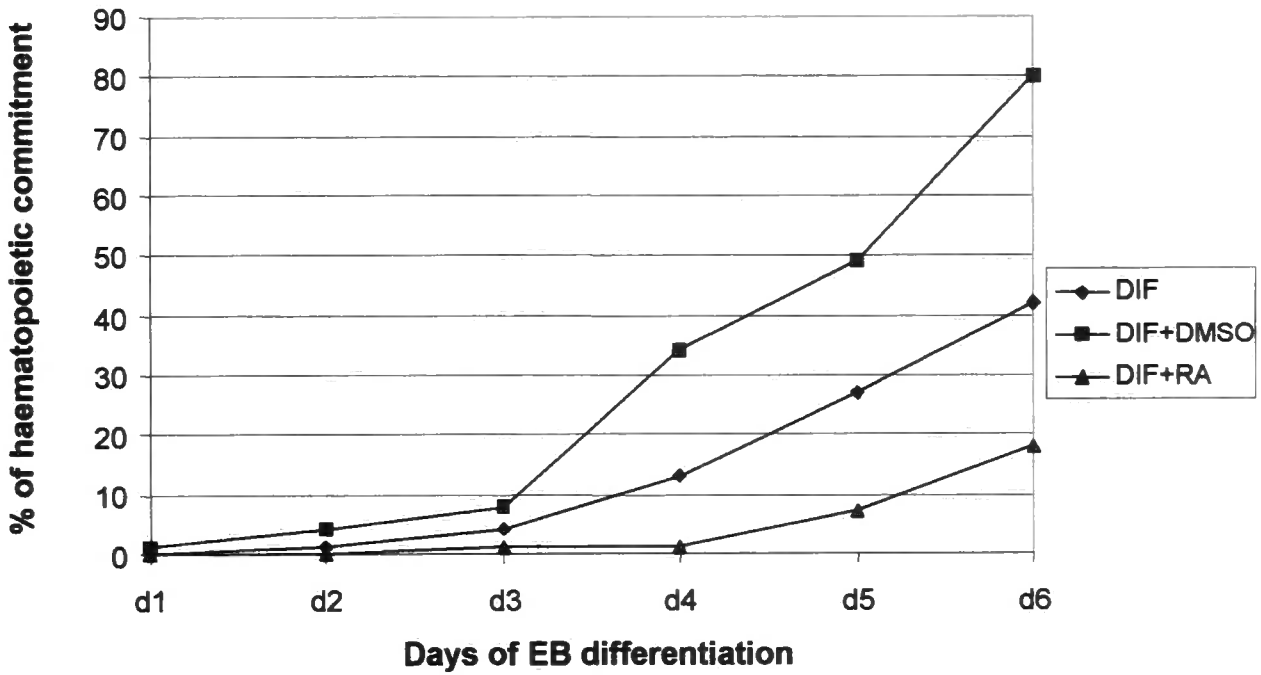


Figure 49 **CFU-A Assay** to show the number of embryoid bodies (EBs) showing haematopoietic commitment.

EBs differentiating in media alone, media + 1% DMSO, and media + 10^{-8} M all-trans retinoic acid

Having determined reproducible haematopoietic commitment of ES cells following differentiation *in vitro*, the molecular events occurring at or around the time points of commitment could be examined. The purpose of this study was to examine the onset of expression of *Hoxa-9* during ES cell differentiation, and therefore a completely quantitative approach was not required. Therefore RT-PCR was used to examine gene expression. A measure of semi-quantitative analysis was offered as long as controls for cDNA production and gel loads were included. To address this, all samples were normalised with respect to RT-PCR of β-actin, a gene commonly used as an internal control for such studies (Lako *et al.*, 2000) (figure 50).

3.3.2 Haematopoietic differentiation: gene expression

The lineages to emerge during development are ectoderm, endoderm and mesoderm. The expression of genes characteristic of these germ layers were examined in this model of haematopoietic differentiation of ES cells *in vitro*. *Activin* β_B as a marker of primitive ectoderm (Albano *et al.*, 1993). In this study, this gene was expressed in the early stages of differentiation (figure 51). However, EBs treated with 1% DMSO showed reduced levels of expression of this ectodermal lineage marker. In contrast, there appeared to be an increase in the level of *Brachyury* expression, a mesoderm marker (Herrman *et al.*, 1990) (figure 52).

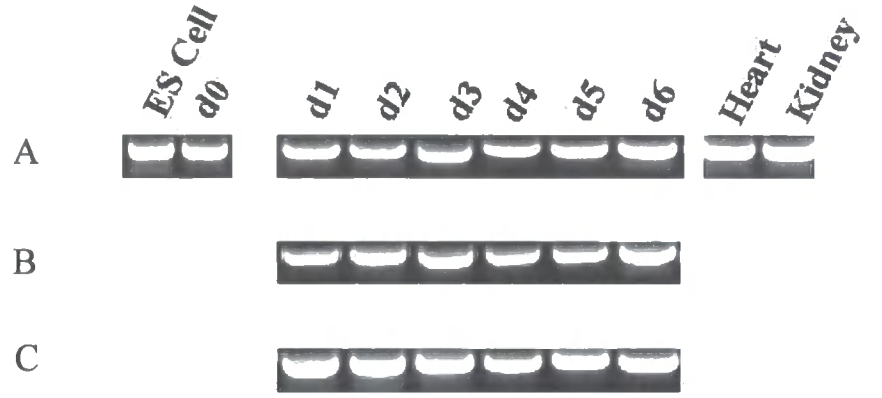


Figure 50 - **PCR analysis of *Beta-Actin*** in ES cells differentiating in: media (A) ; media and 1% dimethyl sulphoxide (B); media and 10^{-8} M retinoic acid (C). This RT-PCR was used to optimise equal amounts of cDNA for further experiments

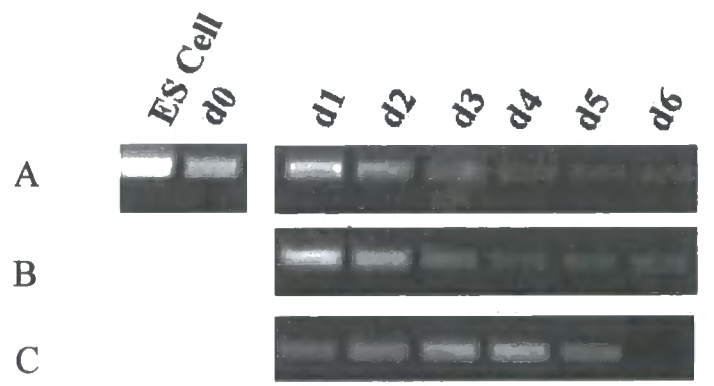


Figure 51 - **PCR analysis of *Activin* β** in ES cells differentiating in: media (A); media and 1% dimethyl sulphoxide (B); media and 10^{-8} M retinoic acid (C).

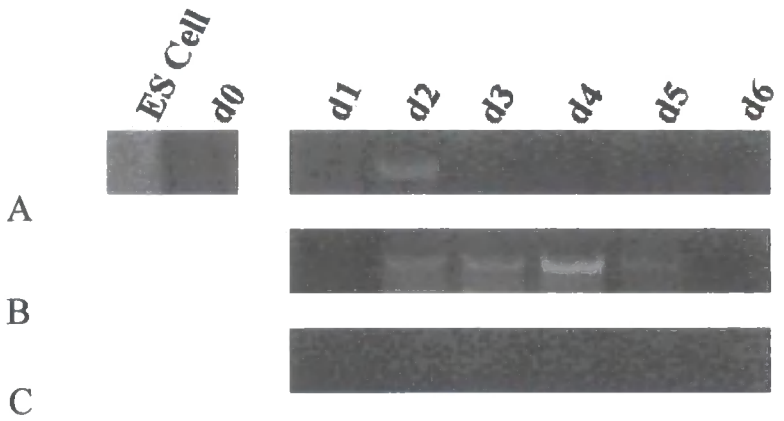


Figure 52 - **PCR analysis of *Brachyury*** in ES cells differentiating in: media (A) ; media and 1% dimethyl sulphoxide (B); media and 10^{-8} M retinoic acid (C).

This apparent down regulation of molecular markers of ectodermal commitment, coupled with an increase in molecular markers of mesoderm is consistent with previous data (Lako *et al.*, 2001).

The endoderm markers, *Collagen αIV* (Adamson and Ayers, 1979), and *AFP* (alpha feta protein) (Dziadek and Andrew, 1983) were only detected in later stages of differentiation in EBs that showed limited haematopoietic (mesoderm) commitment, shown in figures 49, 53 and 54.

Two markers of early haematopoietic progenitors, *c-kit* (Bernex *et al.*, 1996) and *flk-1* (Shalaby *et al.*, 1995) were examined. *c-kit* expression levels in the DMSO treated cells were expected to be higher than demonstrated, as such cells showed haematopoietic commitment in the CFU-A assay. However, it is important to note that the expression of *c-kit* started earlier in these treated cells compared to controls, demonstrating the generation of haematopoietic lineages in the presence of DMSO. The undetectable levels of *c-kit and flk-1* in the RA treated cells, further confirmed the reduced haematopoietic commitment in these cells.

This result from these two lineage markers highlight to an extent some the limitations of using the ES model to study gene expression. The expression of these two early haematopoietic markers in the differentiation alone cultures was as expected, as this mixture of differentiating EBs contain some with mesoderm potential (shown by CFU-A in figure 49), however this is a mixed population. In an effort to generate a more "mesodermally derived" population, 1% DMSO was added to the culture, and by CFU-A analysis there was an increase in the number of EBs showing haematopoietic

commitment. However, not every cell of the EB is committed to haematopoiesis, so although there was up to 80% commitment, it is important to be aware that it is not a pure mesoderm population, demonstrated here by levels of non-mesoderm lineages (Activin) (figure 51) detected in EBs cultured in DMSO.

It was encouraging from the RT-PCR data shown in this thesis that the expression shown in figures 50-66 were reproducible in three independent experiments, suggesting that the experimental procedures were consistent. This reproducibility was important when analysing the expression of *flk-1* (figure 56) in 1% DMSO. The expression of this gene has been shown to be important in successful early haematopoietic commitment (Shalaby *et al.*, 1995), and it might be expected to correlate with the appearance of haematopoietic progenitors detected by CFU-A in this system. However results shown in figure 56 suggest that detectable expression was restricted to day 3 alone. This empathatic result was seen in three independent experiments (data not shown) and is consistent with previously published work (Lako *et al.*, 2000).

GATA-1 is a marker of the more committed haematopoietic cell (Simon *et al.*, 1992).

HSC activity has been previously seen, *in vivo*, at day 4 (Hole *et al.*, 1994). It has been demonstrated in this thesis that high levels of this protein were found at this same time of high HSC activity (figure 57), and when haematopoietic commitment began to increase (figure 49), suggesting that although this ES cell model has limitations, this expression pattern demonstrates some compatibility to *in vivo* systems.

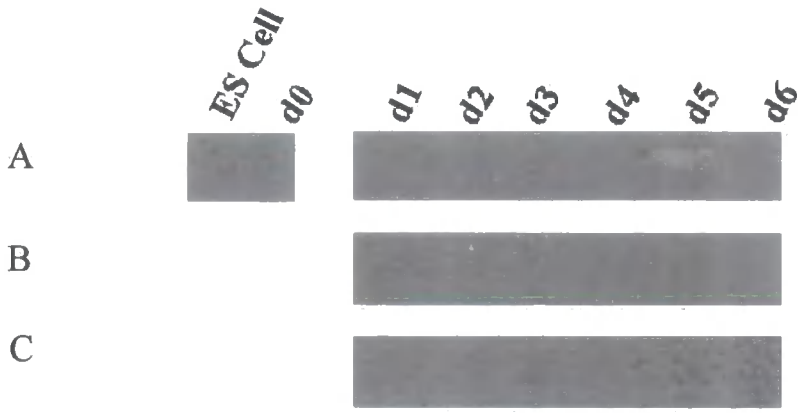


Figure 53 - **PCR analysis of *CollagenαIV*** in ES cells differentiating in: media (A) ; media and 1% dimethyl sulphoxide (B); media and 10^{-8} M retinoic acid (C)

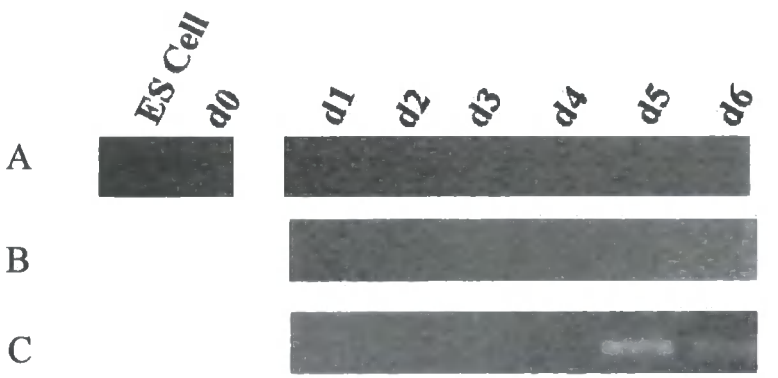


Figure 54 - **PCR analysis of *AFP (alpha feta protein)*** in ES cells differentiating in: media (A) ; media and 1% dimethyl sulphoxide (B); media and 10^{-8} M retinoic acid (C)

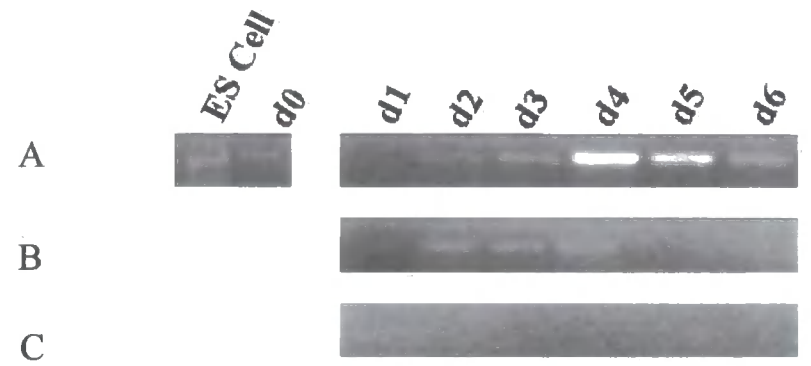


Figure 55 - **PCR analysis of *c-kit*** in ES cells differentiating in: media (A) ; media and 1% dimethyl sulphoxide (B); media and 10^{-8} M retinoic acid (C)

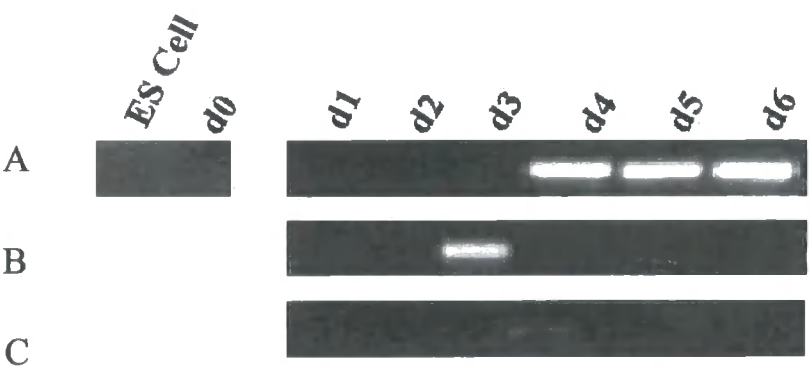


Figure 56 - **PCR analysis of *flk-1*** in ES cells differentiating in: media (A) ; media and 1% dimethyl sulphoxide(B); media and 10^{-8} M retinoic acid (C)

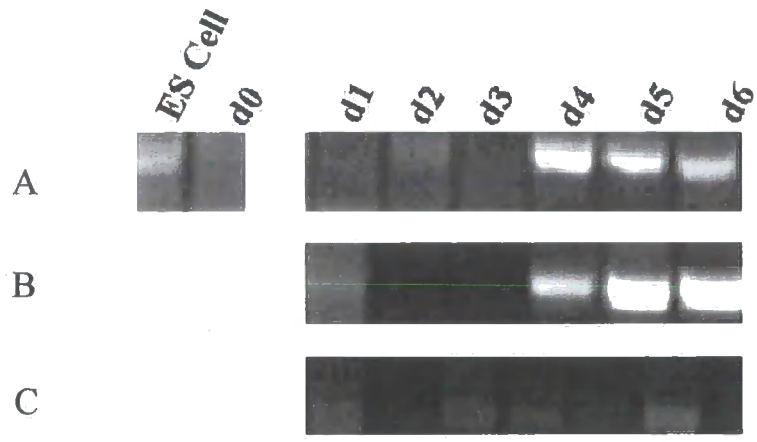


Figure 57 - **PCR analysis of *GATA-1*** in ES cells differentiating in: media (A) ; media and 1% dimethyl sulphoxide (B); media and 10^{-8} M retinoic acid (C)

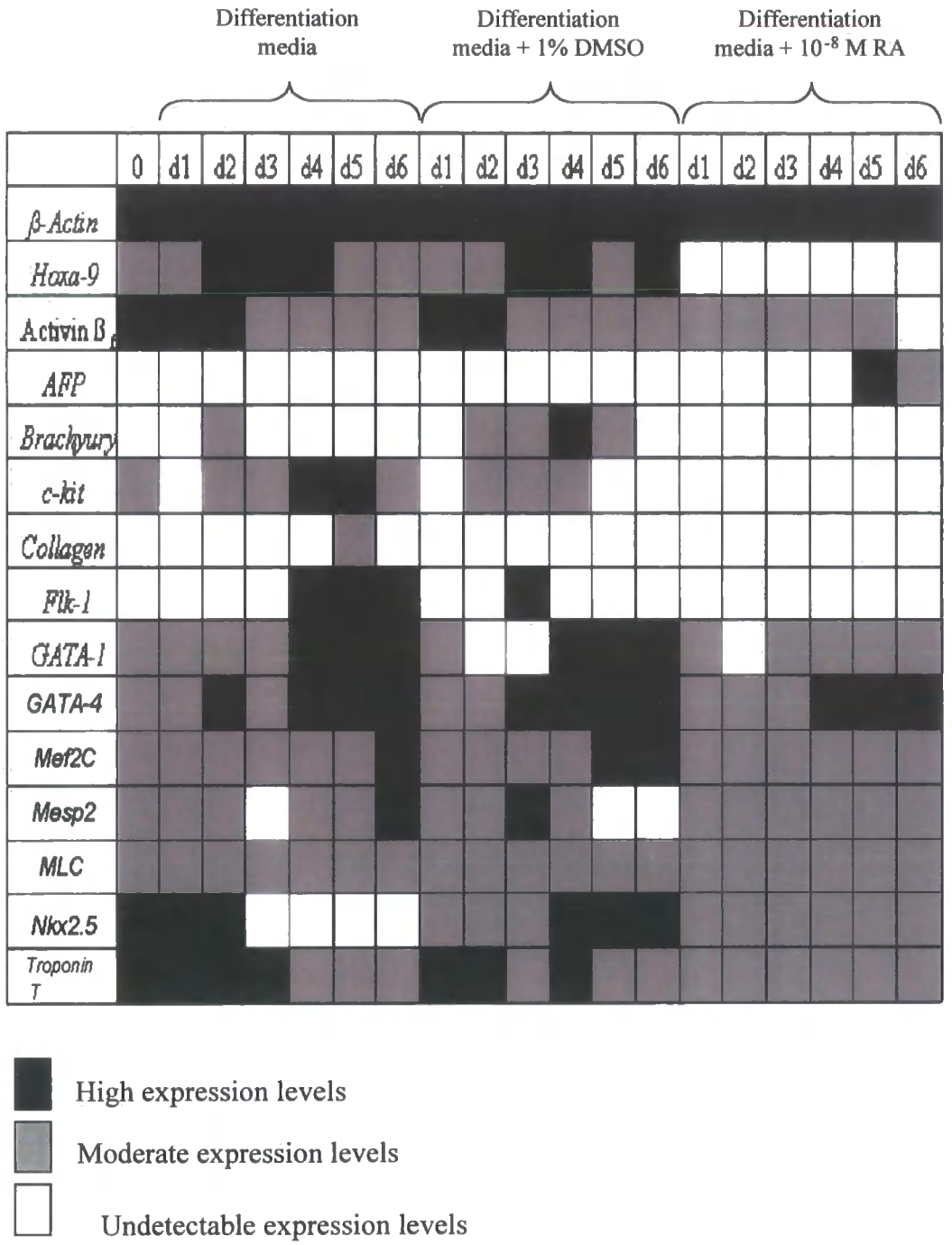


Figure 58 - **Summary of gene expression during the haematopoietic differentiation of ES cells**

The haematopoietic marker genes are shown in normal text, with the cardiac markers shown in **bold** type.

Evidence from CFU-A for haematopoietic progenitors and RT-PCR for differentiation markers indicated that this model of haematopoietic differentiation was appropriate for studying the expression of developmentally regulated genes during haematopoietic commitment. However, this model was not optimised with respect to cardiomyocyte development. The system for *in vitro* differentiation of cardiomyocytes from ES cells was therefore examined.

3.3.3 Cardiomyocyte differentiation: cellular assay

In this differentiation assay ES cells were removed from the conventional suspension cultures, and placed onto pre-gelatinised plates to encourage the differentiation of cardiomyocytes. The formation of functional cardiomyocytes was determined by the appearance of 'twitching' EBs; the rhythmic contraction of these structures. This has previously been used as a measure of cardiomyocyte commitment (Wobus *et al.*, 1997).

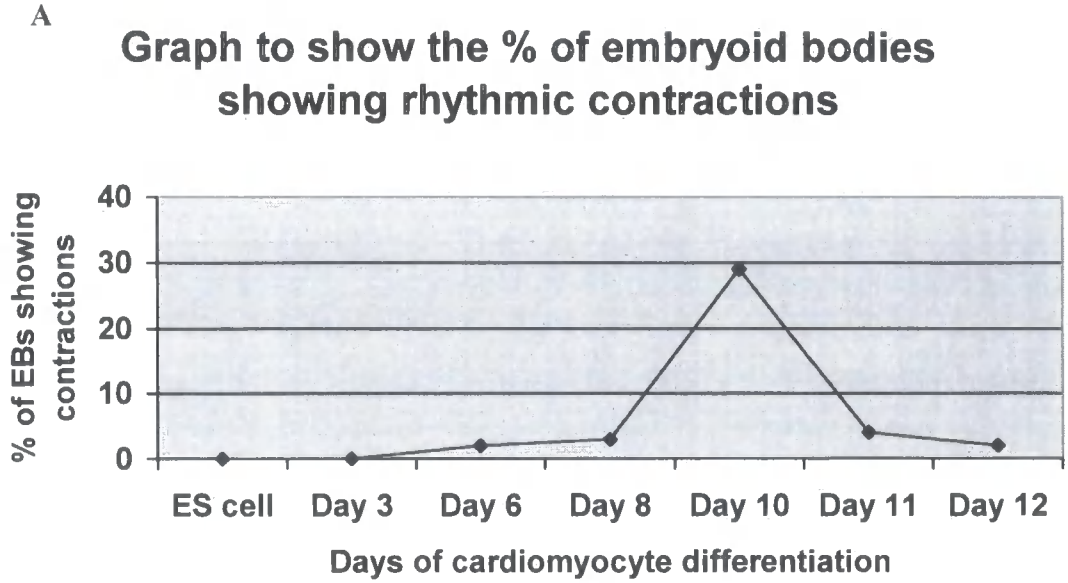
The percentage of EBs demonstrating "twitching" was recorded over the period of differentiation (figure 59). EBs containing functional cardiomyocytes were first detected at day 3 of differentiation. The percentage of EBs demonstrating rhythmic contractions increased over time up to a peak of 29% at day 10.

3.3.4 Cardiomyocyte differentiation: gene expression

A profile of cardiac genes expressed during cardiomyocyte differentiation of ES cells is shown in figure 60 and summarised in figure 61.

For comparison, the same endo, ecto and mesoderm markers studied in the haematopoietic differentiation of ES cells were examined for this data set.

A



B

Day of cardiomyocyte differentiation	% of EBs showing contractions
ES cell	0
Day 3	0
Day 6	2
Day 8	3
Day 10	29
Day 11	4
Day 12	2

Figure 59 – **A** – The number of embryoid bodies showing rhythmic contractions during cardiomyocyte differentiation
B – Graph to show data shown in **A**

The ectoderm marker gene *Activin* β_β consistently showed expression in undifferentiated ES cells which contain lineages from all three germ layers, and demonstrated a continued high level of expression throughout this differentiation process, in contrast to the decreasing levels of expression seen in the haematopoietic differentiation system. In the previous ES cell differentiation pathway, *AFP* expression was undetectable in undifferentiated ES cells, but expression during the cardiomyocyte differentiation increased to higher levels by day 6 where expression was maintained. *Collagen αIV* gene expression could be seen in undifferentiated ES cells, with enhanced levels recorded during days 6-11 of cardiomyocyte differentiation. It could be suggested that the detection of such markers is due to the cardiomyocyte culture containing a more diverse mixed population of all three germ layers, compared to the EBs treated with DMSO.

The expression of *GATA 1-3* are largely confined to the haematopoietic system (Tsai *et al.*, 1994, Pandolfi *et al.*, 1995, Fujiwara *et al.*, 1996, Ting *et al.*, 1996), whilst *GATA 4-6* are expressed predominantly in the heart and gut (Kelley *et al.*, 1993, Grepin *et al.*, 1994, Lavierriere *et al.*, 1994). *GATA-4* is a zinc finger protein found in pre-cardiac mesoderm. Knock out mice show defects in early heart tube formation (reviewed Fishman and Chien 1997).

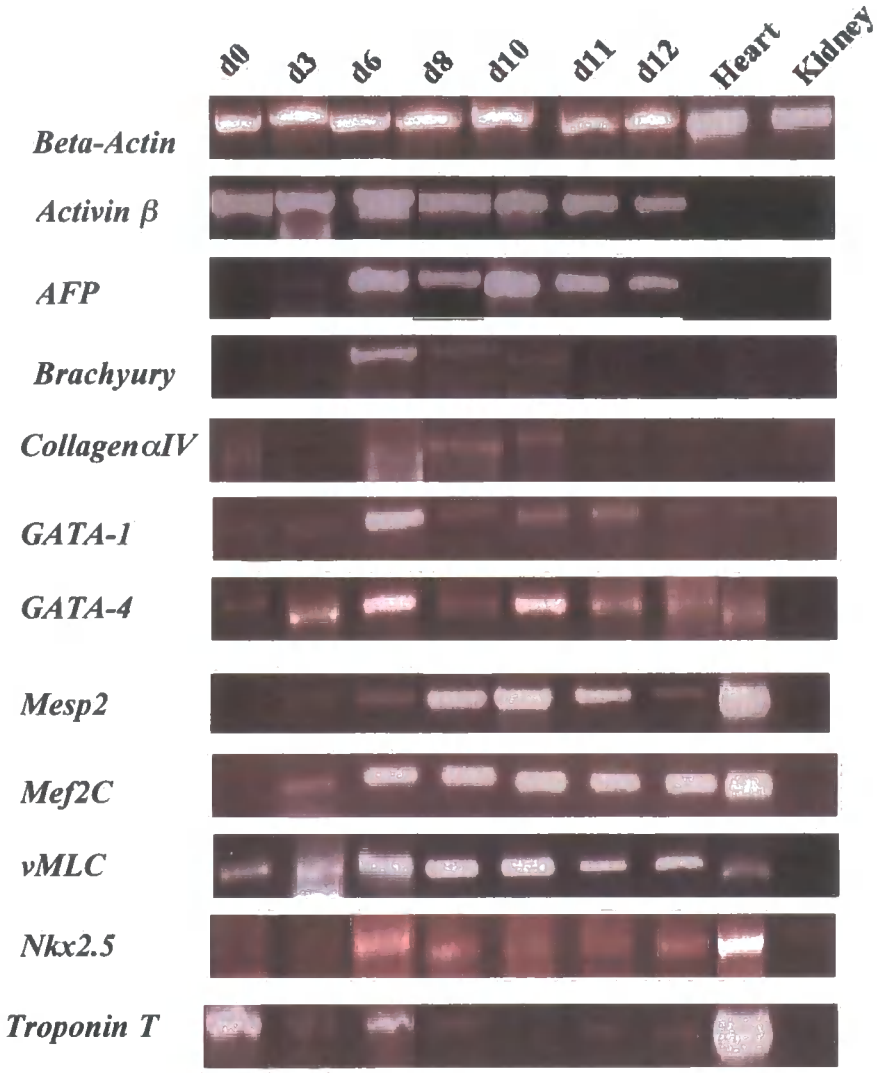


Figure 60 - **Gene expression in ES cells under cardiomyocyte differentiation.** Cell suspensions were collected from hanging drops (see 3.2.1) and LIF was removed at d0. The differentiating EBs were then plated onto gelatinised plates at d3.
Beta-actin was used to optimise equal amounts of cDNA for further RT-PCR analysis

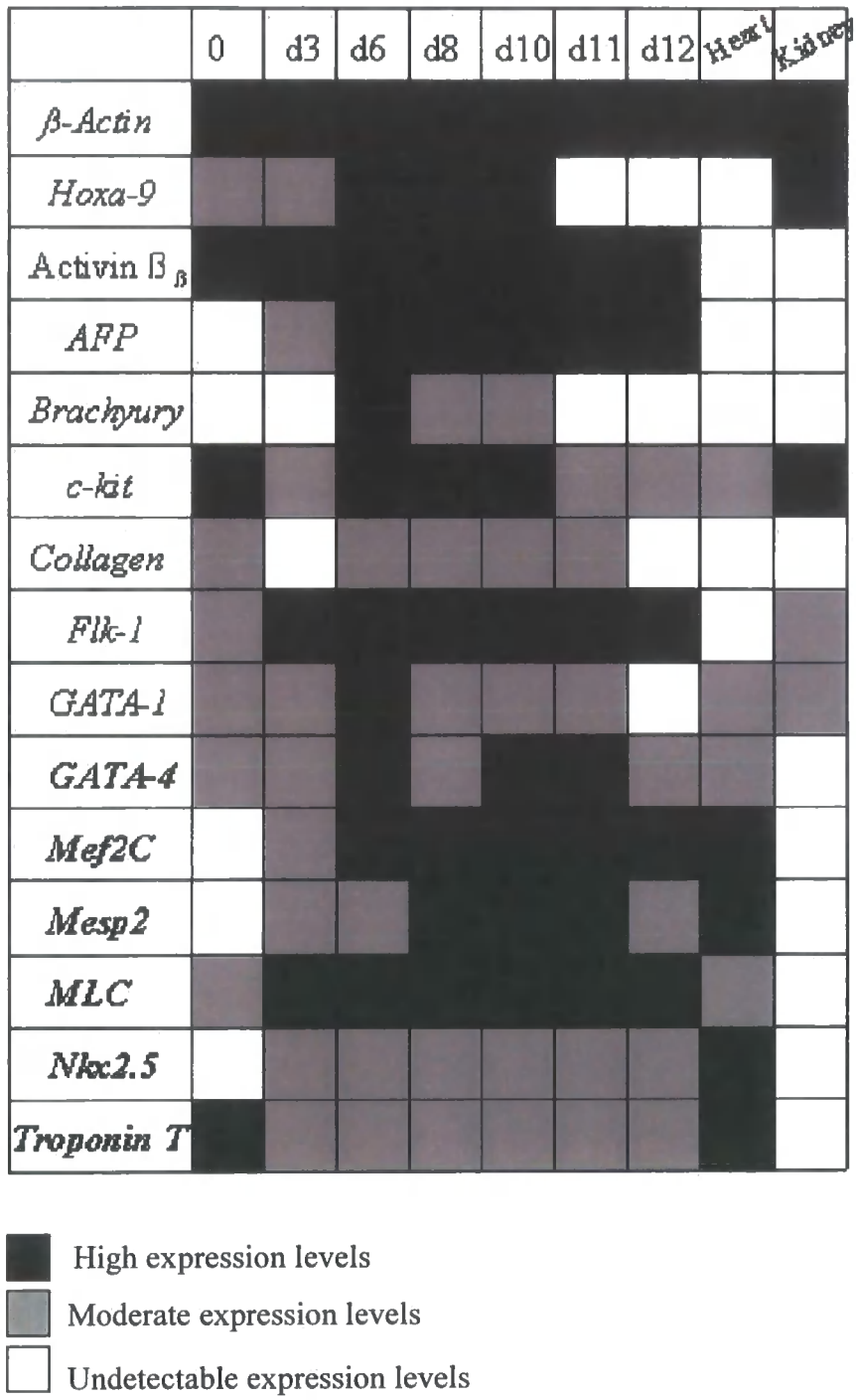


Figure 61 **Summary of gene expression during the cardiac differentiation of ES cells, shown in figure 63**

GATA-4 expression in this investigation has been shown during the differentiation process of cardiomyocytes derived from ES cells. As expected, expression was high and enhanced in populations of EBs showing rhythmic contractions.

Mesp2, like *GATA-4* is an early cardiac marker gene. This basic-helix-loop-helix protein is involved in the development of pre-somite mesoderm tissue and the migration of mesoderm cells (Saga *et al.*, 1997, 1998). Expression of *Mesp2* was found from day 3 of cardiomyocyte differentiation with increasing levels during the period when the rhythmic contractions increased.

It may be expected that being an early marker like *GATA-4* that expression levels of *Mesp2* would be higher at the earlier stages of differentiation. However, this was not a pure cardiomyocyte population, and therefore expression patterns may be different from a cardiomyocyte population alone. It is important to note that the expression levels of both genes appeared to be the greatest when the number of EBs showing rhythmic contractions peaked.

Mef2C is a transcription factor that belongs to the MADS family and primarily expressed in all muscle types (Edmondson *et al.*, 1994, Chambers *et al.*, 1994). It is also expressed in precardiac mesoderm, at E7.5, but mutants show that this gene is required from cardiac looping and right ventricle development as well as being expressed in a subset of cardiac cells (reviewed Lyons, 1996). RT-PCR reveals high levels of expression from day 6 in differentiating embryoid bodies.

vMLC is the earliest ventricular chamber specific gene expressed in the heart (O'Brien *et al.*, 1993). High levels of expression are seen throughout the differentiation process

including early stages, this too was consistent with *in vivo* data in this study, where expression levels increased from day 6 of differentiation.

Nkx2.5 or *Csx*, the *tinman* homologue is a murine specific expressed gene, initially found in cardiac progenitors and pharyngeal endoderm from E7.5 (Lints *et al.*, 1993). Knockout mice show defects in heart tube looping as a result of abnormal muscle growth (Newman *et al.*, 1998). It is therefore critical for early morphogenic determination pathways.

Expression levels appeared comparatively low in this current study as detected by RT-PCR, compared to other cardiac markers that are not as prominent as *Nkx2.5* during heart development, and this could be explained by the technical difficulties reached in optimising PCR conditions for *Nkx2.5*.

Troponin T is expressed in a restricted region of the developing heart. This protein is part of the regulatory system of contractions of skeletal and cardiac muscle (reviewed Ricchiuti *et al.*, 1997).

Having defined the time course of cellular differentiation and appearance of molecular lineage markers in both haematopoietic and cardiomyocyte differentiation models, and validated them with respect to other published work, the expression of *Hoxa-9* in these systems could be examined.

3.3.5 *Hoxa-9* expression during haematopoietic and cardiomyocyte differentiation of ES cells *in vitro*

Studies of *Hox* gene expression have often used the technique of southern blot hybridisation with gene-specific probes following RT-PCR (see 2.2.1). This both allows detection of low level transcripts and confirms the specific nature of the amplified cDNA.

Using this technique, two size variants of *Hoxa-9* were found to expressed in undifferentiated embryonic stem cells (figure 62). The presence of two bands is consistent with alternative splicing of *Hoxa-9* message, as reported previously (Fujimoto *et al.*, 1996).

In the model of ES cell differentiation designed to optimise haematopoietic commitment, *Hoxa-9* expression is shown in EBs cultured in media alone from day 4 onwards. However the presence of 1% DMSO appeared to advance the onset of detectable *Hoxa-9* expression to day 3 of differentiation. In this semi-quantitative assay, levels of expression of *Hoxa-9* appeared to be enhanced. By contrast, expression was down-regulated in the presence of RA. These levels of expression appear to be reasonably well correlated with the development of haematopoiesis as detected by CFU-A assay and RT-PCR of molecular markers. In this model of ES cell differentiation designed to optimise cardiomyocyte commitment, *Hoxa-9* expression tended to increase in expression up to ten days of differentiation. For comparison, the percentage of embryoid bodies showing rhythmic contractions is shown in figure 62.

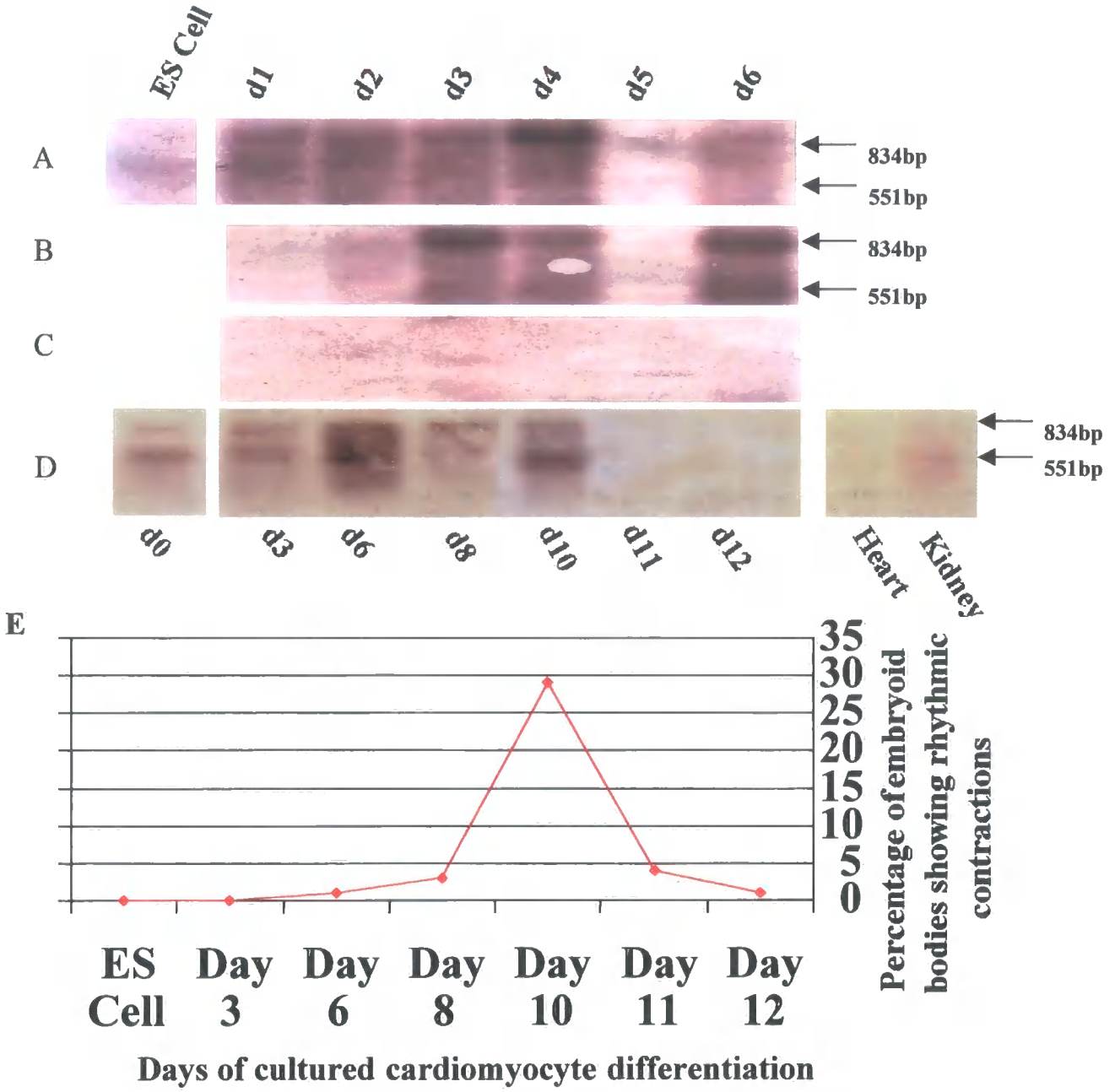


Figure 62 - **Southern blot analysis of *Hoxa-9* expression in differentiating embryonic stem cells**

differentiating in: media (A); media and 1% dimethyl sulphoxide (B); media and

10^{-8} M retinoic acid (C); or under cardiomyocyte differentiation conditions (D), using adult murine heart and kidney as controls.

E shows the number of embryoid bodies showing rhythmic contractions (figure 61) in relation to *Hoxa-9* expression

3.4 Discussion

In this chapter, an *in vitro* ES cell differentiation model has been used to model commitment to haematopoietic and cardiac lineages. The first issue to address was whether the model systems were competent to demonstrate these two lineage commitments.

3.4.1 Confirming the ES cell models

In vitro functional assays and RT-PCR analysis were used to demonstrate lineage commitment and gene expression throughout the two differentiation systems, confirming the validity of using the ES cell models further to study *Hoxa-9* expression.

3.4.2 Haematopoietic differentiation of ES cells

Consistent with previous reports, substantial haematopoietic commitment was detected at day 4, a result consistent with previous published reports (Hole *et al.*, 1996). The enhancement or repression of detectable haematopoietic commitment with DMSO or RA respectively was equally in broad agreement with other studies (Lako *et al.*, 2000). The high levels of expression of early haematopoietic markers such as *Flk-1, c-kit,* and more committed genes (*GATA-1)* in these treated EBs, were in agreement with previous reports and confirmed the derivation of haematopoietic progenitors (Shalaby *et al.*, 1995, Bernex *et al.*, 1996).

3.4.3 Cardiomyocyte differentiation of ES cells

The detection of genes expressed by cardiomyocytes (*Nkx2.5, MespC, Mef2C, vMLC, GATA-4, Troponin T)* in the differentiating ES cell systems was characteristic of the onset of cardiomyogensis. Coincident with the expression of these genes was the appearance of rhythmically beating embryoid bodies, which correlates with the emergence of functional cardiomyocytes (Wobus *et al.,* 1997). The RT-PCR data for pre-cardiac mesoderm markers, and lineage characteristic cardiac genes showed profiles of expression consistent with other reports of cardiac differentiation both *in vitro* and *in vivo* , for example the requirement for *Nkx2.5* throughout cardiogenesis (Lints *et al.,* 1993), and the requirement for *Mesp2* during early stages of cardiac development (Saga *et al.,* 1997, 1998), but more importantly they were expressed consistently in relation to each other, for example, early marker *GATA-4* expression was down regulated after day 10 of differentiation, whilst the chamber specific markers (*vMLC2*) was still being expressed at high levels at these later stages.

3.4.4 *Hoxa-9* expression in the ES cell models

The aim of using ES cell models, was to allow the study of *Hoxa-9* expression in *in vitro* haematopoietic and cardiac systems, in order to detect changes in *Hoxa-9* expression, and potentially to compare the expression with specific lineage marker genes.

Southern blot analysis showed changing levels of *Hoxa-9* expression in both developmental systems. In the haematopoietic system an enhanced *Hoxa-9* expression was found coincident with the emergence of detectable haematopoietic commitment

including more committed markers of *c-kit* (Bernex *et al.*, 1996), and *Flk-1* (Shalaby *et al.*, 1995).

In the cardiomyocyte differentiation system, *Hoxa-9* expression was again detected by RT-PCR in the early developmental stages where ES cell derived cardiomyocytes. Could be first detected Expression became undetectable at the same time during ES cell differentiation when 'twitching' was no longer noticeable.

3.4.5 The comparison of cardiogenesis *in vivo/vitro*

The formation of the functional heart is very complex and is restricted to a specific time course (see figures 38-44). It was difficult to compare our time course of the ES cells that were derived from E5 mouse blastocysts, to what would be occurring in the cardiac system of the embryonic mouse at that time.

However, some evidence has been demonstrated of morphological similarities by the expression of cardiac marker genes at selected time points during our differentiation process; for example *Mef2C* is required for cardiac looping and right ventricle development at around E10 of embryonic development, which respectively could be suggested to be around day 5 in the ES cell model, this expression is shown in figure 60.

However in this study a direct comparison between *in vitro* and *in vivo* systems has been largely avoided.

3.4.6 Potential roles for *Hoxa-9* in development of the heart and blood system

3.4.6.1 *Hoxa-9* in early heart development

On cardiomyocyte initiation, expression levels increased in the early stages of differentiation up until day 10 when comparatively asymmetry of the functional heart is prominent and the chambers have formed in the mouse embryonic heart. The expression patterns were similar to those of early determinating cardiac genes such as *Mesp2* in mesoderm migration to determine cell fate, *Nkx2.5* involved in cardiac looping, and *Troponin T* involved in contractile components. The patterning appeared not to be consistent with the later development morphological specific cardiac genes such as the ventricular chamber specific *vMLC* or *GATA-4* involved in heart tube formation, therefore suggesting a potential functional role for *Hoxa-9* in early cardiac development.

However, 'early' cardiac development is very complex and requires a large amount of interactions between signalling molecules to differentiate cardiac tissue (see figures 38-41), and defining or suggesting a functional role for *Hoxa-9* based on this expression data alone is difficult, and speculations can only be made by comparing *Hox-a9* expression to the RT-PCR data collected from the expression of the lineage markers, and correspond these patterns of expression to morphological changes that occur at those time points in the embryo.

3.4.6.2 *Hoxa-9* as a potential regulator of cell proliferation

It is important at this point to consider the conclusions drawn from the wholemount and *in situ* hybridisation data of a possible role for *Hoxa-9* as a regulator of cell proliferation in the developing atrial and ventricular heart cells. After all, *Hoxa-9* has played several

parts in other proliferation steps during development (Kessel *et al.*, 1991, Yamada *et al.*, 1994).

If there were a potential role for *Hoxa-9* during the proliferation of early cardiomyocytes, then it would be expected to see *Hoxa-9* expression throughout the time point when different structural features are being formed from dividing cells. Coincidentally, detectable levels of *Hoxa-9* expression using RT-PCR are identified from day 0 to day 10 of *in vitro* development, when comparatively *in vivo,* the formation of the four heart chambers has taken place.

It could also seem probable that *Hoxa-9* is fulfilling a similar proliferative role in the early stages of haematopoiesis, as there were high levels of *Hoxa*-9 expression and an increase in haematopoietic commitment around day four of *in vitro* differentiation, when *in vivo,* the number of haematopoietic progenitors begin to expand (reviewed Medvinsky and Dzierzak, 1996).

Hoxa-9 expression levels became undetectable by day 11. In the developing heart respectively, by this time in development the heart is fully formed and functioning, and much of the morphological and terminal differentiation steps of cardiac cells has taken place which may further suggest a functional role for *Hoxa-9* during heart formation, in that expression is no longer detected once formation is complete.

3.4.7 Conclusions

Evidence from *in situ* and wholemount hybridisation data of *Hoxa-9* expression in the developing embryonic heart has been further explored by using an *in vitro* ES cell

differentiation model to derive cardiac and haematopoietic tissue, to further investigate *Hoxa-9* function, during cardiogenesis and haematopoiesis.

The ES cell systems employed and demonstrated consistent and reproducible lineage commitment to cardiomyocyte and haematopoietic cell types. *Hoxa-9* expression was identified throughout early developmental stages of these two processes. Expression of *Hoxa-9* was correlated with the emergence of both cell types, and one interpretation of this data is that expression of this gene may play a role in lineage determination.

However identifying a functional role is difficult from expression data alone, and therefore this study requires the application of functional assays, including gene overexpression, to the *in vitro* ES cell model in order to analyse any phenotypic changes.

Chapter Four – Gene overexpression

4.1 Introduction

In situ and wholemount hybridisation studies along with data collected from *in vitro* ES cell differentiation models have outlined a possible role for *Hoxa-9* during cardiogenesis, and detected expression under conditions where early haematopoietic progenitors were found.

In order to identify a functional role for *Hoxa-9* during these developmental processes, genomic manipulation studies were considered. One approach to examining the functionality of such genes is to study the effect of over- or ectopic expression in somatic or ES cells. Previous findings using these approaches to understand *Hox* gene function will be discussed in this chapter.

4.1.1 Retroviral expression of *Hox* genes in somatic cells: *in vitro* studies

Members of the *Hox* gene family have previously shown to be expressed during haematopoietic differentiation (see figure 12), the expression is, but not exclusively confined to primitive cells (Giampaolo *et al.*, 1994, Sauvageau *et al.*, 1994). Retroviral overexpression studies with *HOX*B3, *HOX*B4 *HOXA9* and *HOX*A10 have all utilised the transduction of primary murine bone marrow cells to study the functional effects on this expression on lineage-specific pathways of the haematopoietic system (Helgason *et al.*, 1996, Nakamura *et al.*, 1996a, Sauvageau *et al.*, 1997, Thorsteinsdottir *et al.*, 1997, 2002, Kroon *et al.*, 1998, Bjornsson *et al.*, 2001, Buske *et al.*, 2001).

Retroviral transduction has successfully highlighted a role for *HOXB3* in proliferation and differentiation of both early myeloid and lymphoid development shown in recipients

of transduced bone marrow cells that demonstrated a decrease in the number of committed T-cells and an increase in thymocytes (Sauvageau *et al.*, 1997).

The overexpression effects of *HOX*B4 on haematopoietic lineage commitment and differentiation have been more closely studied. *In situ* hybridisation studies with murine embryos revealed expression in derivatives of the mesoderm (Graham *et al.*, 1988, Holland *et al.*, 1988), which ultimately give rise to cells in the haematopoietic system and *HOX*B4 mRNA has also been observed in the foetal liver, a major site of embryonic haematopoiesis (Graham *et al.*, 1988). Lethally irradiated recipients of *HOX*B4-transduced bone marrow cells demonstrated an expansion in primitive haematopoietic cells (CD34+), as well as an increase in haematopoietic stem cell (HSC) regeneration which was sustained for up to one year, without haematopoietic differentiation being altered in definitive cell types (Sauvageau *et al.*, 1995, Thorsteinsdottir *et al.*. 1999, Antonchuk *et al.*, 2001), suggesting a role for *HOXB4* during early progenitor stages of haematopoiesis.

There is evidence to suggest that *HOXA10* overexpression in bone marrow cells increases the proliferation of early progenitor cells and can lead to the development of myeloid leukaemia (Thorsteinsdottir *et al.*, 1997).

Another further overexpression study investigated more closely the role of *HOXA10* in normal primitive haematopoietic progenitors. Buske (Buske al., 2001) overexpressed *HOXA10* in human purified $CD34^+$ haematopoietic progenitor cells derived from human cord blood or foetal liver sources, and the impact of the aberrant gene expression on differentiation and proliferation *in vitro* and *in vivo* was analysed. Data from this study provided evidence that the balanced expression of *HOXA10* is important for normal

human haematopoietic development and that aberrant expression contributes to impaired myeloid differentiation and increased proliferation of human progenitor cells (Buske *et al.*, 2001).

As well as studying the effects of *Hox* gene overexpression in cell lines, retroviral gene transfer has been used to investigate the phenotypes of ectopic expression in whole somatic cells *in vivo*.

4.1.2 Retroviral expression of *Hox* genes in somatic cells; *in vivo* studies

Hoxa-9 overexpression in bone marrow is associated with the onset of AML within three to ten months (Kroon *et al.*, 1998, Thorsteinsdottir *et al.*, 1999). To gain a further insight into how *Hoxa-9* affects haematopoietic development at the pre-leukaemic stage, Thorsteinsdottir (Thorsteinsdottir *et al.*, 2002), retrovirally engineered its overexpression in HSC and generated bone marrow chimeras, and also overexpressed *Hoxa-9* in lymphoid cells of transgenic mice. The results showed that *Hoxa-9* had the potential to expand HSCs when overexpressed in a bone marrow transplantation model. The involvement of *Hoxa-9* in chromosomal translocation in human leukaemia together with the evidence that mouse HSCs lacking *Hoxa-9* compete poorly in a bone marrow transplantation model may suggest a role as a regulator of stem cell function (Lawrence *et al.*, 1998).

Hoxa-9 overexpression in lymphoid cells, compared with controls, partially blocked B lymphopoiesis. Interestingly, despite high levels of *Hoxa-9* expression in T and B lymphoid lineages, none of the *Hoxa-9* transgenic mice developed lymphoid malignancies within the eighteen-month observation period in this study. This was in

contrast to previous reports which showed the onset of AML within three to ten months (Kroon *et al.*, 1998, Thorsteinsdottir *et al.*, 1999).

Bjornsson (Bjornsson *et al.*, 2001), generated transgenic mice with a conditional reporter vector. The transgenic mouse model contained the *HOXA10* gene controlled by a tetracycline-responsive element and a minimal promoter. A retroviral vector containing tetracycline transactivator gene was used to induce expression of *HOXA10* in bone marrow cells from the transgenic mice. *HOXA10* induction led to the formation of haematopoietic colonies *in vitro* containing blast-like cells and megakaryocytes. There was also a noticeable increase in the proliferation of primitive haematopoietic progenitors (CFU-S) (Bjornsson *et al.*, 2001).

Previous *HOX*A10 overexpression studies showed that this inducible model was sufficient to give rise to AML in recipient mice after a long latency period (Thorsteinsdottir *et al.*, 1997). Björnsson and Buske's studies (discussed in 4.1.1) suggest that this was not a primary event, but that it requires a secondary mutation to take place. This argument was supported by the latency period before AML onset. There may be a need for the additional overexpression of *HOX*A10 cofactors in order to increase the levels of *HOX*A10 expression to the desired threshold for AML onset.

An alternative approach to investigate early differentiation is to examine overexpression in ES cells.

4.1.3 Overexpression of transgenes in ES cells

The *HOX*B4 overexpression study by Helgason (Helgason *et al.*, 1996) contributes to the data using ES cells as targets for *Hox* gene overexpression. In this study ES cells were

retroviral-transduced with *HOXB4* to study the effects on haematopoiesis. The overexpression significantly enhanced the number of haematopoietic progenitors detected at various times in the ES differentiation cultures. There was enhanced early proliferation of erythroid and myeloid progenitors, without a reduction in granulocyte or macrophage yield. Therefore suggesting a more primitive proliferative role for *HOX*B4 in a lineage-specific manner (Helgason *et al.*, 1996).

Jackson (Jackson and Baird *et al.*, 2002) used episomal vectors to study the effects of both loss of function and overexpression of a novel homeobox gene *Ehox* in *in vitro* ES cell differentiation. Data suggested a role for *Ehox* in the regulation of early stages of murine ES cell differentiation that were not restricted to haematopoiesis.

4.1.4 *Hoxa-9* overexpression and acute myeloid leukaemia:

One investigation in studying the functions of *Hoxa-9* in normal biology, is to look at the close association with acute myeloid leukaemia.

4.1.4.1 *Hoxa-9* and *Meis1* associations;

Myeloid leukaemia was induced in leukaemic cell line mice (BXH-2) by the insertional mutation of cellular proto-oncogenes or tumour suppressor genes. Following proviral integration into the genome of BXH-2 mice (leukaemic mice), there was correlation to high expression levels of *Hoxa-9* or *Hoxa-7*, associated with *Meis1*. The proviral activation of each of the *Hox* genes was strongly correlated with the proviral activation of *Meis1* suggesting that they may cooperate to produce myeloid leukaemia formation. This

was early genetic evidence that *Meis1*, a Pbx associated protein, cooperates with *Hox* genes in the onset of AML (Moskow *et al.*, 1995, Nakamura *et al.*, 1996a).

Further studies have shown that the retroviral mediated overexpression of *Hoxa-9, Pbx1* or *Meis1* alone are incapable of transforming primary bone marrow cells, nor the combination of *Hoxa-9* and *Pbx1*. Only the overexpression of *Hoxa-9* and *Meis1a* simultaneously induced growth factor dependent AML in < 3 months when transplanted into syngenic mice (Kroon *et al.*, 1998).

Overexpression of *Hoxa-9* and *Meis1* in mouse primary bone marrow cells lead to AML (Kroon *et al.*, 1998). The protein products of these genes can form heterodimeric complexes that show DNA binding, suggesting that both form similar complexes with *PBX* proteins, so which is their preferred binding partner in inducing leukaemia? Three-way DNA site selection experiments were set up in order to ascertain which proteins would preferentially bind (*Hoxa-7, Hoxa-9, Meis1*). In every experiment, a similar *HOXA9-PBX* complex resulted (Shen *et al.*, 1999). Electrophoretic mobility shift assay showed a trimeric complex also formed in the presence of *MEIS1*, suggesting that *MEIS1* had enhanced the *in vitro HOX*A9-*PBX* protein complex formation in the absence of DNA through a specific recognition site (Shen *et al.*, 1999). It was of interest that coexpression of *HOX*A9 and *PBX1* does not elucidate leukaemic transformation (Kroon *et al.*, 1998), and that it is the combined gene expression with *MEIS1*, it's preferred binding partner that results in disease.

This may be due to the overexpression levels of *MEIS1* and *HOX*A9 that changed the normal levels of *PBX*, leading to the formation of different DNA-protein complexes, or the high levels of *PBX* in the myeloid cells of previous experiment may have reflected

levels of this protein sufficient for interaction with exogenous *HOX*A9 and *MEIS1* (Kroon *et al.*, 1998).

Constitutive overexpression of *Hoxa-9* in primary bone marrow evoked a block in promyelocyte differentiation in the absence of *Meis* gene expression, through a mechanism that requires DNA binding, but not at the same binding consensus as *Pbx* or *Meis* (Calvo *et al.*, 2000). This suggested that *Hoxa-9* immortalisation is independent of *Meis* gene expression(Calvo *et al.*, 2000).

As well as the association with *Meis1, Hoxa-9* also binds other proteins.

4.1.4.2 *Hox* and *PBX* interactions

A number of studies have demonstrated that *HOX* proteins collaborate in the in vitro DNA binding with members of the TALE (three-amino-acid loop extension) subclass of homeodomain-containing proteins comprising PBC (mammalian *PBX* and Drosophila extradenticle (exd)) and *MEIS* (mammalian *MEIS* and *PREP1* and Drosophila HTH proteins) families (Mann 1995). Interactions with *PBX* provide both increased DNA binding affinity for many *HOX* proteins (Shen *et al.*, 1997, 1999) and substantial DNA selectivity for *HOX* proteins from paralogue groups 1 to 10 (Chang *et al.*, 1996). *HOX* proteins from paralogue groups 1 to 8 require tryptophan within a conserved YPWM motif in order to interact with *PBX,* while paralogue groups 9 and 10 proteins use a tryptophan in a conserved ANW sequence. The remaining paralogue groups from 9 to 13 form a cooperative DNA binding complex with another homeodomain protein, *MEIS1* (Shen *et al.*, 1997). Therefore *HOX* proteins from paralogue groups 9 to 10, possess the unique capacity to form DNA binding complexes with either *PBX* or *MEIS* on distinct

DNA target sequences as they are located at the transition between the YPWM-containing proteins and the ANW-containing proteins. There are also studies that have identified binding complexes showing PBX-MEIS binding sites (Chang *et al.*, 1997, Knoepfler *et al.*, 1997).

4.1.5 Aims

Three novel approaches to analysing the effects of *Hox* gene overexpression have been discussed. It has been shown that retroviral transduction using *Hox* genes have been previously successful in determining a function role in haematopoiesis, in both *in vitro* somatic cells and *in vivo* animal studies. The application of two expression vector vehicles – retroviral and episomal have also been shown to be successful in overexpressing candidate genes in ES cell models. Previous *Hoxa-9* overexpression studies, with one exception (Thorsteinsdottir *et al.*, 2002), have explored the onset of AML following *Hoxa-9* transduction in bone marrow transplantation models. However, unlike the intentions of the studies in this thesis, these reports used the transplantation models to additionally analyse the expression levels of *Hoxa-9* interaction molecules, to evaluate the leukemogenic mechanisms. It is the intention in this thesis to expand the findings observed in the atrial and ventricular of the embryonic heart (figures 18-32) and identify a function for *Hoxa-9* in cardiac development using retroviral mediated overexpression of *Hoxa-9* in *in vitro* ES cells.

4.2 Materials and methods

All cells were maintained in 10ml/25cm^2 flask in the described media, unless otherwise stated.

4.2.1 Cell maintenance

C0S-7 cell maintenance

The COS-7 cell line was purchased from American Type Culture Collection (ATCC), and maintained in 50% (v/v) HAMS F12 (GibcoBRL #27165-029), 40% v/v GMEM (GibcoBRL #21710-025), 10% foetal calf serum in a humidified 5% CO_2 atmosphere at 37°C.

3T3 cell maintenance

The 3T3 cell line was a kind gift from Prof. Chris Hutchinson at the University of Durham. The cells were passaged when confluent and maintained in DMEM (GibcoBRL #11966-025), 10% (v/v) heat inactivated foetal calf serum, 1% (v/v) 5000u/ml Penicillin/streptomyocin in a humidified 5% CO_2 atmosphere at 37°C.

Phoenix packaging cell maintenance

The Phoenix cell line were purchased from ATCC and maintained as necessary in Glutamax-1 (GibcoBRL #31966-021), 10% (v/v) heat inactivated foetal calf serum, 1% (v/v) 5000u/ml penicillin/streptomyocin in a humidified 5% CO_2 atmosphere at 37°C.

4.2.2 Vector construction

The LX-GFP retroviral vector (Miller and Rossman, 1989) was a kind gift from Dr. L Fairbairn at the University of Manchester. This retroviral vector allows stable gene expression in the host cell. DNA was ligated into this vector following the appropriate treatment of the vector with digestive enzymes. Briefly, for LX-GFP retrovirus vector, 10 µg of vector DNA was incubated with 5 µl of the appropriate digestive enzyme buffer (10mM Tris-HCl, 300mM KCL, 5mM $MgCl_2$, 0.1mM EDTA,
1 mM DTT, 0.5 mg/ml BSA, 50% Glycerol), 40 units of BamHI enzyme (Promega), and sterile water up to the total volume of 50 µl. This solution was then incubated at 37°C for 3 hours, and then a sample was electrophoresed on a 1.8% agarose gel (as described in *2.2.1*) to confirm the linearisation.

The DNA to be inserted into the LX-GFP vector was treated as before (2.2.6), in that PCR primers were designed to add the appropriate BamHI sequence to the beginning of the 3' sequence of the *Hoxa-9* insert, to allow for efficient ligation in the LX-GFP vector.

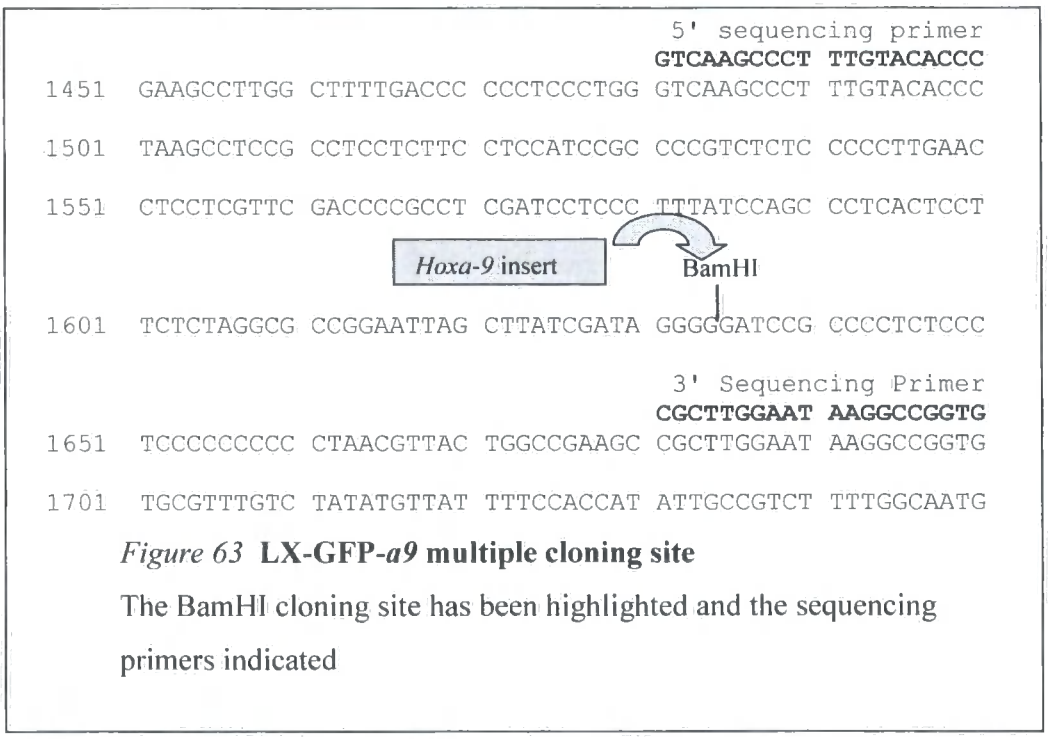


Figure 63 **LX-GFP-*a9* multiple cloning site**
The BamHI cloning site has been highlighted and the sequencing primers indicated

4.2.3 Vector dephosphorylation

To prevent re-ligation of the linearised cloning vehicle, phosphate groups from both termini were removed, using alkaline phosphatase. 1 µg of linearised vector made up to 20 µl with sterile water, was added to 2 µl (1 unit/µl) calf intestinal alkaline phosphatase (CIAP) and 2.5 µl 10x CIAP buffer (0.5 M Tris-HCl, 10 mM $MgCl_2$, 1 mM $ZnCl_2$, 10 mM spermidine). The solution was incubated at 37°C for thirty minutes, before a further 2 µl of CIAP was added and incubated for a further 30 minutes at 37°C.

The phosphorylated DNA was purified by extraction with an equal volume of phenol:chloroform (50:50 v/v), and centrifuged at 4000*g* (Sigma, 2-15) for ten minutes. The upper phase was collected, and added to 3x volume of 100% ethanol, and 1/10 volume of 3 M sodium acetate. The solution was incubated at -20°C for fifteen minutes, before being centrifuged at 4000*g* (Sigma, 2-15) for ten minutes. The pellet was collected and washed in 70% (v/v) ethanol, before being air dried and resuspended in 10 µl of TE buffer (see appendix A.20).

4.2.4 Vector-DNA ligation

Following the removal of the phosphate ends, the vector was ligated by adding 1 µg of dephosphorylated vector DNA and 1 µg of desired insert DNA (as described in 4.2.4) to 1 µl (3 u/µl) T4 DNA Ligase, 1µl 10x T4 DNA Ligase buffer, and sterile water to a final volume of 10 µl. This solution was incubated overnight, and run a sample on agarose gel to confirm ligation (2.2.1).

4.2.5 Vector transformation into bacterial cells

The cloning protocols used for each vector were slightly different. The LX-GFP retroviral generation requires the insertion of DNA into the vector via a digestion, dephosphorylation and ligation pathway, as described previous 4.2.2-5), whereas the CT-TOPO-GFP acquires the insert as described in 2.2.6.

The following transformation protocols for each of the vectors are similar.

2 µl of the LX-GFP vector-DNA mix or the CT-TOPO-GFP-DNA mix was added to MAX efficiency Stbl2 (Invitrogen, Paisley, Scotland #10268-019) and one shot TOP10 *E.coli* (Invitrogen, #C4040-10) competent cells respectively, and incubated on ice for thirty minutes. 500 µl of SOC medium (Invitrogen) was added and agitated on a platform shaker for thirty minutes at 37°C. Following incubation, 50-100 µl of mix was plated out onto LB agar plates (see appendix A.9) containing 50 µg/ml ampicillin, and incubated overnight at 37°C. Colonies that developed were analysed by RT-PCR to confirm inserts (*2.2*).

4.2.6 DNA extraction from bacterial cells

Clones containing the required inserts were inoculated into 10ml LB broth (see appendix A.10) containing 50 µg/ml ampicillin and incubated overnight at 37°C on an orbital shaker. Plasmid DNA was extracted from the bacterial cells using Promega miniprep kit, following the manufacturer's instructions. Briefly, the cultures were centrifuged at 4000*g* (Sigma 2-15) for ten minutes, and the pellet of bacterial cells were resuspended in 250 µl resuspension buffer and transferred to a microcentrifuge tube containing 250 µl of lysing buffer. 350 µl of neutralisation buffer was added to this mix, before the solution was centrifuged at 4000*g* (Sigma 2-15) for ten minutes and the supernatant transferred to the

QIAprep column. After the supernatant had been applied, the column was centrifuged at 4000*g* (Sigma, 2-15) for 60 seconds, and the flow-through discarded. 0.5 ml of wash buffer was added into the column and a further centrifugation step was carried out as before. The spin column was washed further, the flow-through discarded and 50 µl TE buffer (A.20) was applied to the column, following an identical centrifugation step as before, the eluted DNA was stored at –20°C.

The vector DNA was then sequenced using DNA sequence specific forward and reverse sequencing primers particular for that vector, as shown in B.1-5.

4.2.7 Calcium phosphate transfection

Vector constructs were incorporated into cultured cells in some experiments by using a calcium phosphate transfection technique. This procedure was carried out using the calcium transfection kit (Sigma, Poole, Dorset, England), following the manufacturers instructions. Briefly, COS-7 (CT-TOPO-GFP) or Phoenix cells (LX-GFP) were plated out in sterile 6 well plates at a density of $2x10^5$ cells per well, 24 hours prior to transfection. Under sterile conditions, 5 µg (in 10 µl sterile water) of purified DNA (4.2.5) was mixed with 6 µl of calcium phosphate, made up to 50 µl with sterile water that was added drop wise into 60 µl of HEPES that was being vortexed (see appendix A.5) (HATI Rotamixer). This was left at room temperature for ten minutes, before being transferred to the tissue culture plates containing the pre-plated cells, in 5ml of fresh media. These were incubated at 37°C at 5% CO_2 for twelve hours when a full media change was carried out. After a total of forty-eight hours following transfection, the cells were analysed for GFP fluorescence by flow cytometry. Each set of transfection

experiments using CT-TOPO-GFP(*a9*) was carried out twice to generate two independent sets of data in each cell type.

4.2.8 Electroporation

CGR8 ES cells were grown in the presence of LIF, and harvested (3.1). These cells were collected and centrifuged at 1000*g* for five minutes (Sigma, 2-15). The supernatant was removed and $3x10^6$ cells were resuspended in 1ml ice cold PBS (see appendix A.15).

10 µg of purified linearised vector DNA (see 4.2.2) was added to the cell suspension and incubated for five minutes. The 1 ml mix was placed into pre-chilled electroporation cuvettes and shocked at 240 volts (BTX electronic genetics – ECM395 electrocell manipulator). The cells were than incubated on ice for ten minutes before being added to 10 ml fresh CGR8 media, and transferred to culture flasks. Twenty-four hours following electroporation, the cells were put under antibiotic selection. This selection was achieved 400 µg/mL of G418 (a neomycin analogue). Previous work had shown that for these cells lines, this concentration of antibiotic gave optimal selective properties (data not shown).

4.2.9 Flow cytometry

This facility was used at The University of Durham, operated by Ms. Trudy Horton (Beckman Coulter Diagnostics, using EXPO Cytometer software). For each set of flow cytometry data, 10,000 events were analysed.

Flow cytometry is carried out in addition to using fluorescent microscopy to detect expression if GFP, as lower levels of expression can be detected using flow cytometry then microscopy, which relies on the human eye.

4.2.10 Retroviral transduction of cultured cells

Following the calcium phosphate transfection of the LX-GFP vector containing the insert (see 4.2.7) into the packaging cell lines (Phoenix), the cells were further incubated in tissue culture flasks containing fresh media, until confluent. When confluent, the media was replaced with 5 ml of medium and cells were incubated for a further forty-eight hours. The supernatant was then removed and centrifuged for five minutes at 1000*g* (Sigma, 2-15), to remove any remaining packaging cells. The supernatant was then filtered (0.45 μm), and collected in a sterile universal tube.

2ml of supernatant was added to 3ml of fresh media on top of $2x10^5$ previously grown 3T3 or CGR8 cells giving a total of 5ml of media in the tissue culture flask, 4 μg/ml of polybrene (GibcoBRL) was added to aid the transduction process. The media was changed and the presence of GFP was analysed by flowcytometry after forty-eight hours. Each set of transduction experiments using LX-GFP(*a9*) was carried out twice to generate two independent sets of data in each cell type.

4.2.11 Genomic analysis of cultured cells

Genomic DNA was extracted using the Promega, (Southampton, Hampshire, England) Wizard genomic DNA purification kit. Briefly, the cells were harvested, washed in 1 ml PBS (see appendix A.15) and collected in a 1.5ml eppendorf tube. 600 μl of nuclei lysis solution was added, and the solution was agitated using a pipette until no cell clumps remained. 3 μl of RNase solution was added to the nuclear lysate and the tube mixed by inverting 3-4 times before being incubated at 37°C for fifteen minutes. 200 μl of protein

precipitation solution was added and vortexed at high speed for twenty seconds (HATI rotamixer). The sample was then spun at 13,000*g* (Sigma 2-15) for four minutes.

The supernatant was collected and DNA precipitated with 600 µl of propan-2-ol. The solution was gently mixed by inversion until threads of DNA appeared. Precipitated DNA was pelleted by centrifugation for one minute at 4000*g* (Sigma, 2-15). The supernatant was carefully removed and discarded and the remaining pellet was washed in 70% (v/v) ethanol. Once the ethanol was removed, the pellet was air-dried and resuspended in 100 µl rehydration solution.

4.3 Results

Evidence presented in this thesis from *in situ* and wholemount data suggested that *Hoxa-9* is expressed in a spatial (in the heart atria and ventricle), and temporal (at E9.5 and 10.5) manner in developing cardiomyocytes within the embryonic heart.

The expression of *Hoxa-9* in differentiating ES cells suggested that *Hoxa-9* is expressed during early stages of haematopoietic commitment.

In order to answer the question as to why *Hoxa-9* is being expressed at specific sites during these critical times in development, this study must further investigate a functional role. To identify a functional role for *Hoxa-9* during these developmental processes, genomic manipulation techniques were adapted to overexpress *Hoxa-9* in ES cells.

The overexpression of genes in host cells can be achieved in a temporary (transient) or permanent (stable) manner. Transient transfection allows a rapid high level of expression, but then the expression disappears as the cells fail to stably integrate the DNA, and hence they lyse. Stable transfection obtains moderate levels of expression over a continuous period of time in culture following stable integration of the expression vector in the host cell genome.

4.3.1 Stable transfection of CT-TOPO-GFP in embryonic stem cells:

4.3.1.1 Electroporation of CT-TOPO-GFP in embryonic stem cells;

The full length coding sequence of *Hoxa-9* cloned in the CT-TOPO-GFP reporter vector (see figure 13, page 46) was electroporated into ES cells as described in 4.2.8. Following transfection, the CGR8 ES cells were cultured (see 3.2.1) for forty-eight hours before

GFP expression was analysed using flow cytometry and fluorescent microscopy, shown in figure 64.

For each set of overexpression experiments in this study, a control transfection was carried out using an 'empty vector' to account for any false positive data following *Hoxa-9* transfections, and a mock transfection containing no DNA, shown in figures 64-70 as 'negative'. This latter cell population was used to set up logical gate margins for the flow cytometry data.

4.3.1.2 GFP expression analysis;

The CGR8 ES cells transfected with the empty vector (CT-TOPO-GFP), showed an expected high level of GFP expression seen using fluorescent microscopy (figure 64), compared to mock transfected cells where GFP expression was not visible.

These GFP expression findings using fluorescent microscopy were confirmed using flow cytometry. The empty vector transfected cells generated a high peak of fluorescence along the FL-1 y-axis (figure 64), demonstrating that 98.6% of transfected cells expressed GFP within the gates (figure 71). This result confirmed the efficiency of using electroporation to transfect ES cells with CT-TOPO-GFP constructs. However, flow cytometry confirmed that the ES cells transfected with the *Hoxa-9* reporter gene (CT-TOPO-GFP-*a9*) were not overexpressing GFP and therefore *Hoxa-9*, confirmed using PCR.

One possibility is that transcription of *Hoxa-9/GFP* may take longer to be detected, and therefore cells were analysed using the same methods following six further passages; and similar undetectable levels were obtained (figure 65).

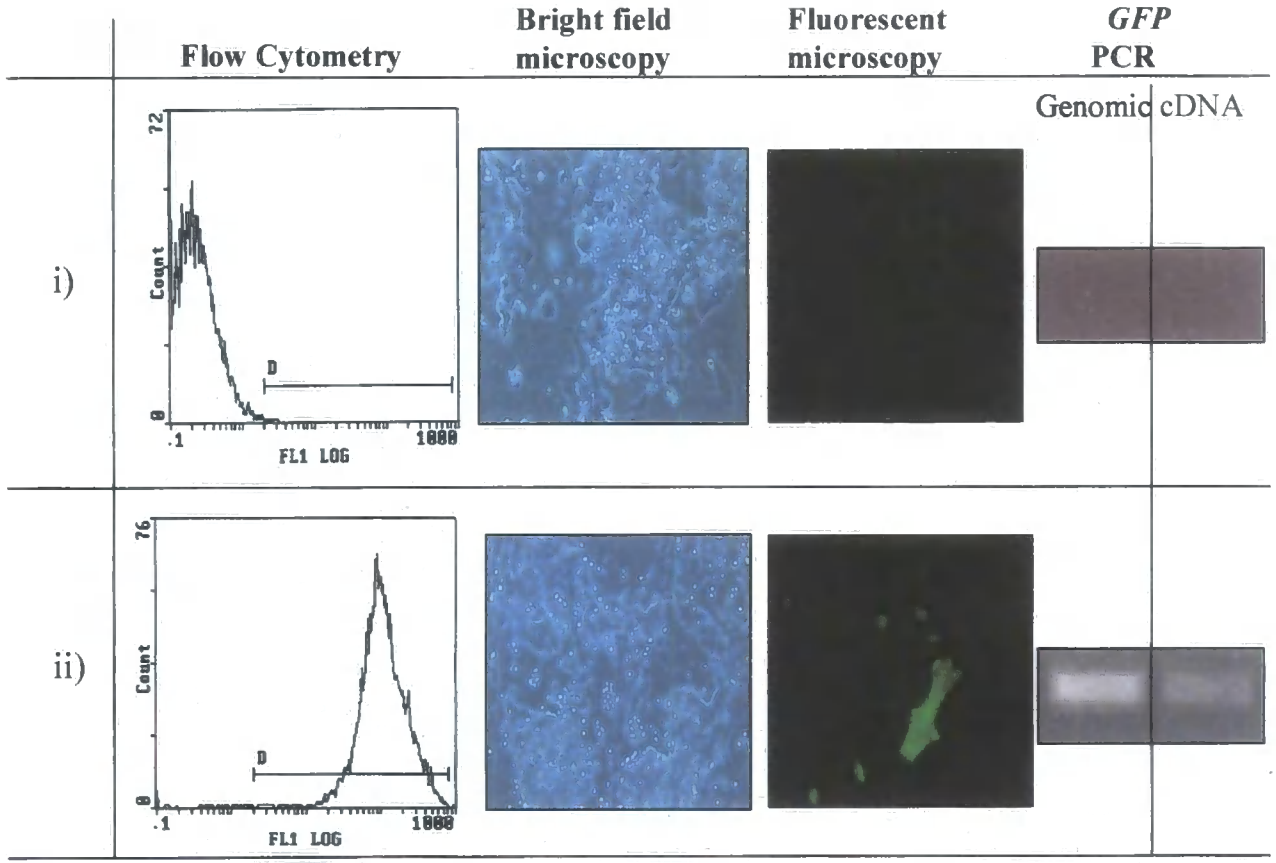


Figure 64 – **Stable transfection of CGR8 cells with heterologous promoter expression vector**
Flow cytometry, microscopy and PCR data from CGR8 ES cells transfected stably with: i) No DNA ii) CT-TOPO-GFP empty vector

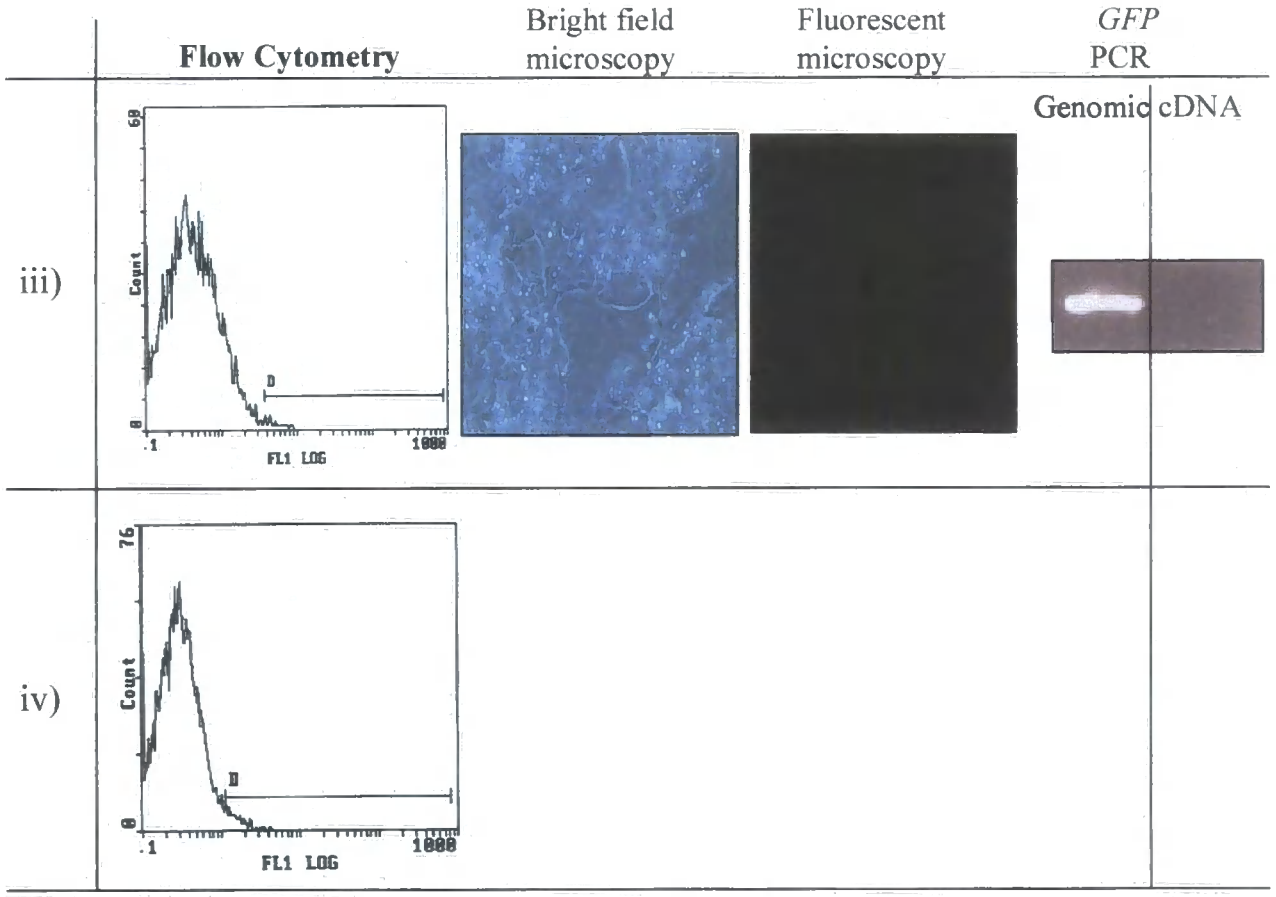


Figure 65 – **Stable transfection of CGR8 cells with heterologous promoter expression vector**
Flow cytometry, microscopy and PCR data from CGR8 ES cells transfected stably with iii) CT-TOPO-GFP-*a9* after forty-eight hours iv) CT-TOPO-GFP-*a9* (passage 7)

In order to examine whether CT-TOPO-GFP-*a9* had been incorporated into the genome, genomic DNA and RNA (treated with DNase to exclude genomic contamination) was extracted from the cells forty- eight hours after transfection and at passage 7 to analyse incorporation and expression. PCR analysis (described 2.2.1) was carried out to detect the presence of CT-TOPO-GFP-*a9* construct by using primers specific for the *Hoxa-9* sequence (shown in B.1).

The genomic DNA data showed incorporation of CT-TOPO-GFP-*a9,* but consistent with the poorly observed GFP expression, *Hoxa-9* mRNA was undetectable in RT-cDNA (figures 64-70). These cells, although not showing evidence of transgene transcription, were further expanded under antibiotic selection (see 4.2.8). These cells grew very slowly in comparison to controls (data not shown), and GFP could not be visualised using microscopy at this stage. The poor growth of the ES cells transfected with CT-TOPO-GFP-*a9* compromised further analysis using flow cytometry and RT-PCR.

As stable expression (random integration of the cloning vector into the host cell genome) of *Hoxa-9* could not be obtained in CGR8 cells, it followed to test CT-TOPO-GFP-*a9* transfection using transient expression methods. To confirm the initial efficiency of this method, transient expression was carried out in COS-7 cells.

4.3.2 Transient transfection of CT-TOPO-GFP:

4.3.2.1 Transient transfection of CT-TOPO-GFP in COS-7 cells;

COS-7 cells were transientally transfected as before with the empty vector, the mock transfection and the CT-TOPO-GFP-*a9.*

4.3.2.2 GFP expression analysis;

Forty-eight hours post calcium phosphate transfection with the CT-TOPO-GFP constructs, GFP expression was examined using flow cytometry in the COS-7 cells. As seen using fluorescent microscopy, COS-7 cells transfected with CT-TOPO-GFP-*a9* and the empty vector (CT-TOPO-GFP) showed high levels of GFP expression (6.70% and 31.90% respectively) within the gate margins (figures 66, 71). As can be seen in figure 66, levels of fluorescence in both empty vector and CT-TOPO-GFP-*a9* confirmed transfection and vector efficiency and suggested vector integration and consequently *Hoxa-9* expression. To be consistent, PCR analysis was carried out to confirm these COS-7 cell findings, and figure 66 demonstrates both genomic integration and *Hoxa-9* transcription.

4.3.2.3 Transient expression using the CT-TOPO-GFP construct in ES cells;

This transient method of CT-TOPO-GFP-*a9* in COS-7 cells confirmed the efficiency of the transfection method and the vector, and therefore the procedure was repeated using CGR8 ES cells, in place of the COS-7 cells.

4.3.2.4 GFP expression analysis;

Forty-eight hours after calcium phosphate transfection in ES cells, GFP expression could be seen using fluorescent microscopy, in ES cells transfected with CT-TOPO-GFP-*a9* compared to controls. The fluorescence levels were not as intense as those cells transfected with the empty vector, and this was confirmed using flow cytometry (figure 70). 21% of cells transfected with the empty vector showed fluorescence within the gate

margins along the FL-1 scale, whilst only 2.99% showed fluorescence following transfection with CT-TOPO-GFP-*a9* (figure 74). Compared with the control and 1.61% of cells showing fluorescence in mock transfected cells, CT-TOPO-GFP-*a9* transfected ES cells showed a comparatively lower level of GFP and hence *Hoxa-9* overexpression.

4.3.2.5 Problems using the CT-TOPO-GFP-*a9* construct in ES cells

ES cells transientally transfected with CT-TOPO-GFP-*a9* demonstrated a higher percentage of cells showing fluorescence within the gates (figure 67) than those stably transfected with the same expression vector (figure 65). However, the purpose of this overexpression study, is to differentiate ES cells that overexpress *Hoxa-9* to compare changes in lineage commitment, and therefore transient expression is not sufficient, in that there is only short term overexpression. The purpose of these transient transfections in both ES cells and COS-7 cells was to confirm that the vector was capable of overexpressing *Hoxa-9* transcription in these cell lines. The data shown in figures 66-67, compared to controls, confirm the efficiency.

Transient expression brought about minimal levels of expression (2.99% of cells showing fluorescence) compared to controls, confirming that the CT-TOPO-GFP-*a9* construct is functioning (at low levels) in ES cells, however this method is insufficient as it only overexpresses *Hoxa-9* short-term and therefore alternative overexpression systems were investigated.

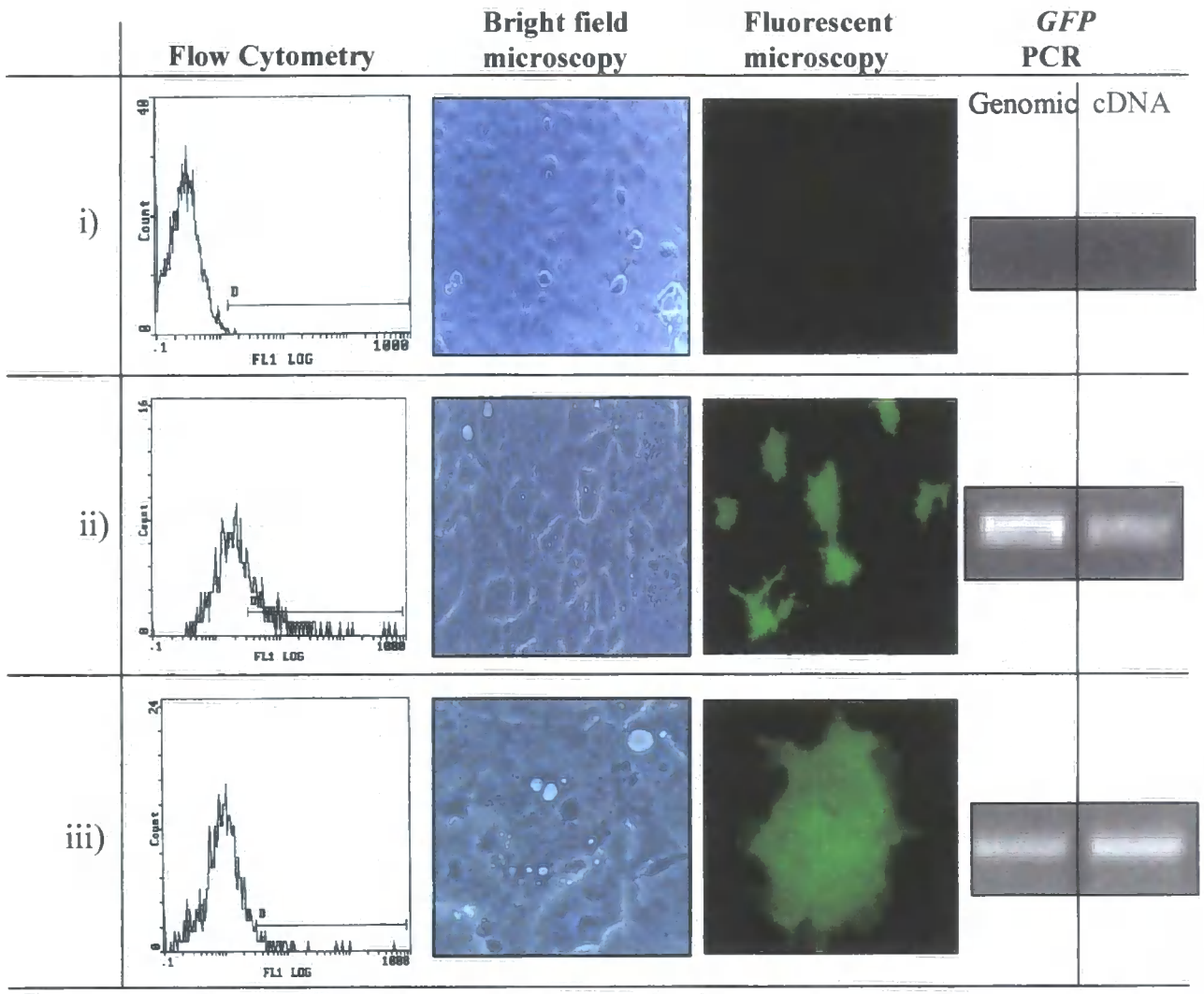


Figure 66 – **Transient transfection of COS-7 cells with heterologous promoter expression vector**
Flow cytometry, microscopy and PCR data from COS-7 cells transiently transfected with i) No DNA ii) CT-TOPO-GFP iii) CT-TOPO-GFP-*a9*

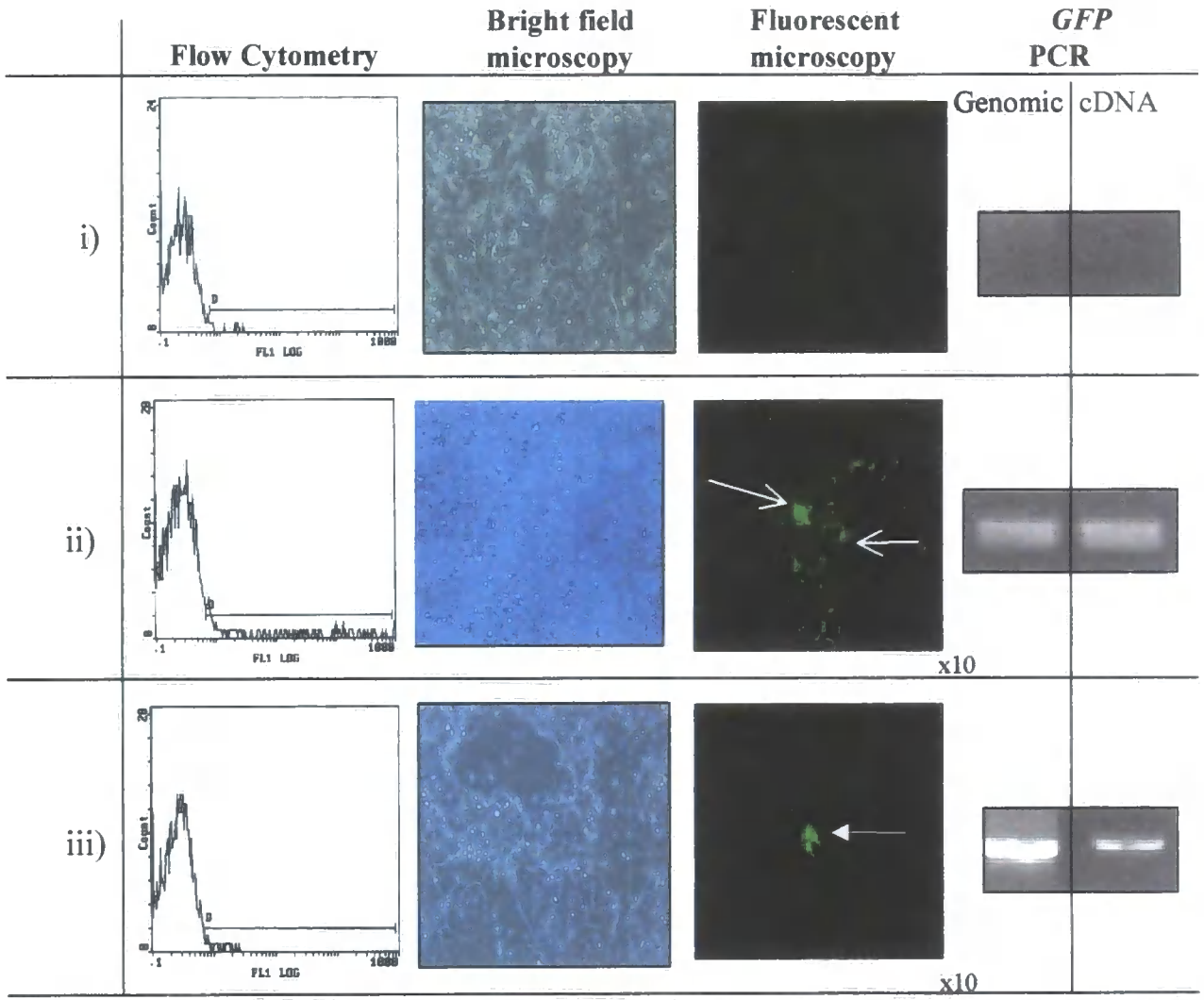


Figure 67 – **Transient transfection of CGR8 cells with heterologous promoter expression vector**
Flow cytometry, microscopy and PCR data from CGR8 ES cells transiently transfected with i) No DNA ii) CT-TOPO-GFP iii) CT-TOPO-GFP-*a9*

4.3.3 Retroviral overexpression studies:

4.3.3.1 Retroviral overexpression of *Hox* genes;

Previous *Hox* gene overexpression reports have used retroviral gene transduction to produce the stable expression of *Hox/HOX* genes into target cells. For *Hoxa-9*, the use of retroviral gene transfer has been used to overexpress these *Hox* genes into bone marrow cells to assess a functional role in the haematopoietic system of recipient mice (Thorsteinsdottir *et al.*, 1997, 2001, Lawrence *et al.*, 1997, Izon *et al.*, 1998, Kappen *et al.*, 2000), like *HOXA10* (Thorsteinsdottir *et al.*, 1997, Bjornsson *et al.*, 2001, Buske *et al.*, 2001), and *HOXB3* (Sauvageau *et al.*, 1995, 1997).

Other unrelated studies, have successfully used retroviruses to overexpress other *Hox* genes into ES cells (Helgason *et al.*, 1996, Keller *et al.*, 1998, Jackson and Baird *et al.*, 2002). In order to overcome the previous issues of insufficient stable transfection in ES cells using the SV40-driven expression vector a MoMLV-based retroviral vector was constructed containing the full length *Hoxa-9* coding sequence (as described in 4.2.5-10).

4.3.4 The application of the LX-GFP-*a9* construct:

4.3.4.1 GFP expression analysis in the Phoenix packaging cells;

Phoenix packaging cells were transfected with: empty vector (LX-GFP) where after forty-eight hours 24.1% of cells showed fluorescence within the gate margins (figure 68); LX-GFP-*a9* construct (22.5% fluorescence) and a mock transfection (0.90% fluorescence). PCR analysis verified this flow cytometry data, and demonstrated that the LX-GFP-*a9* construct had been incorporated into the phoenix cell genome and that these

cells were showing *Hoxa-9* transcription, and therefore supernatant was collected from the phoenix packaging cells to infect CGR8 ES cells, as described in 4.2.10.

4.3.4.2 GFP expression analysis following the retroviral transduction of CGR8 ES cells;

Forty-eight hours following application of the phoenix cell supernatant to the ES cells, the GFP expression was analysed as described previously in section 4.3.1.2. Cytometry showed very low levels of fluorescence in ES cells transduced with the empty vector and with LX-GFP-*a9* (figure 69). Further PCR analysis from genomic DNA and cDNA suggested that the construct had been integrated into the genome of the ES cells, but had failed to show GFP transcription consistent with the low levels of GFP expression shown by flow cytometry (figure 72). To confirm the viability of the retroviral vector system, 3T3 cells were transduced.

4.3.4.3 GFP expression analysis following the retroviral transduction of 3T3 cells;

Following application of the phoenix cell supernatant, the 3T3 cells were analysed for fluorescence and GFP transcription. Flow cytometry showed fluorescence in the cells transduced with the empty vector (3.02% of cells showing fluorescence), and in cells transduced with the LX-GFP-*a9* construct (4.83%) (see figure 70). PCR analysis confirmed the incorporation and transcription of the transgene using the LX-GFP-*a9* construct in 3T3 cells.

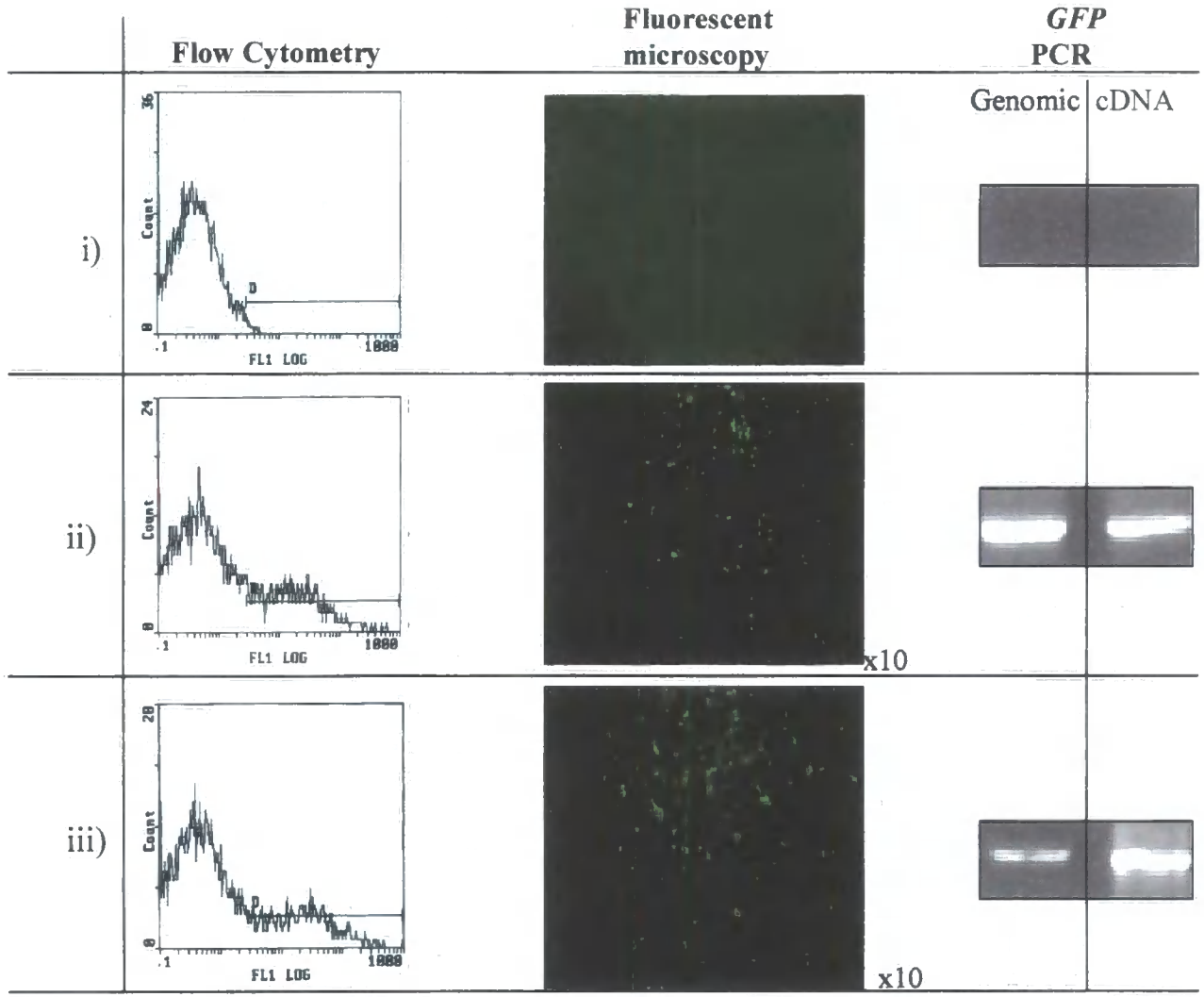


Figure 68 – **Transfection of Phoenix cells with retrovirus**
Flow cytometry, microscopy and PCR data from Phoenix cells transfected with i) No DNA ii) LX-GFP empty vector iii) LX-GFP-*a9*

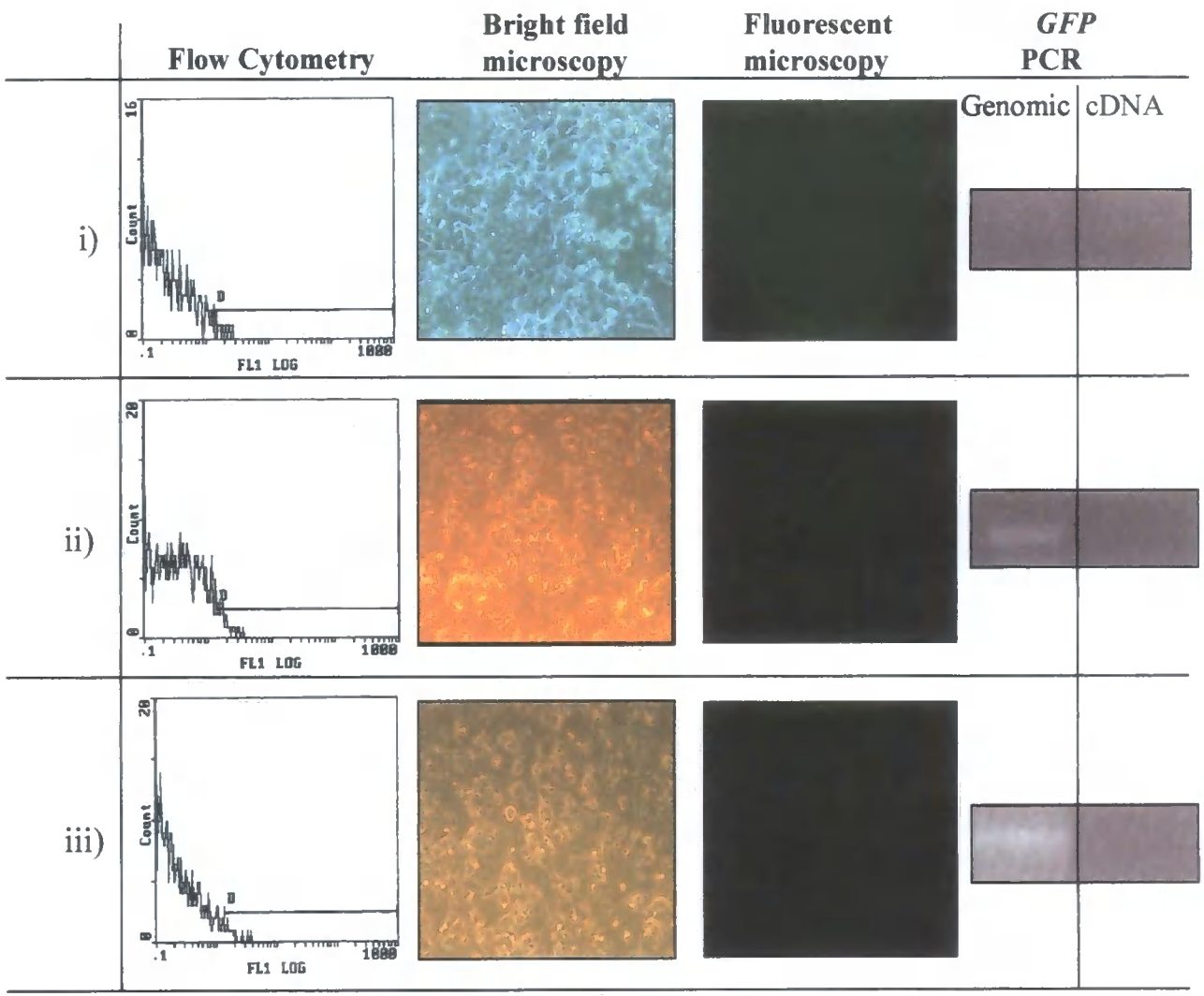


Figure 69 – **Retroviral transduction of CGR8 cells with retrovirus**
Flow cytometry, microscopy and PCR data from CGR8 ES cells transduced with i) No DNA ii) LX-GFP empty vector iii) LX-GFP-*a9*

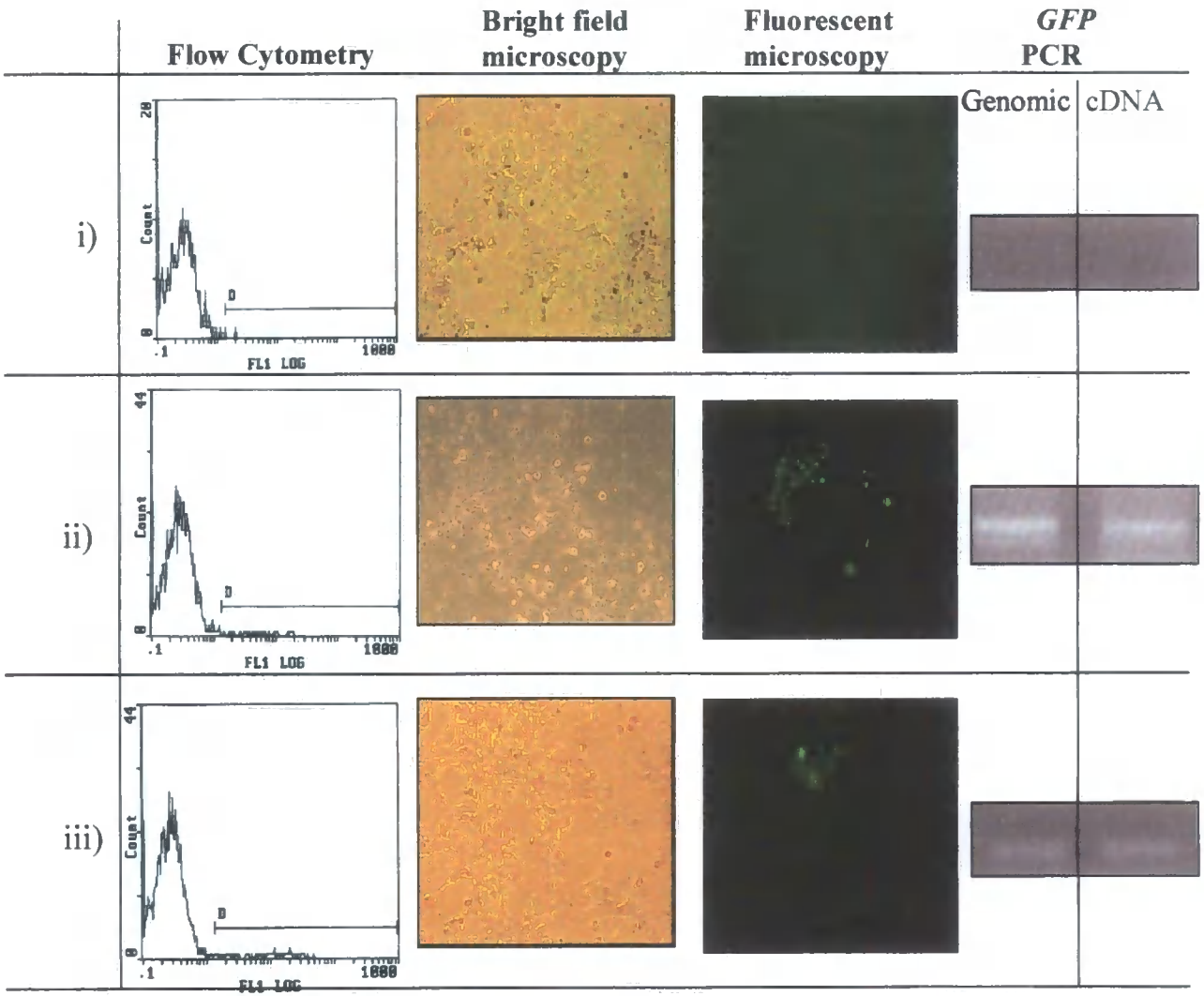


Figure 70 – **Retroviral transduction of 3T3 cells retrovirus**
Flow cytometry, microscopy and PCR data from 3T3 cells transduced with i) No DNA ii) LX-GFP empty vector iii) LX-GFP-*a9*

	Cell only	Empty vector	Vector + *Hoxa-9* insert
Stable CGR8 with TOPO-GFP *Figure 4.3a*	1.25%	98.6%	0.36%
Transient CGR8 with TOPO-GFP *Figure 4.3b*	1.61%	21.1%	2.99%
Transient COS-7 with TOPO-GFP *Figure 4.3c*	0.16%	31.9%	6.70%
Phoenix with LX retroviral *Figure 4.3d*	0.90%	24.1%	22.5%
CGR8 with S/N from Phoenix *Figure 4.3e*	0.61%	3.22%	1.2%
3T3 with S/N from Phoenix *Figure 4.3f*	0.48%	3.02%	4.83%

Figure 71 **Summary of GFP expression using flow cytometry**
A summary table showing the percentage of GFP-expressing cells in 10,000 events analysed

4.3.5 Summary

In summary, initial stable expression in CGR8 ES cells using a conventional CT-TOPO-GFP vector with the *Hoxa-9* full coding sequence insert (CT-TOPO-GFP-*a9*), failed to transcribe the transgene, although genomic DNA showed incorporation of the construct into the ES cell genome. Following this unsuccessful method, the efficiency of the CT-TOPO-GFP-*a9* construct in ES cells was tested by transient transfections, initially in COS-7 cells and subsequently in CGR8 ES cells, and was confirmed using flow cytometry, however the products of this transient method were insufficient for further use in this study.

Previous studies using retroviral gene transfer to overexpress *Hox* and other genes in ES cells had been successful, and therefore a MoMLV-GFP (LX-GFP) based retrovirus was constructed containing the same *Hoxa-9* insert (LX-GFP-*a9*). The initial viral packaging cells showed a high proportion of cells showing fluorescence, and GFP transcription, and therefore CGR8 ES cells were infected with the active viral particles collected from the supernatant of the packaging cells. However, as before in ES cells, genomic incorporation was demonstrated but transgene transcription levels were undetectable.

4.4 Discussion

ES cell overexpression data involving conventional transfection techniques and retroviral constructs have been successfully used to identify functional roles for candidate genes, including members of the *Hox* gene family in several developmental processes. However, these studies have not to date reported on the overexpression effects of *Hoxa-9* in ES cell differentiation models and there is no data on the functional roles for *Hoxa-9* in embryonic cardiogenic and haematopoietic differentiation pathways. In order to address this, two different methods of engineering overexpression of *Hoxa-9* in ES cells; calcium chloride transfection and retroviral tranduction were employed in this study. Although low level transient transfection was observed in a limited number of experiments, no stable overexpressing clones were isolated. There could be a number of possible explanations; these include the suitability of the vector, the competence of ES cells to be retrovirally transduced and the capacity of ES cells to overexpress *Hoxa-9*.

4.4.1 The vector construct

It is possible that the expression vectors used in this study were incompatible to overexpressing in ES cells, as transgene transcription was undetectable in these cells following transfection. However the evidence shown in this study, of high levels of fluorescence shown by flow cytometry following transfection with the control empty vector, against the mock transfections, demonstrate the vector efficacy for transgene transcription, as measured by GFP expression using flow cytometry, but does not eliminate the incompatibility of the retrovirus vector as control experiments (LX-GFP), like the LX-GFP-*a9* infection showed undetectable GFP levels. However it is important

to note that there is evidence of such vectors including those used here in successful heterologous gene overexpression in ES cells (reviewed Friedrich and Soriano, 1993, Helgason *et al.*, 1996). Therefore an alternative to the vehicle construct is the desired gene insert.

4.4.2 The vector insert

It is possible that the inefficient overexpression of *Hox-9* in ES cells was due to the function of the insert. There was insufficient cell growth observed in ES cells stably transfected with the CT-TOPO-GFP-*a9* construct, in comparison to control transfections, which may suggest that it was the addition of the *Hoxa-9* insert in the vector that resulted in this poor growth phase.

There is evidence however, to suggest that the *Hoxa-9* coding sequence insert in the expression construct was not in itself preventing overexpression, as there was successful overexpression in transfected COS-7 cells, and evidence of transient expression in ES cells (Figure 66-67). Also similar levels of fluorescence were observed in ES cells following retroviral infection of the empty vector (LX-GFP), and LX-GFP-*a9* respectively, suggesting further that it is not the *Hoxa-9* insert causing transcription repression.

4.4.3 The target cells

Evidence has been shown to eliminate the possibility of the construct and the *Hoxa-9* insert preventing overexpression in ES cells. It is therefore possible that the ES cells that were incompetent in overexpressing *Hoxa-9,* using a conventional heterologous promoter

and retroviral tranduction. However, there is evidence to suggest otherwise that retroviral mediated transduction has been used successfully to manipulate ES cells *in vitro* and shown to be a convenient means to perform insertional mutagenesis in the mouse (Hoeben *et al.*, 1991, Prince *et al.*, 1991, Tsukiyama *et al.*, 1992). This previous evidence suggests that ES cells are largely compatible to ES cell overexpression, and other repressive mechanisms should be considered as potential causes in this study.

4.4.4 DNA methylation

It has been shown that retroviral expression can be repressed in numerous cell types, including ES cells and HSC (Asche *et al.*, 1984, Challita *et al.*, 1994). For example, retroviral expression vectors that are functional in mature haematopoietic cells fail to express the transgene in animals transplanted with infected stem cells (John, 1995, Krall and Kohn, 1996, Lei *et al.*, 1996). Transcriptional repression is thought to be mediated by both *cis*-type-de novo methylation of the integrated proviruses and cell-type-specific *trans*-acting transcriptional repressors (Gautsch, 1980, Loh *et al.*, 1990, Hoeben *et al.*, 1991). The effect of *trans*-acting factors on retroviral expression through binding of specific sequences within the promoters of retroviruses has been examined in many studies (Petersen *et al.*, 1991, Prince and Rigby, 1991, Tsukiyama *et al.*, 1992). In contrast the role of methylation in silencing has been less clear. DNA methylation is thought to be a general mechanism used by cells to silence foreign DNA and may be involved in the cell defence against transposable elements (Yoder *et al.*, 1997). DNA methylation has also been associated with the repression of gene expression and the silencing of viral control elements (Jahner *et al.*, 1985, Cedar *et al.*, 1988, Walsh *et al.*,

1998). ES cells have provided a good model to study the role of DNA methylation in retroviral silencing. First, it was demonstrated that ES cells have high *de novo* methylation activity, which leads to effective methylation of integrated retroviral vectors, while little or no *de novo* methylation activity was detected in differentiated cells (Lei *et al.*, 1996). In addition ES cells have been genetically modified to alter the endogenous level of DNA methylation by the targeted disruption of the maintenance methyltransferase gene *Dnmt1*. ES cells homologous for this mutation proliferate normally with their genomic DNA highly demethylated, while differentiated cells and mice die due to the loss of genomic methylation (Li *et al.*, 1992, Lei *et al.*, 1996). Therefore these modified ES cells are useful to study the effect of DNA methylation on retroviral gene expression.

4.4.5 Conclusions

In situ, wholemount and *in vitro* ES cell expression data have suggested potential roles for *Hoxa-9* during the development of the cardiac and haematopoietic system. It followed that this study should attempt to identify a functional role for *Hoxa-9* during these developmental processes.

Two previously used expression vector systems, including a CT-TOPO-GFP construct under the control of a heterologous promoter, and a retroviral vector (LX-GFP) were adapted to overexpress *Hoxa-9* in ES cells.

Initial stable transfections into ES cells appeared inconclusive, with *Hoxa-9* transcription levels undetectable, however control transfections confirmed vector efficiency in ES cells.

The LX-GFP-*a9* retroviral infection in non-ES cells (3T3 cells) showed moderate levels of fluorescence compared to mock experiments, confirming transduction efficiency using the retrovirus system, but infected ES cells with either LX-GFP or LX-GFP-*a9* showed undetectable fluorescence using flow cytometry. Evidence has been demonstrated from this report and previous findings to eliminate the possibilities of neither the vector constructs, not the *Hoxa-9* insert causing transcription repression in ES cells, and possible causes have been discussed.

The expression pattern in the embryonic heart demonstrated using wholemount and *in situ* hybridisation techniques in chapter two, may have possible implications in heart development and function studies. It will therefore be important to continue to look at alternative genomic manipulation studies using *Hoxa-9* to identify a function for this gene cardiac development, where a potential association has been initially highlighted in this thesis.

Chapter five – Final discussion

5.1 Summary

This study has incorporated a range of techniques to investigate the expression patterns of *Hoxa-9* during embryonic development. One purpose was to explore the potential role of *Hoxa-9* during normal haematopoiesis driven by evidence for its involvement in abnormal haematopoiesis. In addition to finding *Hoxa-9* expression in differentiated ES cell populations that demonstrated haematopoietic commitment, *Hoxa-9* was also shown to be expressed in a spatial and temporal manner in the embryonic heart. Overexpression techniques were applied to the ES cell model, to identify a functional role for *Hoxa-9*.

There are several approaches that could facilitate finding the functions and regulatory roles of *Hoxa-9* during these developmental processes. Such approaches have successfully been used in earlier reports to explore the role of several other *Hox* genes during various developmental processes, including haematopoiesis. Broadly speaking they result in either loss-of-gene-function, or gain-of-gene-function. (Shen *et al.*, 1989, Takeshita *et al.*, 1993, Lawrence *et al.*, 1995, 1997, Lill *et al.*, 1995, Helgason *et al.*, 1996, Thorsteinsdottir *et al.*, 1997, 2002, Crooks *et al.*, 1999, Kappen *et al.*, 2000, Buske *et al.*, 2001, Yaron *et al.*, 2001).

5.1.1 Future work

Given more time, there are numerous alternative experiments that could be carried out to identify a functional role for *Hoxa-9* during cardiogenesis. These future experiments are important as a potential association between developmental stages of cardiogenesis and *Hoxa-9* expression has been shown, which have the potential to suggest a functional role

for *Hoxa-9* during the development of the embryonic heart. This work would allow a new insight into the role of *Hoxa-9* during development, other than those areas previously studied as well as providing potential therapeutic applications in heart development malformations.

5.1.1.1 Testing DNA methylation

It has been suggested that DNA methylation may be a potential cause of repression in the transduced ES cells in this study. Future work could investigate this problem by applying bisulphite based cytosine methylation analysis to the genomic DNA extracted from the ES cells to test for methylation. This method is based on a chemical modification of cytosine residues (methylation) in the presence of sodium bisulphite (Frommer *et al.*, 1992). In the first step of the bisulphite reaction, cytosines are sulfonated and deaminated converting them to uracil sulphonate. A subsequent desulphonation at a basic pH completes the conversion from cytosine to uracils. After bisulphite treatment the chromosomal region of interest is PCR-amplified and the PCR products sequenced. Only methyl-cytosines are detected as cytosines in this sequencing reaction, whereas all unmethylated cytosines appear as thymidines (Olek *et al.*, 1996). This sequence could be compared to controls to identify areas of methylation in the DNA of infected ES cells.

Other future work could include alternative genomic manipulation techniques using *Hoxa-9* in the ES cell model. Overexpression techniques are invaluable in identifying functional roles for candidate genes throughout development, and their advances in understanding the role of *Hox* genes in haematopoiesis has been invaluable. In this thesis

two different previously successful, overexpression methods were applied to *Hoxa-9* in an *in vitro* ES cell model, however, these results were inconclusive. One approach for overexpressing *Hoxa-9* in ES cells could be carried out using conditional expression vectors – an alternative vector system to those used previously in this thesis which allows a regulatable method of overexpressing transgene under the control of a selected antibiotic promoter.

5.1.1.2 Conditional overexpression

This method allows the regulatable gene expression in a wide variety of cell types, which could include the ES cell model previously used in this manuscript. One convenient method is to use a tetracylin-inducible high-level gene expression system as described by Gossen and Bujard (Gossen and Bujard, 1992). In conjunction with a packaging cell line as before, the retroviral 'Tet' vectors produce infectious, replication-incompetent retrovirus that can be used to introduce a gene of interest into a selected cell line.

This method would allow the controlled overexpression of *Hoxa-9* at selected times during ES cell differentiation *in vitro,* by the addition (activation) or removal (suppression) of tetracycline to the culture. This expression could be implicated into the equivalent stages during differentiation at crucial times of *Hoxa-9* expression during development e.g. E9.5 during cardiogenesis differentiation. This could also be applied to the haematopoietic pathway of differentiation. Any changes in *Hoxa-9* gene expression compared to control experiments, or changes in commitment could then be monitored to identify a functional effect.

Bjornsson (Bjornsson *et al.*, 2001) used a Tet-retroviral vector system to generate transgenic mice overexpressing *HOXA10* that could be induced or repressed depending on the presence or absence of tetracycline. Consequently, a vector containing the tetracycline transactivating gene was used to induce expression of the *HOXA10* gene in bone marrow cells from the transgenic mice. This method of using a conditional expression vector under the control of a transactivating gene, could be applied using *Hoxa-9*. This method would not only identify a potential function for the gene in the whole animal model, as a transgenic, but the model could be used to identify target genes of *Hoxa-9* other than in leukaemia, by screening and analysing changes in alternative candidate gene expression following the generation of the *Hoxa-9* transgenic.

As well as overexpression techniques to identity functions for *Hoxa-9* during embryonic development, loss-of-function studies could also be used.

5.1.1.3 Anti sense

Several other loss-of function *Hox* gene studies, excluding *Hoxa-9,* have used the anti-mRNA, or anti sense method to silence gene expression (Shen *et al.*, 1989, Takeshita *et al.*, 1993, Lill *et al.*, 1995).

The principals of one anti sense approach could also be applied to this investigation. Loss of function would be achieved by employing an oligonucleotide that acts as an alternative binding site or decoy, for protein stabilising elements that normally interact with a given mRNA (Beelman *et al.*, 1995, Liebhaber *et al.*, 1996). By attracting away mRNA-stabilising protein, the decoy induces instability, and ultimately destruction of the mRNA.

However, this technique has been replaced recently by the advanced RNAi (RNA interference) method.

5.1.1.4 RNAi

A newly developed approach would be to affect RNA interference or post-transcriptional gene silencing (Sharp, 1999, Gura, 2000). RNAi employs a gene-specific double-stranded RNA which, when introduced into a cell, leads to diminution of the targeted mRNA. The actual mechanism whereby this is accomplished is poorly understood but reports suggest that the it lies with the size and necessity for processing the targeted dsRNA (Grishok *et al.*, 2000, Zamore *et al.*, 2000). In *C.elegans* and *Drosophila* this is a highly reproducible method for disrupting gene expression. One obstacle in mammals is that dsRNAs longer than 30nt will activate antiviral response, leading to non-specific degradation of RNA transcripts and a general shutdown of host cell protein translation (Baglioni and Nilsen, 1983, Williams, 1997). As a result, the long dsRNA generally does not produce RNAi activity, and RNAi therefore is not a general method used in silencing genes in mammalian cells. This obstacle was overcome by Tuschl and colleagues (Elbashir *et al.*, 2001) who found that gene-specific suppression in mammalian cells can be achieved by *vitro*-synthesised siRNA (small interfering RNA) that are 21nt in length, long enough to induce gene-specific suppression, but short enough to evade the host interferon response. Sui (Sui *et al.*, 2002), developed a DNA vector-based approach to achieve RNAi in mammalian cells, by which small RNAs are predicted to be synthesised from a DNA template under the control of RNA polymerase III promoter in transfected cells. RNA polymerase III (pol III) has the advantage of directing the synthesis of small non-coding

transcripts whose 3' ends are defined by termination within a stretch of 4-5 thymidines (Bogen hagen *et al.*, 1980). These properties make it possible to use DNA templates to synthesis, *in vivo,* small RNAs with structural features close to what has been found to be required for active siRNAs synthesis *in vitro* (Elbashir *et al.*, 2001).

Using this DNA-vector based RNAi approach, siRNA coding sequences (<21nt) of *Hoxa-9* would have to be determined, and demonstrate that it shares no homology with any other *Hox* gene, this fragment would then be inserted into the reporter plasmid, which ideally would contain GFP and transfected into the ES cells. The level of GFP expression from the empty vector without the siRNA insert, could be compared to that of the siRNA containing plasmid to confirm the efficiency of silencing. Once confirmed changes in commitment in ES cell derived-haematopoietic and cardogenesis would be compared with controls.

One method in generating loss of function is the Cre-*loxP* recombination method. Unlike the other method mentioned, Cre-*loxP* recombination allows gene silencing in specific tissues. Out of all the potential methods discussed, this method appears most suitable for identifying *Hoxa-9* function in the cardiac and haematopoietic systems, as *Hoxa-9* expression could be under the control of either *Nkx2.5* (heart) or *SCL* (primitive haematopoietic) promoters, which are specific to those tissues and the effects can be studied in these organs respectively.

5.1.1.5 Cre-*loxP* recombination

The natural function of the Cre recombinase is to mediate recombination between two *LoxP* sequences that are the same orientation, leading to the excision of the intervening sequence between the two sites. Using gene targeting, *loxP* sequences can be stitched into a desired gene or chromosomal location, and the subsequent provision of a gene encoding the Cre product can result in an artificially generated site-directed recombination event. In lethal knockouts, methods have been developed to inactivate expression of the target gene in only selected predetermined cells of the animal, and therefore the animal can survive. However, previous *Hoxa-9* knockout studies have not shown lethality (Fromental-Ramain *et al.*, 1996, Lawrence *et al.*, 1997, Izon *et al.*, 1998) and therefore this method would not be suitable in this study.

The following diagrams suggest potential ways of identifying a functional role of *Hoxa-9* in the differentiating ES cell model, and tissue specific deletion of *Hoxa-9* using Cre-*loxP* recombination techniques.

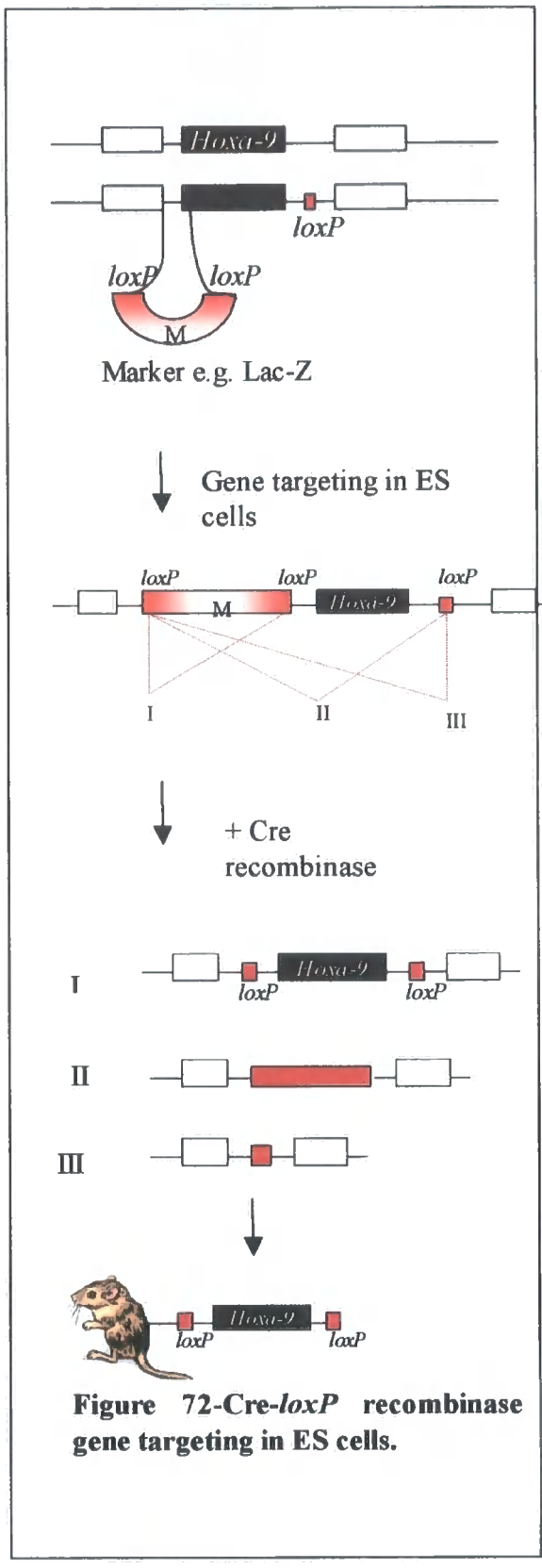


Figure 72-Cre-*loxP* recombinase gene targeting in ES cells.

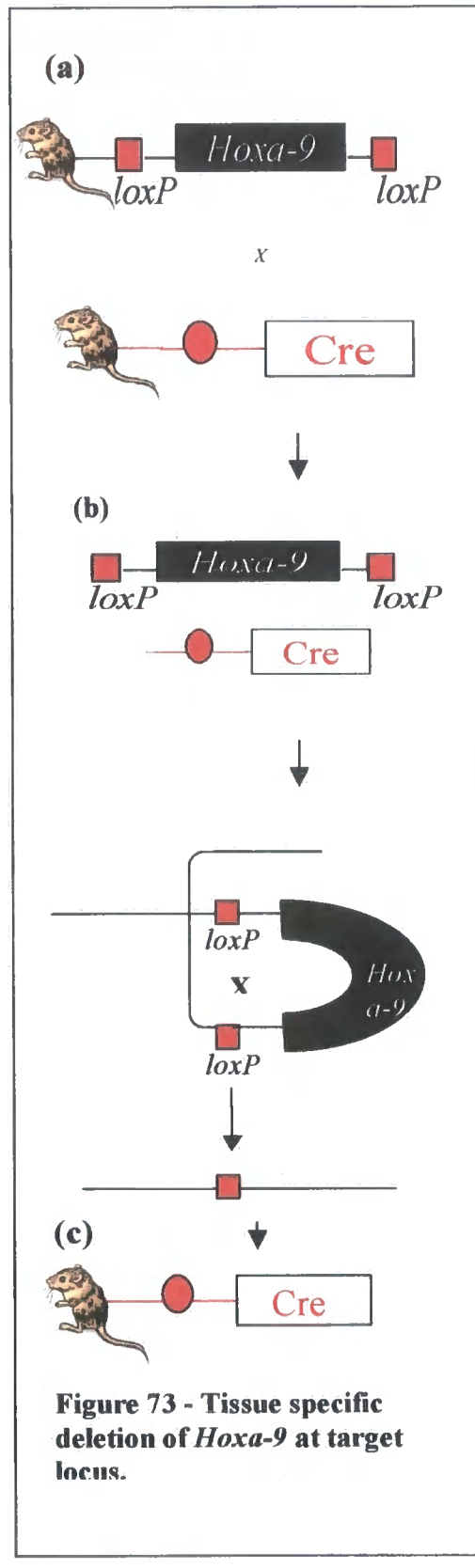


Figure 73 - Tissue specific deletion of *Hoxa-9* at target locus.

Adapted from Human Molecular Genetics, Strachan and Read, 1996.

Figures 72-73 illustrate methods in which three *loxP* sites are introduced along with a marker e.g. *Lac-Z* at the target locus of *Hoxa-9*. *Cre* recombinase is consequently transfected, and transient expression results in the recombination between the introduced *loxP* sites to give different products. Type I recombinants are used to generate mice in which the target locus is flanked with *loxP* sites. Such mice can be mated with previously constructed transgenic mice which carry an integrated construct consisting of the *Cre* recombinase gene linked to a tissue specific promoter (shown in figure 5.1b). Offspring which contain both the *loxP*-flanked target locus plus the *Cre* gene will express the *Cre* gene in the desired tissue type, and the resulting recombination between the *loxP* sites in these cells results in tissue-specific inactivation of the *Hoxa-9* gene. The specificity of the promoter using the Cre-*LoxP* method, is critical, and it is important to ensure that the promoter used is specific to the target tissue only, and therefore the promoters suggested would be suitable for this study.

5.1.2 Further potential roles for *Hoxa-9* in development from this study

These potential future work experiments may identify the function of *Hoxa-9* in heart development to associate with the previously unreported expression pattern shown in this thesis.

The reason for this spatial and temporal pattern in the embryonic heart is difficult to conclude from expression data alone, without any supporting functional data, and therefore the implications of *Hoxa-9* in heart development can only be speculated at this stage by analysing previously functional roles of *Hox* genes in other developmental

processes and comparing this data with the expression patterns of *Hoxa-9* in the developing heart in conjunction with the morphological events that occur at those times.

Hox genes have been expressed and implemented in several proliferation processes during development for example, blood cell production, including HSC expansion (see figure 4), gut organogenesis (Duluc *et al.*, 1997, Sekimoto *et al.*, 1998), mammalian limb development (reviewed Krumlauf, 1992), and chondrocyte proliferation (Selleri *et al.*, 2001). Previous examples of *Hoxa-9* expression in proliferating myeloid and T-cells (Lawrence *et al*, 1997, Anderson *et al.*, 1998, Izon *et al.*, 1998), and in the uncontrollable diseased state of myeloid cells – acute myeloid leukaemia have been highlighted (Nakamura *et al.*, 1996a, Kroon 2000).

Regarding these previous studies there may be a possibility that in the developing embryonic heart, *Hoxa-9* is differentially regulating cell proliferation, first in atria cells between E9.5-E10.5 and then in both the atria and the ventricle cells at E12. During heart development at E9.5 the atria and ventricular chambers are identified and become more established. There is also separation of the cardiac tube into the two chambers and the outflow tract, which is another feature that we see staining for *Hoxa-9* expression.

It is also likely that *Hoxa-9* has a similar regulatory proliferation role in the early haematopoietic cells. It was at this early stage when *Hoxa*-9 was demonstrated being expressed in early differentiating ES cells, and coincidentally HSCs at this time, show high proliferation potential in becoming the different blood cell precursors, and therefore require the expression of such genes.

This potential proliferative role, as well as defining if changes in *Hoxa-9* expression are a requirement or a by-product of heart development could be determined by all the

methods discussed in this chapter, but more specifically using the Cre-*loxP* recombinase study in heart specific tissue (controlled by an *Nkx2.5* promoter).

This spatial and temporal patterning of *Hoxa-9* demonstrated in this thesis in the embryonic heart has previously been seen with *Hoxa-9* in the developing digestive tract of the mouse (Sekimoto *et al.*, 1998) using wholemount *in situ* hybridisation. At E9.5 *Hoxa-9* (and *Hoxa-7*), signals were detected in visceral mesoderm at the mid and hind gut levels, and showed sharp rostral borders at the ileum/caecum boundary. This mesoderm-associated *Hox* gene expression is consistent and might be upstream of inductive signals transmitted from the mesenchymal layer (Sekimoto *et al.*, 1998). The molecules responsible for the above epithelial-mesenchymal inductive signalling remain unclear, although a number of genes have been identified as candidates, such as *Shh,* the *TGF-β* superfamily, the *Wnt* family, the *FGF* family, and several cell adhesion molecules. It remains to be determined which of these genes are components of the downstream *Hox* code in the gut. These molecules could also be responsible for inductive *Hoxa-9* signalling in the developing heart. It would be of interest in this study, to analyse their expression levels in conjunction with those of *Hoxa-9,* and potentially apply the Cre-*loxP* technique to these genes in the heart using the *Nkx2.5* promoter, to identify any responsive changes in *Hoxa-9* expression. This method may identify potential control mechanisms of *Hoxa-9* in the heart, and suggest possible activation pathways that could be further manipulated to investigate further *Hoxa-9* in heart development. The involvement of such candidates control genes in the upstream signals for the establishment of the *Hox* code in the heart will be important for understanding tissue morphogenesis.

The mechanisms involved in *Hox* gene regulation in tissues are relatively unclear, and require further investigation. It would be possible to apply gene trap screening to the *in vitro* ES cell model used in this thesis to screen for candidate genes expressed during cardiomyocyte and haematopoietic progenitor differentiation. Kluppel (Kluppel *et al.*, 2002) described a novel gene trap protocol to screen for target genes that are regulated during inductive events in undifferentiated and differentiated mouse embryonic stem cells. This approach integrated several features that allowed *in vitro* screening of large numbers of gene trap clones prior to generating lines of mutant mice. Moreover, targets of spatially and temporally restricted signalling pathways were analysed by screening undifferentiated ES cells versus ES cells differentiated into embryoid bodies. This method could be employed to screen candidate *Hox* target genes and mediators of signalling that function during different stages of heart development.

During embryonic heart development, there are several genes identified that control heart morphogenesis and function which include *Nkx2.5, GATA-4, Mesp2C, Mef2,* and *BMP-2. BMP-2* has been previously implicated in the activation pathway of *Hox* genes in response to *Shh* regulate the transcription of *Hox* in the growth stage of skeletal and mesenchyme tissue development (Iimura *et al.*, 1994, Roberts *et al.*, 1995, Watanabe *et al.*, 1998). In the heart *BMP-2* is expressed in early cardiac tissue and is thought to initialise early cardiac commitment stages (see figures 2.4f-l). In comparative terms, this early *in vivo* E7.5 stage is approximately equivalent to days 2-3 of *in vitro* ES cell differentiation. Incidentally, it is from around this stage that *Hoxa-9* expression was detected to this model, and continued up to day 10 of cardiomyocyte differentiation. It is

therefore possible that candidate genes such as *BMP-2* could be regulating *Hoxa-9* expression during embryonic heart development. This theory could be tested as before by carrying out *BMP-2* knockouts using Cre-*loxP* recombination with *BMP-2* in the heart, and analysing effects on *Hoxa-9* expression. The use of conditional expression vectors could also be used to regulate *BMP-2* expression in differentiating ES cells at specific stages when *Hoxa-9* was initially detected and undetected during cardiogenesis (d2 and d10) to examine the effects on *Hoxa-9* expression. Alternatively, *Hoxa-9* could be overexpressed in a similar manner during early stages when *BMP-2* has been previously identified in heart development (d2-4). Following *Hoxa-9* overexpression, *BMP-2* expression at these developmental stages could be examined in response to *Hoxa-9* activation. These suggested methods would help identify the regulation mechanisms between *Hoxa-9* and *BMP-2* in cardiomyocytes and heart development. The identification of *Hox* gene regulatory molecules is crucial in understanding the temporal and spatial expression patterns of these genes during normal development, and further understanding developmental malformations as a result of *Hox* gene dysregulation.

The *Hoxa-9* loss of function may produce a phenotype in the developing heart that may have large implications on heart development and future therapeutic malformation studies. Although previous loss of function studies have shown no lethality (Fromental-Ramain *et al.*, 1996, Lawrence *et al.*, 1997, Izon *et al.*, 1998), the structure and function of the resulting chimeras was not analysed, however, just because there was no lethality does not mean that *Hoxa-9* does not have an influencing function in heart development, it

is important however to understand and apply *Hox* gene redundancy and substitution to all these single gene knockout studies.

5.1.3 Loss of function in vertebrates

Homologous recombination in mouse ES cells, transgenic mice with mutated *Hox* genes have generated data in vertebrates on loss of function phenotypes (reviewed McGinnis and Krumlauff, 1992). These experiments provide direct support for the roles of *Hox* genes in patterning regional diversity in several developmental processes including the brachial region of the head (Wilkinson *et al.*, 1989, Lumsden, 1990, Hunt and Krumlauf, 1991a). Null alleles of *Hox-1.5 (Hoxa-3)* (Chisaka and Capecchi, 1991) and *Hox-1.6 (Hoxa-1)* (Lufkin *et al.*, 1991), display recessive lethal phenotypes in neonatal animals resulting from complex morphological defects first apparent in early embryogenesis. The mutant phenotypes are largely confined to the head and thorax, but there are no obvious transformations of axial structures. Instead, malformations appear that are not restricted to a single tissue or cell type and can comprise derivatives from several germ layers (Chisaka and Capechhi, 1991, Lufkin *et al.*, 1991). These loss of function mutant phenotypes therefore resemble those of *Lab* and *Dfd* genes in that they are not homeotic transformations but structural deficiencies.

5.1.3.1 Hox gene compensatory mechanisms and redundancy

The observation of loss of function transformations in *Drosophila* is dependent on the overlapping domains of expression of other *HOM-C* genes. This is more complex in the vertebrates in light of the *Hox* cluster duplications, which have generated paralogous

genes with nearly identical domains of expression (Gaunt *et al.*, 1989, Hunt *et al.*, 1991b). The absence of expression of one paralogue may be compensated by another. In the *Hox-1.5, Hox-1.6* mutants, within the affected regions of the head, not all structures that normally express the mutated genes have abnormal features. In particular, the rhombomeres, and major domains of *Hox* expression, appear completely normal, despite the fact that neural crest derivatives migrating from the rhombomeres are affected. Paralogous genes do not have identical rhombomere boundaries of expression, and levels are uniformly distributed at early stages, with differences in expression. This mutant study provides support for the concept of limited functional compensation or redundancy in some, but not all tissues, and this may be one of the reasons for the lack of obvious homeotic transformations observed in mice.

Loss of function studies of *Hox* genes have provided invaluable evidence for functional roles for several *Hox* genes during crucial developmental stages. However, in considering *Hox* gene redundancy and compensatory mechanisms it may be important to study the *Hoxa-9* paralogues, including *Hoxb-9, c-9 and d-9* either as double mutants with *Hoxa-9,* or as comparable single knock outs. Incidentally none of these paralogues have been implicated in leukaemia, haematopoiesis or cardiogenesis.

5.1.4 Summary

Although a functional role for *Hoxa-9* could not be found in the embryonic heart and primitive haematopoiesis from the data in this current study, prospective future work has been suggested and discussed. However, this thesis has identified a spatial and temporal

expression pattern of *Hoxa-9* during murine embryonic development in the heart atria, ventricles and vasculature that has not been previously reported. This expression data has been related to previous findings of *Hox* genes in spatial and temporal expression patterning, and potential roles suggested. It is therefore important to understand further the functions of *Hoxa-9* during cardiomyocyte development in embryogenesis and in particular to examine any influential implications that the data discovered in this thesis may have on the future of heart development studies.

References

Adamson, E.D. and Ayers, S.E. (1979) The localization and synthesis of some collagen types in developing mouse embryos. Cell 16, 953-65.

Akam M (1989) Hox and HOM: homologous gene clusters in insects and vertebrates. Cell 57, 347-9.

Akam ME (1987) The molecular basis for metameric pattern in the *Drosophila* embryo. Development 101, 1-22.

Alkema, M.J., van der Lugt, N.M., Bobeldijk, R.C., Berns, A. and van Lohuizen, M. (1995) Transformation of axial skeleton due to overexpression of bmi-1 in transgenic mice. Nature 374, 724-7.

Antonchuk, J., Sauvageau, G. and Humphries, R.K. (2001) HOXB4 overexpression mediates very rapid stem cell regeneration and competitive hematopoietic repopulation. Experimental Haematology 29, 1125-1134.

Appel, B. and Sakonju, S. (1993) Cell-type-specific mechanisms of transcriptional repression by the homeotic gene products UBX and ABD-A in Drosophila embryos. EMBO Journal 12, 1099-109.

Asche, W., Colletta, G., Warnecke, G., Nobis, P., Pennie, S., King, R.M. and Ostertag, W. (1984) Lack of retrovirus gene expression in somatic cell hybrids of friend cells and teratocarcinoma cells with a teratocarcinoma phenotype. Molecular and Cellular Biology 4, 923-30.

Averof, M. and Akam, M. (1995) Hox genes and the diversification of insect and crustacean body plans. Current Opinions in Biology 376, 73-78.

Azpiazu, N. and Frasch, M. (1993) tinman and bagpipe: two homeo box genes that determine cell fates in the dorsal mesoderm of Drosophila. Genes Development 7, 1325-1340.

Balling, R., Mutter, G., Gruss, P. and Kessel, M. (1989) Craniofacial abnormalities induced by ectopic expression of the homeobox gene Hox-1.1 in transgenic mice. Cell 58, 237-47.

Bao, Z., Bruneau, B., Seidman, J., Seidman, C.E. and Cepko, C.L. (1999) Regulation of chamber-specific gene expression in the developing heart by Irx4. Science 283, 1161-4.

Barinaga, M. (1994) Looking to development's future. Science 266, 562.

Beachy, P.A., Krasnow, M.A., Gavis, E.R. and Hogness, D.S. (1988) An Ultrabithorax protein binds sequences near its own and the Antennapedia P1 promoters. Cell 55, 1069-81.

Beelman, C.A. and Parker, R. (1995) Degradation of mRNA in eukaryotes. Cell 81, 179-83.

Beeman, R.W., Stuart, J.J., Haas, M.S. and Denell, R.E. (1989) Genetic analysis of the homeotic gene complex (HOM-C) in the beetle Tribolium castaneum. Developmental Biology 133, 196-209.

Bender, W., Akam, M., Karch, F., Beachy, P.A., Peifer, M., Spierer, P., Lewis, E.B. and Hogness, D.S. (1983) Molecular Genetics of the Bithorax complex in *Drosophila melanogaster*. Science 221, 23-29.

Bermingham Jr, J.R., Martinez-Arias, A., Petitt, M.G. and Scott, M.P. (1990) Different patterns of transcription from the two Antennapedia promoters during Drosophila embryogenesis. Development 109, 553-66.

Bernex, F., De Sepulveda, P., Kress, C., Elbaz, C., Delouis, C. and Panthier, J.J. (1996) Spatial and temporal patterns of c-kit-expressing cells in WlacZ/+ and WlacZ/WlacZ mouse embryos. Development 122, 3023-33.

Berthelsen, J., Zappavigna, V., Ferretti, E., Mavilio, F. and Blasi, F. (1998) The novel homeoprotein Prep1 modulates Pbx-Hox protein cooperativity. EMBO Journal 17, 1423-1433.

Bieberich, C.J.U., M.F. Awgulewitsch, A. Ruddle, F.H. (1990) Evidence for positive and negative regulation of the Hox-3.1 gene. Proc. Natl. Acad. Sci. U S A 87, 8462-8466.

Bigas, A., Martin, D.I. and Bernstein, I.D. (1995) Generation of hematopoietic colony-forming cells from embryonic stem cells: synergy between a soluble factor from NIH-3T3 cells and hematopoietic growth factors. Blood 85, 3127-33.

Bijl, J., van Oostveen, J.W., Kreike, M., Rieger, E., van der Raaij-Helmer, L.M., Walboomers, J.M., Corte, G., Boncinelli, E., van den Brule, A.J. and Meijer, C.J. (1996) Expression of HOXC4, HOXC5, and HOXC6 in human lymphoid cell lines, leukemias, and benign and malignant lymphoid tissue. Blood 87, 1737-45.

Bjornsson, J.M., Andersson, E., Lundstrom, P., Larsson, N., Xu, X., Repetowska, E., Humphries, R.K. and Karlsson, S. (2001) Proliferation of primitive myeloid progenitors can be reversibly induced by HOXA10. Blood 98, 3301-8.

Blatt, C., Aberdam, D., Schwartz, R. and Sachs, L. (1988) DNA rearrangement of a homeobox gene in myeloid leukaemic cells. EMBO J 7, 4283-90.

Blatt, C., Lotem, J. and Sachs, L. (1992) Inhibition of specific pathways of myeloid cell differentiation by an activated Hox-2.4 homeobox gene. Cell Growth and Differentiation 3, 671-6.

Bodmer, R. (1993) The gene tinman is required for specification of the heart and visceral muscles in *Drosophila*. Development 118.

Bogue, C.W., Lou, L.J., Vasavada, H., Wilson, C.M. and Jacobs, H.C. (1996) Expression of Hoxb genes in the developing mouse foregut and lung. American Journal of Respiratory Cell Molecular Biology 15, 163-71.

Boncinelli, E., Simeone, A., Acampora, D. and Mavilio, F. (1991) HOX gene activation by retinoic acid. Trends in Genetics 7, 329-334.

Bonifer, C., Faust, N., Geiger, H. and Muller, A.M. (1998) Developmental changes in the differentiation capacity of haematopoietic stem cells. Immunology Today 19, 236-41.

Borrow, J., Shearman, A.M., Stanton Jr, V.P., Becher, R., Collins, T., Williams, A.J., Dube, I., Katz, F. and Kwong, Y., Morris C, Ohyashiki K, Toyama K, Rowley J, Housman DE. (1996) The t(7;11)(p15;p15) translocation in acute myeloid leukaemia fuses the genes for nucleoporin NUP98 and class I homeoprotein HOXA9. Nature Genetics 12, 159-167.

Bour, B.A., O'Brien, M.A., Lockwood, W.L., Goldstein, E.S., Bodmer, R., Taghert, P.H., Abmayr, S.M. and Nguyen, H.T. (1995) *Drosophila MEF2*, a transcription factor that is essential for myogenesis. Genes Development 9, 730-741.

Breen, T.R. and Harte, P.J. (1993) Trithorax regulates multiple homeotic genes in the bithorax and Antennapedia complexes and exerts different tissue-specific, parasegment-specific and promoter-specific effects on each. Development 117, 119-34.

Brook, F.A. and Gardner, R.L. (1997) The origin and efficient derivation of embryonic stem cells in the mouse. Proc. Natl. Acad. Sci. U S A 94, 5709-12.

Brown, S. and Denell, R.E. (1996) Segmentation and dorsoventral patterning in *Tribolium*. Cellular Developmental Biology 7, 553-560.

Brustle, O., Jones, K.N., Learish, R.D., Karram, K., Choudhary, K., Wiestler, O.D., Duncan, I.D. and McKay, R.D. (1999) Embryonic stem cell-derived glial precursors: a source of myelinating transplants. Science 285, 754-6.

Burkert, U., von Ruden, T. and Wagner, E.F. (1991) Early fetal hematopoietic development from in vitro differentiated embryonic stem cells. New Biology 3, 698-708.

Buske, C., Feuring-Buske, M., Antonchuk, J., Rosten, P., Hogge, D.E., Eaves, C.J. and Humphries, R.K. (2001) Overexpression of HOXA10 perturbs human lymphomyelopoiesis in vitro and in vivo. Blood 97, 2286-92.

Calvo, K.R., Sykes, D.B., Pasillas, M. and Kamps, M.P. (2000) Hoxa9 immortalizes a granulocyte-macrophage colony-stimulating factor-dependent promyelocyte capable of biphenotypic differentiation to neutrophils or macrophages, independent of enforced meis expression. Molecular Cell Biology 20, 3274-3285.

Campione, M., Steinbeisser, H., Schweicker, t.A., Deissler, K., van Bebber, F., Lowe, L.A., Nowotschin, S., Viebahn, C., Haffter, P., Kuehn, M.R. and Blum, M. (1999) The homeobox gene Pitx2: mediator of asymmetric left-right signaling in vertebrate heart and gut looping. Development 126, 1225-1234.

Cantor, A.B. and Orkin, S.H. (2001) Hematopoietic development: a balancing act. Current Opinions in Genetics and Development 11, 513-9.

Care, A., Silvani, A., Meccia, E., Mattia, G., Peschle, C. and Colombo, M.P. (1998) Transduction of the SkBr3 breast carcinoma cell line with the HOXB7 gene induces bFGF expression, increases cell proliferation and reduces growth factor dependence. Oncogene 16, 3285-9.

Care, A., Silvani, A., Meccia, E., Mattia, G., Stoppacciaro, A., Parmiani, G., Peschle, C. and Colombo, M.P. (1996) HOXB7 constitutively activates basic fibroblast growth factor in melanomas. Molecular and Cellular Biology 16, 4842-51.

Carroll, S.B., Laymon, R.A., McCutcheon, M.A., Riley, P.D. and Scott, M.P. (1986) The localization and regulation of Antennapedia protein expression in Drosophila embryos. Cell 47, 113-122.

Castelli-Gair, J. and Akam, M. (1995) How the Hox gene Ultrabithorax specifies two different segments: the significance of spatial and temporal regulation within metameres. Development 121, 2973-2982.

Castelli-Gair, J., Greig, S., Micklem, G. and Akam, M. (1994) Dissecting the temporal requirements for homeotic gene function. Development 120, 1983-1995.

Cedar, H. (1988) DNA methylation and gene activity. Cell 53, 3-4.

Challita, P.M. and Kohn, D.B. (1994) Lack of expression from a retroviral vector after transduction of murine hematopoietic stem cells is associated with methylation in vivo. Proc. Natl. Acad. Sci. U S A 91, 2567-71.

Chambers, A.E., Logan, M., Kotecha, S., Towers, N., Sparrow, D. and Mohun, T.J. (1994) The RSRF/MEF2 protein SL1 regulates cardiac muscle-specific transcription of a myosin light-chain gene in Xenopus embryos. Genes Development 8, 1324.

Chan, S.K., Popperl, H., Krumlauf, R. and Mann, R.S. (1996) An extradenticle-induced conformational change in a HOX protein overcomes an inhibitory function of the conserved hexapeptide motif. EMBO J 15, 2476-87.

Chang, C.P., Brocchieri, L., Shen, W.F., Largman, C. and Cleary, M.L. (1996) Pbx modulation of Hox homeodomain amino-terminal arms establishes different DNA-binding specificities across the Hox locus. Molecular Cell Biology 16, 1734-1745.

Chang, C.P., de Vivo, I. and Cleary, M.L. (1997) The Hox cooperativity motif of the chimeric oncoprotein E2a-Pbx1 is necessary and sufficient for oncogenesis. Molecular and Cellular Biology 17, 81-88.

Chang, C.P., Jacobs, Y., Nakamura, T., Jenkins, N.A., Copeland, N.G. and Cleary, M.L. (1997) Meis proteins are major in vivo DNA binding partners for wild-type but not chimeric Pbx proteins. Molecular Cell Biology 17, 5679-5687.

Chang, P.Y., Kozono, T., Chida, K., Kurok, i.T. and Huh, N. (1998) Differential expression of Hox genes in multistage carcinogenesis of mouse skin. Biochemical and Biophysical Research Communications 248, 749-52.

Chang, Y., Paige, C.J. and Wu, G.E. (1992) Enumeration and characterization of DJH structures in mouse fetal liver. EMBO J 11, 1891-1899.

Chan-Thomas, P.S., Thompson, R.P., Robert, B., Yacoub, M.H. and Barton, P.J. (1993) Expression of homeobox genes Msx-1 (Hox-7) and Msx-2 (Hox-8) during cardiac development in the chick. Developmental Dynamics 197, 203-216.

Chavrier, P., Zerial, M., Lemaire, P., Almendral, J., Bravo, R. and Charnay, P. (1988) A gene encoding a protein with zinc fingers is activated during G0/G1 transition in cultured cells. EMBO J 7, 29-35.

Chen, F. and Capecchi, M.R. (1997) Targeted mutations in hoxa-9 and hoxb-9 reveal synergistic interactions. Developmental Biology 181, 186-196.

Chen, F. and Capecchi, M.R. (1999) Paralogous mouse Hox genes, Hoxa9, Hoxb9, and Hoxd9, function together to control development of the mammary gland in response to pregnancy. Proc. Natl. Acad. Sci. U S A 96, 541-6.

Chen, U. (1992) Differentiation of mouse embryonic stem cells to lympho-hematopoietic lineages in vitro. Developmental Immunology 2, 29-50.

Chen, U., Kosco, M. and Staerz, U. (1992) Establishment and characterization of lymphoid and myeloid mixed-cell populations from mouse late embryoid bodies, "embryonic-stem-cell fetuses". Proc. Natl. Acad. Sci. U S A 89, 2541-5.

Chisaka, O. and Capecchi, M.R. (1991) Regionally restricted developmental defects resulting from targeted disruption of the mouse homeobox gene hox-1.5. Nature 350, 473-479.

Cho, K.W., Goetz, J., Wright, C.V., Fritz, A., Hardwicke, J. and De Robertis, E.M. (1988) Differential utilization of the same reading frame in a Xenopus homeobox gene encodes two related proteins sharing the same DNA-binding specificity. EMBO J 7, 2139-49.

Choi, D., Oh, H.J., Chang, U.J., Koo, S.K., Jiang, J.X., Hwang, S.Y., Lee, J.D., Yeoh, G.C., Shin, H.S., Lee, J.S. and Oh, B. (2002) In vivo differentiation of mouse embryonic stem cells into hepatocytes. Cell transplantation 11, 359-68.

Chouinard, S. and Kaufman, T.C. (1991) Control of expression of the homeotic labial (lab) locus of Drosophila melanogaster: evidence for both positive and negative autogenous regulation. Development 113, 1267-80.

Christiansen, J.H., Coles, E.G., Robinson, V., Pasini, A. and Wilkinson, D.G. (2001) Screening from a subtracted embryonic chick hindbrain cDNA library: identification of genes expressed during hindbrain, midbrain and cranial neural crest development. Mechanisms of Development 102, 119-33.

Christoffels, V.M., Habets, P.E., Franco, D., Campione, M., de Jong, F., Lamers, W.H., Bao, Z.Z., Palmer, S., Biben, C., Harvey, R.P. and Moorman, A.F. (2000) Chamber formation and morphogenesis in the developing mammalian heart. Developmental Biology 223, 266-78.

Cillo, C., Barba, P., Freschi, G., Bucciarelli, G., Magli, M.C. and Boncinelli, E. (1992) HOX gene expression in normal and neoplastic human kidney. International Journal of Cancer 51, 892-7.

Cillo, C., Wilmore, H.P., Barba, P. and Brown, K.W. (1995) Expression and structure of HOX genes in Wilms' tumour. International Journal of Oncology 7, 1145-1150.

Colberg-Poley, A.M., Voss, S.D., Chowdhury, K., Stewart, C.L., Wagner, E.F. and Gruss, P. (1985) Clustered homeo boxes are differentially expressed during murine development. Cell 43, 39-45.

Crispino, J.D., Lodish, M.B., MacKay, J.P. and Orkin, S.H. (1999) Use of altered specificity mutants to probe a specific protein-protein interaction in differentiation: the GATA-1:FOG complex. Molecular and Cellular Biology 3, 219-28.

Crooks, G.M., Fuller, J., Petersen, D., Izadi, P., Malik, P., Pattengale, P.K., Kohn, D.B. and Gasson, J.C. (1999) Constitutive HOXA5 expression inhibits erythropoiesis and increases myelopoiesis from human hematopoietic progenitors. Blood 94, 519-28.

Cumano, A., Dieterlen-Lievre, F. and Godin, I. (1996) Lymphoid potential, probed before circulation in mouse, is restricted to caudal intraembryonic splanchnopleura. Cell 96, 907-16.

Dani, C., Smith, A.G., Dessolin, S., Leroy, P., Staccini, L., Villageois, P., Darimont, C. and Ailhaud, G. (1997) Differentiation of embryonic stem cells into adipocytes in vitro. Journal of Cell Science 110, 1279-85.

Dash, A.B., Williams, I.R., Kutok, J., Tomasson, M.H., Anastasiadou, E., Lindahl, K., Li, S., Van Etten, R.A. and Borrow J, H.D., Druker B, Gilliland DG (2002) A murine model of CML blast crisis induced by cooperation between BCR/ABL and NUP98/HOXA9. Proc. Natl. Acad. Sci. U S A 99, 7622-7.

De Robertis, E.M., Oliver, G. and Wright, C.V. (1990) Homeobox genes and the vertebrate body plan. Scientific American 263, 46-52.

De Vita, G., Barba, P., Odartchenko, N., Givel, J.C., Freschi, G., Bucciarelli, G., Magli, M.C., Boncinelli, E. and Cillo, C. (1993) Expression of homeobox-containing genes in primary and metastatic colorectal cancer. European Journal of Cancer 29A, 887-93.

Dedera, D.A., Waller, E.K., LeBrun, D.P., Sen-Majumdar, A., Stevens, M.E., Barsh, G.S. and Cleary, M.L. (1993) Chimeric homeobox gene E2A-PBX1 induces proliferation, apoptosis, and malignant lymphomas in transgenic mice. Cell 74, 833-843.

Dekker, E.J., Pannese, M., Houtzager, E., Timmermans, A., Boncinelli, E. and A., D. (1992) Xenopus Hox-2 genes are expressed sequentially after the onset of gastrulation and are differentially inducible by retinoic acid. Development Supplement, 195-202.

Desplan, C.T., J. and O'Farrell, P.H. (1985) The Drosophila developmental gene, engrailed, encodes a sequence-specific DNA binding activity. Nature 318, 630-5.

Doetschman, T.C., Eistetter, H., Katz, M., Schmidt, W. and Kemler, R. (1985) The in vitro development of blastocyst-derived embryonic stem cell lines: formation of visceral yolk sac, blood islands and myocardium. Journal of embryology and experimental morphology 87, 27-45.

Douboule, D. (1994) Guidebook to the Homeobox genes. Oxford University Press, London.

Drab, M., Haller, H., Bychkov, R., Erdmann, B., Lindschau, C., Haase, H., Morano, I., Luft, F.C. and Wobus, A.M. (1997) From totipotent embryonic stem cells to spontaneously contracting smooth muscle cells: a retinoic acid and db-cAMP in vitro differentiation model. FASEB Journal 11, 905-15.

Duboule, D. and Dollè, P. (1989) The structural and functional organization of the murine HOX gene family resembles that of *Drosophila* homeotic genes. EMBO J 8, 1497-1505.

Duluc, I., Lorentz, O., Fritsch, C., Leberquier, C., Kedinger, M. and Freund, J.N. (1997) Changing intestinal connective tissue interactions alters homeobox gene expression in epithelial cells. Journal of Cell Science 110, 1317-24.

Dziadek, M. and Andrews, G.K. (1983) Tissue specificity of alpha-fetoprotein messenger RNA expression during mouse embryogenesis. EMBO J 2, 549-554.

Dzierzak, E., Sanchez, M.J., Muller, A., Miles, C., Holmes, A., Tidcombe, H. and Medvinsky, A. (1997) Hematopoietic stem cells: embryonic beginning. Journal of Cell Physiology 173, 216-8.

Dziezak, E. and Medvinsky, A. (1995) Mouse embryonic hematopoiesis. Trends in Genetics 11, 359-365.

Dziezak, E., Medvinsky, A.L. and de Bruijn, M. (1998) Qualitative and quantitatve aspects of haematopoietic cell development in the mammalian embryo. Immunology Today 19, 228-241.

Edmondson, D.G., Lyons, G., Martin, J.F. and Olson, E.N. (1994) Mef2 gene expression marks the cardiac and skeletal muscle lineages during mouse embryogenesis. Development 120, 1251.

Elbashir, S.M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K. and Tuschl, T. (2001) Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature 411, 494-8.

Emerson, S. (1991) Basic Principles and Practice. Churchill Livingstone, New York.

Evans, D., Miller, J.B. and Stockdale, F.E. (1988) Developmental patterns of expression and coexpression of myosin heavy chains in atria and ventricles of the avian heart. Developmental Biology 127, 376-83.

Evans, M.J. and Kaufman, M.H. (1981) Establishment in culture of pluripotential cells from mouse embryos. Nature 292, 154-6.

Evans, S.M. and O'Brien, T.X. (1993) Expression of the helix-loop-helix factor Id during mouse embryonic development. Developmental Biology 159, 485-499.

Fainsod, A., Bogarad, L.D., Ruusala, T., Lubin, M., Crothers, D.M. and Ruddle, F.H. (1986) The homeo domain of a murine protein binds 5' to its own homeo box. Proc. Natl. Acad. Sci. U S A 83, 9532-5.

Fishman, M.C. and Olson, E.N. (1997) Parsing the heart: genetic modules for organ assembly. Cell 91, 153-156.

Franke, A., DeCamillis, M., Zink, D., Cheng, N., Brock, H.W. and Paro, R. (1992) Polycomb and polyhomeotic are constituents of a multimeric protein complex in chromatin of Drosophila melanogaster. EMBO J 11, 2941-2950.

Friedmann, Y., Daniel, C.A., Strickland, P. and Daniel, C.W. (1994) Hox genes in normal and neoplastic mouse mammary gland. Cancer Research 54, 5981-5.

Friedrich, G. and Soriano, P. (1993) Insertional mutagenesis by retroviruses and promoter traps in embryonic stem cells. Methods in Enzymology 225, 681-701.

Frohnhöfer, H.G. and Nüsslein-Volhard, C. (1986) Organization of anterior pattern in the *Drosophila* embryo by the maternal gene *Bicoid*. Nature 324, 120-125.

Fromental-Ramain, C., Warot, X., Lakkaraju, S., Favier, B., Haack, H., Birling, C., Dierich, A., Doll e, P. and Chambon, P. (1996) Specific and redundant functions of the paralogous Hoxa-9 and Hoxd-9 genes in forelimb and axial skeleton patterning. Development 122, 461-472.

Frommer, M., McDonald, L.E., Millar, D.S., Collis, C.M., Watt, F., Grigg, G.W. and Molloy PL, P.C. (1992) A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands. Proc. Natl. Acad. Sci. U S A 89, 1827-31.

Fujimoto, S., Araki, K., Chisaka, O., Araki, M., Takagi, K., Yamamura, K., (1998) Analysis of the murine *Hoxa-9* cDNA: an alternatively spliced transcript encodes a truncated protein lacking the homeodomain. Gene 209, 77-85

Fujiwara, Y., Browne, C.P., Cunniff, K., Goff, S.C. and Orkin, S.H. (1996) Arrested development of embryonic red cell precursors in mouse embryos lacking transcription factor GATA-. Proc. Natl. Acad. Sci. U S A 93, 12355-8.

Fuller, J.F., McAdara, J., Yaron, Y., Sakaguchi, M., Fraser, J.K. and Gasson, J.C. (1999) Characterization of HOX gene expression during myelopoiesis: role of HOX A5 in lineage commitment and maturation. Blood 93, 3391-400.

Gage, P.J., Suh, H. and Camper, S.A. (1999) Dosage requirement of Pitx2 for development of multiple organs. Development 126, 4643-4651.

Garcia-Bellido, A. and Capdevila, M.P. (1978) The initiaition and maintenance of gene activity in a developmental pathway of *Drosophila*. 36th Symp. Developmental Biology, 3-21.

Gaunt, S.J., Krumlauf, R. and Duboule, D. (1989) Mouse homeo-genes within a subfamily, Hox-1.4, -2.6 and -5.1, display similar anteroposterior domains of expression in the embryo, but show stage- and tissue-dependent differences in their regulation. Development 107, 131-41.

Gautsch, J.W. (1980) Embryonal carcinoma stem cells lack a function required for virus replication. Nature 285, 100-2.

Gehring, W.J. (1987) Homeo boxes in the study of development. Science 236, 1245-52.

Geissler, E.N., Liao, M., Brook, J.D., Martin, F.H., Zsebo, K.M., Housman, D.E. and Galli, S.J. (1981) Stem cell factor (SCF), a novel hematopoietic growth factor and ligand for c-kit tyrosine kinase receptor, maps on human chromosome 12 between 12q14.3 and 12qter. Somatic cell molecular genetics 17, 207-14.

Georgopoulos, K., Moore, D.D. and Derfler, B. (1992) Ikaros, an early lymphoid-specific transcription factor and a putative mediator for T cell commitment. Science 258, 808-12.

Giampaolo, A., Sterpetti, P., Bulgarini, D., Samoggia, P., Pelosi, E., Valtieri, M. and Peschle, C. (1994) Key functional role and lineage-specific expression of selected HOXB genes in purified hematopoietic progenitor differentiation. Blood 84, 4872.

Golub, T.R., Slonim, D.K., Tamayo, P., Huard, C., Gaasenbeek, M., Mesirov, J.P., Coller, H. and Loh, M.L., Downing JR, Caligiuri MA, Bloomfield CD, Lander ES. (1999) Molecular classification of cancer: class discovery and class prediction by gene expression monitoring. Science 286, 531-537.

Gordon, S., Fraser, I., Nath, D., Hughes, D. and Clark, S. (1992) Macrophages in tissues and in vitro. Current Opinions in Immunology 4, 25-32.

Gossen, M. and Bujard, H. (1992) Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. Proc. Natl. Acad. Sci. U S A 89, 5547-51.

Gould, A., Morrison, A., Sproat, G., White, R.A. and Krumlauf, R. (1997) Positive cross-regulation and enhancer sharing: two mechanisms for specifying overlapping Hox expression patterns. Genes Development 11, 900-13.

Graham, A., Papalopulu, N., Lorimer, J., McVey, J.H., Tuddenham, E.G.D. and Krumlauf, R. (1988) Characterization of a murine homeobox gene, Hox-2.6, related to drosophila deformed gene. Genes Development 2, 1424.

Graham, G.J. and Pragnell, I.B. (1992) The haemopoietic stem cell: properties and control mechanisms. Seminars in Cell Biology 3, 423.

Grepin, C., Robitaille, L., Antakly, T. and Nemer, M. (1995) Inhibition of transcription factor GATA-4 expression blocks in vitro cardiac muscle differentiation. Molecular Cell Biology 15, 4095-4102.

Grishok, A., Tabara, H. and Mello, C.C. (2000) Genetic requirements for inheritance of RNAi in C. elegans. Science 287, 2494-7.

Gura, T. (2000) A silence that speaks volumes. Nature 404, 804-8.

Gutierrez-Ramos, J.C. and Palacios, R. (1992) In vitro differentiation of embryonic stem cells into lymphocyte precursors able to generate T and B lymphocytes in vivo. Proc. Natl. Acad. Sci. U S A 89, 9171-5.

Haack, H. and Gruss, P. (1993) The establishment of murine Hox-1 expression domains during patterning of the limb. Developmental Biology 157, 410-22.

Hafen, E., Levine, M. and Gehring, W.J. (1984) Regulation of Antennapedia transcript distribution by the bithorax complex in Drosophila. Nature 307, 287-9.

Hanson, R.D., Hess, J.L., Yu, B.D., Ernst, P., van Lohuizen, M., Berns, A., van der Lugt, N.M., Shashikant, C.S. and Ruddle FH, S.M., Korsmeyer SJ. (1999) Mammalian Trithorax and polycomb-group homologues are antagonistic regulators of homeotic development. Proc. Natl. Acad. Sci. U S A 96, 14372-14377.

Harding, K. and Levine, M. (1988) Gap genes define the limits of antennapedia and bithorax gene expression during early development in Drosophila. EMBO J 7, 205-14.

Harvey, R.P. (1996) NK-2 homeobox genes and heart development. Developmental Biology 178, 203-216.

Hayes, P.H., Sato, T. and Denell, R.E. (1984) Homoeosis in Drosophila: the ultrabithorax larval syndrome. Proc. Natl. Acad. Sci. U S A 81, 545-9.

Heikinheimo, M., Scandrett, J.M. and Wilson, D.B. (1994) Localization of transcription factor GATA-4 to regions of the mouse embryo involved in cardiac development. Developmental Biology 164, 361.

Helgason, C.D., Sauvageau, G., Lawrence, H.J., Largman, C. and Humphries, R.K. (1996) Overexpression of HOXB4 enhances the hematopoietic potential of embryonic stem cells differentiated in vitro. Blood 87, 2740-9.

Hoeben, R.C., Migchielsen, A.A., van der Jagt, R.C., van Ormondt, H. and van der Eb, A.J. (1991) Inactivation of the Moloney murine leukemia virus long terminal repeat in murine fibroblast cell lines is associated with methylation and dependent on its chromosomal position. Journal of Virology 65, 904-12.

Hoey, T. and Levine, M. (1988) Divergent homeo box proteins recognize similar DNA sequences in Drosophila. Nature 332, 858-61.

Hogan, B., Beddington, R., Costantini, F. and Lacy, E. (1994) Manipulating the mouse embryo: a laboratory manual. Cold Spring Harbor publications, Cold Spring Harbor, New York.

Hole, N., Graham, G.J., Menzel, U. and Ansel, I.J.D. (1996) A limited temporal window for the derivation of multilineage repopulating hematopoietic progenitors during embryonal stem cell differentiation in vitro. Blood 88, 1266-76.

Hole, N. and Smith, A.G. (1994) Culture of Hematopoietic Cells - Embryonic Stem cells and Hematopoiesis. Wiley-Liss, pp. 235-249.

Holland, P.W.H., Garica-Fernandez, J., Holland, L.Z., Williams, N.A. and Holland, N.D. (1994) The molecular control of spatial patterning in amphioxus. Journal of Marine Biology Association 74, 49-60.

Holland, P.W.H. and Hogan, B.L.M. (1988) Expression of homeo box genes during mouse development. Genes Development 2, 773.

Hromas, R., Orazi, A., Neiman, R.S., Maki, R., Van Beveran, C., Moore, J. and Klemsz, M. (1993) Hematopoietic lineage- and stage-restricted expression of the ETS oncogene family member PU.1. Blood 82, 2998-3004.

Hunt, P., Gulisano, M., Cook, M., Sham, M.H., Faiella, A., Wilkinson, D., Boncinelli, E. and R, K. (1991) A distinct Hox code for the branchial region of the vertebrate head. Nature 353, 861-4.

Hunt, P. and Krumlauf, R. (1991a) Deciphering the Hox code: clues to patterning branchial regions of the head. Cell 66, 1075-8.

Iimura, T., Oida, S., Takeda, K., Maruoka, Y. and Sasaki, S. (1994) Changes in homeobox-containing gene expression during ectopic bone formation induced by bone morphogenetic protein. Biochemical and Biophysical Research Communications 201, 980-7.

Ikuta, K., Kina, T., MacNeil, I., Uchida, N., Peault, B., Chien, Y.H. and Weissman, I.L. (1990) A developmental switch in thymic lymphocyte maturation potential occurs at the level of hematopoietic stem cells. Cell 62, 863-74.

Ip, H.S., Wilson, D.B., Heikinheimo, M., Tang, Z., Ting, C.N., Simon, M.C., Leiden, J.M. and Parmacek, M.S. (1994) The GATA-4 transcription factor transactivates the cardiac muscle-specific troponin C promoter-enhancer in nonmuscle cells. Molecular Cell Biology 14, 7517.

Irish, V.F., Martinez-Arias, A. and Akam, M. (1989) Spatial regulation of the Antennapedia and Ultrabithorax homeotic genes during Drosophila early development. EMBO J 8, 1527-1537.

Irving, C. and Mason, I. (2000) Signalling by FGF8 from the isthmus patterns anterior hindbrain and establishes the anterior limit of Hox gene expression. Development 127, 177-86.

Izon, D.J., Rozenfeld, S., Fong, S.T., Komuves, L., Largman, C. and Lawrence, H.J. (1998) Loss of function of the homeobox gene Hoxa-9 perturbs early T-cell development and induces apoptosis in primitive thymocytes. Blood 92, 383-393.

Jackson, I.J., Schofield, P. and Hogan, B. (1985) A mouse homoeo box gene is expressed during embryogenesis and in adult kidney. Nature 317, 745-748.

Jackson, M., Baird, J.W., Cambray, N., Ansell, J.D., Forrester, L.M. and Graham, G.J. (2002) Cloning and characterisation of EHOX, a novel homeobox gene essential for ES cell differentiation. Journal of Biological Chemistry epub ahead of print.

Jahner, D. and Jaenisch, R. (1985) Retrovirus-induced de novo methylation of flanking host sequences correlates with gene inactivity. Nature 315, 594-7.

James, R. and Kazenwadel, J. (1991) Homeobox gene expression in the intestinal epithelium of adult mice. Journal of Biological Chemistry 226, 3246-3251.

Jiang, J., Hoey, T. and M., L. (1991) Autoregulation of a segmentation gene in Drosophila: combinatorial interaction of the even-skipped homeo box protein with a distal enhancer element. Genes Development 5, 265-77.

Jiang, Y. and Evans, T. (1996) The Xenopus GATA-4/5/6 genes are associated with cardiac specification and can regulate cardiac-specific transcription during embryogenesis. Developmental Biology 174, 258-270.

Johnson, A.D. and Herskowitz, I. (1985) A repressor (MAT alpha 2 Product) and its operator control expression of a set of cell type specific genes in yeast. Cell 42, 237-47.

Johnson, G.R. and Barker, D.C. (1985) Erythroid progenitor cells and stimulating factors during murine embryonic and fetal development. Experimental Haematology 13, 200-8.

Johnson, G.R. and Moore, M.A. (1975) Role of stem cell migration in initiation of mouse foetal liver haemopoiesis. Nature 258, 726-728.

Jurgens, G. (1985) A group of genes controlling the spatial expression of the bithorax complex in *Drosophila*. Nature 316, 153-55.

Kappen, C. (2000) Disruption of the homeobox gene Hoxb-6 in mice results in increased numbers of early erythrocyte progenitors. American Journal of Haematology 65, 111-8.

Kaufman, M.H. (1992) The Atlas of Mouse Development. Academic Press Limited, LondonVol. 1.

Kaufman, T.C., Lewis, R. and Wakimoto, B. (1980) Cytogenetic analysis of chromosome 3 in *Drosophila melanogaster*, the homeotic gene complex in polytene chromosome interval 84A-B. Genetics 94, 115-133.

Kaufman, T.C., Seeger, M.A. and Olsen, G. (1990) Molecular and genetic organization of the antennapedia gene complex of *Drosophila melanogaster.* Advances in Genetics 27, 309-362.

Keller, G., Kennedy, M., Papayannopoulou, T. and Wiles, M.V. (1993) Hematopoietic commitment during embryonic stem cell differentiation in culture. Molecular and Cellular Biology 13, 473-86.

Keller, G., Lacaud, G. and Robertson, S. (1999) Development of the hematopoietic system in the mouse. Experimental Haematology 27, 777-787.

Kelley, C., Blumberg, H., Zon, L.I. and Evans, T. (1993) GATA-4 is a novel transcription factor expressed in endocardium of the developing heart. Development 118, 817-27.

Kennison, J.A. (1993) Transcriptional activation of Drosophila homeotic genes from distant regulatory elements. Trends in Genetics 9, 75-9.

Kenyon, C. and Wang, B. (1991) A cluster of *Antennapedia*- class homeobox genes in a nonsegmented animal. Science 253, 516-517.

Kessel, M. and Gruss, P. (1990) Murine developmental control genes. Science 249, 374-9.

Kessel, M. and Gruss, P. (1991) Homeotic transformations of murine prevertebrae and concommitant alteration of *Hox* codes induced by retinoic acid. Cell 67, 89-104.

Kitamura, K., Miura, H., Miyagawa-Tomita, S., Yanazawa, M., Katoh-Fukui, Y., Suzuki, R. and Ohuchi H, S.A., Motegi Y, Nakahara Y, Kondo S, Yokoyama M. (1999) Mouse Pitx2 deficiency leads to anomalies of the ventral body wall, heart, extra- and periocular mesoderm and right pulmonary isomerism. Development 126, 5749-5758.

Knoepfler, P.S., Calvo, K.R., Chen, H., Antonarakis, S.E. and Kamps, M.P. (1997) Meis1 and pKnox1 bind DNA cooperatively with Pbx1 utilizing an interaction surface disrupted in oncoprotein E2a-Pbx1. Proc. Natl. Acad. Sci. U S A 94, 14553-14558.

Knoepfler, P.S. and Kamps, M.P. (1997) The highest affinity DNA element bound by Pbx complexes in t(1;19) leukemic cells fails to mediate cooperative DNA-binding or cooperative transactivation by E2a-Pbx1 and class I Hox proteins - evidence for selective targetting of E2a-Pbx1 to a subset of Pbx-recognition elements. Oncogene 14, 2521-2531.

Kolossov, E., Fleischmann, B.K., Liu, Q., Bloch, W., Viatchenko-Karpinski, S., Manzke, O., Ji, G.J., Bohlen, H., Addicks, K. and Hescheler, J. (1998) Functional characteristics of ES cell-derived cardiac precursor cells identified by tissue-specific expression of the green fluorescent protein. Journal of Cell Biology 143, 2045-56.

Kongsuwan, K., Allen, J. and Adams, J.M. (1989) Expression of Hox-2.4 homeobox gene directed by proviral insertion in a myeloid leukemia. Nucleic Acids Research 17, 1881-92.

Kongsuwan, K., Webb, E., Housiaux, P. and Adams, J.M. (1988) Expression of multiple homeobox genes within diverse mammalian haemopoietic lineages. EMBO J 7, 2131.

Kornberg, T.B. (1993) Understanding the homeodomain. Journal of Biological Chemistry 268, 26813-6.

Krall, W. and Kohn, D.B. (1996) Expression levels by retroviral vectors based upon the N2 and the MFG backbones. Gene Therapy 4, 365.

Kroon, E., Krosl, J., Thorsteinsdottir, U., Baban, S., Buchberg, A.M. and Sauvageau, G. (1998) Hoxa9 transforms primary bone marrow cells through specific collaboration with Meis1a but not Pbx1b. EMBO J 17, 3714-3725.

Kroon, E., Thorsteinsdottir, U., Mayotte, N., Nakamura, T. and Sauvageau, G. (2001) NUP98-HOXA9 expression in hemopoietic stem cells induces chronic and acute myeloid leukemias in mice. EMBO J 20, 350-361.

Krosl, G., He, G., Lefrancois, M., Charron, F., Romeo, P.H., Jolicoeur, P., Kirsch, I.R., Nemer, M. and Hoang, T. (1998) Transcription factor SCL is required for c-kit expression and c-Kit function in hemopoietic cells. Journal of Experimental Medicine 188, 439-50.

Krumlauf, R. (1992) Evolution of the vertebrate Hox homeobox genes. Bioessays 14, 245-52.

Krumlauf, R. (1994) Hox genes in vertebrate development. Cell 78, 191-201.

Kulessa, H., Frampton, J. and Graf, T. (1992) GATA-1 reprograms avian myelomonocytic cell lines into eosinophils, thromboblasts, and erythroblasts. Genes in Development 9, 1250-62.

Ladd, A.N., Yatskievych, T.A. and Antin, P.B. (1998) Regulation of avian cardiac myogenesis by activin/TGFbeta and bone morphogenetic proteins. Developmental Biology 204, 407-419.

Lako, M., Lindsay, S., Lincoln, J., Cairns, P.M., Armstrong, L. and Hole, N. (2001) Characterisation of Wnt gene expression during the differentiation of murine embryonic stem cells in vitro: role of Wnt3 in enhancing haematopoietic differentiation. Mechanisms of Development 103, 49-59.

Laughon, A. and Scott, M.P. (1984) Sequence of a Drosophila segmentation gene: protein structure homology with DNA-binding proteins. Nature 310, 25-31.

Laverriere, A.C., MacNeill, C., Mueller, C., Poelmann, R.E., Burch, J.B. and Evans, T. (1994) GATA-4/5/6, a subfamily of three transcription factors transcribed in developing heart and gut. Journal of Biological Chemistry 269, 23177.

Lawrence, H.J., Helgason, C.D., Sauvageau, G., Fong, S., Izon, D.J., Humphries, R.K. and Largman, C. (1997) Mice bearing a targeted interruption of the homeobox gene HOXA9 have defects in myeloid, erythroid, and lymphoid hematopoiesis. Blood 89, 1922-1930.

Lawrence, H.J., Rozenfeld, S., Cruz, C., Matsukuma, K., Kwong, A., Komuves, L., Buchberg, A.M. and Largman, C. (1999) Frequent co-expression of the HOXA9 and MEIS1 homeobox genes in human myeloid leukemias. Leukemia 13, 1993-9.

Le Mouellic, H., Lallemand, Y. and Brulet, P. (1992) Homeosis in the mouse induced by a null mutation in the Hox-3.1 gene. Cell 69, 251-64.

LeBrun, D.P. and Cleary, M.L. (1994) Fusion with E2A alters the transcriptional properties of the homeodomain protein PBX1 in t(1;19) leukemias. Oncogene 9, 1641-7.

Lei, H., Oh, S.P., Okano, M., Juttermann, R., Goss, K.A., Jaenisch, R. and Li, E. (1996) De novo DNA cytosine methyltransferase activities in mouse embryonic stem cells. Development 122, 3195-205.

Leimeister, C., Externbrink, A., B., K. and Gessler, M. (1999) Hey genes: a novel subfamily of hairy- and Enhancer of split related genes specifically expressed during mouse embryogenesis. Mechanisms of Development 85, 173-7.

Lessard, J., Baban, S. and Sauvageau, G. (1998) Stage-specific expression of polycomb group genes in human bone marrow cells. Blood 91, 1216-24.

Levine, M. and Hoey, T. (1988) Homeobox proteins as sequence-specific transcription factors. Cell 55, 537-40.

Lewis, E.B. (1978) A gene complex controlling segmentation in *Drosophila*. Nature 276, 565-570.

Liebhaber, S.A., Wang, Z., Cash, F.E., Monks, B. and Russell, J.E. (1996) Developmental silencing of the embryonic zeta-globin gene: concerted action of the promoter and the 3'-flanking region combined with stage-specific silencing by the transcribed segment. Molecular and Cellular Biology 16, 2637-46.

Lill, M.C., Fuller, J.F., Herzig, R., Crooks, G.M. and Gasson, J.C. (1995) The role of the homeobox gene, HOX B7, in human myelomonocytic differentiation. Blood 85, 692-7.

Lilly, B., Zhao, B., Ranganayakulu, G., Paterson, B.M., Schulz, R.A. and Olson, E.N. (1995) Requirement of MADS domain transcription factor D-MEF2 for muscle formation in *Drosophila*. Science 267, 688-693.

Lints, T.J., Parsons, L.M., Hartley, L., Lyons, I. and Harvey, R.P. (1993) Nkx-2.5: a novel murine homeobox gene expressed in early heart progenitor cells and their myogenic descendants. Development 119, 419-431.

Logan, M., Pagan-Westphal, S.M., Smith, D.M., Paganessi, L. and Tabin, C.J. (1998) The transcription factor Pitx2 mediates situs-specific morphogenesis in response to left-right asymmetric signals. Cell 94, 307-317.

Lough, J., Barron, M., Brogley, M., Sugi, Y., Bolender, D.L. and Zhu, X. (1996) Combined BMP-2 and FGF-4, but neither factor alone, induces cardiogenesis in non-precardiac embryonic mesoderm. Developmental Biology 178, 198-202.

Lowney, P., Corral, J., Detmer, K., LeBeau, M.M., Deaven, L., Lawrence, H.J. and Largman, C. (1991) A human Hox 1 homeobox gene exhibits myeloid-specific expression of alternative transcripts in human hematopoietic cells. Nucleic Acids Research 19, 3443-9.

Lu, M.F., Pressman, C., Dyer, R., Johnson, R.L. and Martin, J.F. (1999) Function of Rieger syndrome gene in left-right asymmetry and craniofacial development. Nature 401, 276-278.

Lufkin, T. (1997) Transcriptional regulation of vertebrate Hox genes during embryogenesis. Critical Review in Eukaryotic Gene Expression 7, 195-213.

Lumsden, A. (1990) The cellular basis of segmentation in the developing hindbrain. Trends in Neuroscience 13, 329-35.

Lumsden, A. and Krumlauf, R. (1996) Patterning the vertebrate neuraxis. Science 274, 1109-15.

Lyons, G.E. (1996) Vertebrate Heart Development. Current Opinions in Genetic Development 6, 454-60.

MacKenzie, A., Ferguson, M.W. and Sharpe, P.T. (1991) Hox-7 expression during murine craniofacial development. Development 113, 601-11.

MacKenzie, A., Ferguson, M.W. and Sharpe, P.T. (1992) Expression patterns of the homeobox gene, Hox-8, in the mouse embryo suggest a role in specifying tooth initiation and shape. Development 115, 403-20.

Magli, M.C., Barba, P., Celetti, A., De Vita, G., Cillo, C. and Boncinelli, E. (1991) Coordinate regulation of HOX genes in human hematopoietic cells. Proc. Natl. Acad. Sci. U S A 88, 6348-52.

Mann, R.S. (1994) Engrailed-mediated repression of Ultrabithorax is necessary for the parasegment 6 identity in Drosophila. Development 120, 3205-3212.

Mann, R.S. (1995) The specificity of homeotic gene function. Bioessays 17, 855-863.

Marshall, H., Nonchev, S., Sham, M.H., Muchamore, I., Lumsden, A. and Krumlauf, R. (1992) Retinoic acid alters hindbrain Hox code and induces transformation of rhombomeres 2/3 into a 4/5 identity. Nature 360, 737-41.

Marshall, H., Studer, M., Popperl, H., Aparicio, S., Kuroiwa, A. and Brenner, S.K.R. (1994) A conserved retinoic acid response element required for early expression of the homeobox gene Hoxb-1. Nature 370, 567-71.

Martin, E.C. and Adler, P.N. (1993) The Polycomb group gene Posterior Sex Combs encodes a chromosomal protein. Development 117, 641-55.

Martin, G.R. (1981) Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. Proc. Natl. Acad. Sci. U S A 78, 7634-8.

Mathews, C.H., Detmer, K., Boncinelli, E., Lawrence, H.J. and Largman, C. (1991) Erythroid-restricted expression of homeobox genes of the human HOX 2 locus. Blood 78, 2248-52.

Mathews, C.H., Detmer, K., Lawrence, H.J. and Largman, C. (1993) Expression of the Hox 2.2 homeobox gene in murine embryonic epidermis. Differentiation 52, 117-84.

McClanahan, T., Dalrymple, S., Barkett, M. and Lee, F. (1993) Hematopoietic growth factor receptor genes as markers of lineage commitment during in vitro development of hematopoietic cells. Blood 81, 2903-2915.

McCormick, A., Brady, H., Theill, L.E. and Karin, M. (1990) Regulation of the pituitary-specific homeobox gene GHF1 by cell-autonomous and environmental cues. Nature 345, 829-32.

McGinnis, W. and Krumlauf, R. (1992) Homeobox genes and axial patterning. Cell 68, 283-302.

McKercher, S.R., Torbett, B.E., Anderson, K.L., Henkel, G.W., Vestal, D.J., Baribault, H. and Klemsz M, F.A., Wu GE, Paige CJ, Maki RA. (1996) Targeted disruption of the PU.1 gene results in multiple hematopoietic abnormalities. EMBO J 15, 5647-58.

Meazza, R., Faiella, A., Corsetti, M.T., Airoldi, I., Ferrini, S., Boncinelli, E. and Corte, G. (1995) Expression of HOXC4 homeoprotein in the nucleus of activated human lymphocytes. Blood 85, 2084-90.

Medvinsky, A. (1993) Ontogeny of the mouse hematopoietic system. Seminars in Developmental Biology 4, 333-340.

Medvinsky, A. and Dziezak, E. (1996) Definitive hematopoiesis is autonomously initiated by the AGM region. Cell 86, 897.

Medvinsky, A., Samoylina, N.L., Muller, A.M. and Dzierzak, E.A. (1996) Development of day-8-colony-forming units-spleen hematopoietic progenitors during early murine embryogenesis: spatial and temporal mapping. Blood 87, 557-566.

Miller, A.D. and Rosman, G.J. (1989) Improved retroviral vectors for gene transfer and expression. Biotechniques 7, 980-990.

Miller-Hance, W.C., LaCorbiere, M., Fuller, S.J., Evans, S.M., Lyons, G., Schmidt, C. and Robbins J, C.K. (1993) In vitro chamber specification during embryonic stem cell cardiogenesis. Expression of the ventricular myosin light chain-2 gene is independent of heart tube formation. Journal of Biological Chemistry 268, 25244-52.

Monica, K., LeBrun, D.P., Dedera, D.A., Brown, R. and Cleary, M.L. (1994) Transformation properties of the E2a-Pbx1 chimeric oncoprotein: fusion with E2a is essential, but the Pbx1 homeodomain is dispensable. Molecular and Cellular Biology 14, 8304-8313.

Moore, M.A. and Metcalf, D. (1970) Ontogeny of the haemopoietic system: yolk sac origin of in vivo and in vitro colony forming cells in the developing mouse embryo. British Journal of Haematology 18, 279-96.

Moreau-Gachelin, F., Wendling, F., Molina, T., Denis, N., Titeux, M., Grimber, G., Briand, P., Vainchenker, W. and Tavitian, A. (1996) Spi-1/PU.1 transgenic mice develop multistep erythroleukemias. Molecular and Cellular Biology 16, 2453-63.

Morrison, S.J., Hemmati, H.D., Wandycz, A.M. and Weissman, I.L. (1995) The purification and characterization of fetal liver hematopoietic stem cells. Proc. Natl. Acad. Sci. U S A 72, 10302-6.

Moskow, J.J., Bullrich, F., Huebner, K., Daar, I. and Buchberg, A.M. (1995) Meis1, a PBX1-related homeobox gene involved in myeloid leukemia in BXH-2 mice. Molecular and Cellular Biology 15, 5434-43.

Moss, J.B., Xavier-Neto, J., Shapiro, M.D., Nayeem, S.M., McCaffery, P., Drager, U.C. and Rosenthal, N. (1998) Dynamic patterns of retinoic acid synthesis and response in the developing mammalian heart. Developmental Biology 199, 55-71.

Muller, A., Medvinsky, A.L., Strouboulis, J., Grosveld, F. and Dzierazak, E. (1994) Development of hematopoietic stem cell activity in the mouse embryo. Immunity 1, 291-301.

Muller, A.M. and Dzierzak, E.A. (1993) ES cells have only a limited lymphopoietic potential after adoptive transfer into mouse recipients. Development 118, 1343-51.

Muller, J. and Bienz, M. (1991) Long range repression conferring boundaries of Ultrabithorax expression in the Drosophila embryo. EMBO J 10, 3147-3155.

Muller, J., Gaunt, S. and Lawrence, P.A. (1995) Function of the Polycomb protein is conserved in mice and flies. Development 121, 2847-52.

Muller, M., Affolter, M., Leupin, W., Otting, G., Wuthrich, K. and Gehring, W.J. (1988) Isolation and sequence-specific DNA binding of the Antennapedia homeodomain. EMBO J 7, 4299-304.

Muller, M., Fleischmann, B.K., Selbert, S., Ji, G.J., Endl, E., Middeler, G., Muller, O.J., Schlenke, P. and Frese, S.W.A., Hescheler J, Katus HA, Franz WM (2000) Selection of ventricular-like cardiomyocytes from ES cells in vitro. FASEB Journal 14, 2540-8.

Muthuchamy, M., Pajak, L., Howles, P., Doetschman, T. and Wieczorek, D.F. (1993) Developmental analysis of tropomyosin gene expression in embryonic stem cells and mouse embryos. Molecular and Cellular Biology 13, 3311-23.

Nakagawa, O., Nakagawa, M., Richardson, J.A., Olson, E.N. and Srivastava, D. (1999) HRT1, HRT2, and HRT3: a new subclass of bHLH transcription factors marking specific cardiac, somitic, and pharyngeal arch segments. Developmental Biology 216, 72-84.

Nakamura, T., Largaespada, D.A., Lee, M.P., Johnson, L.A., Ohyashiki, K., Toyama, K., Chen, S.J., Willman, C.L., Chen, I.M. and Feinberg AP, J.N., Copeland NG, Shaughnessy JD Jr. (1996) Fusion of the nucleoporin gene NUP98 to HOXA9 by the chromosome translocation t(7;11)(p15;p15) in human myeloid leukaemia.
Nat Genet. 1996 Feb;12(2):154-8. Nature Genetics 12, 154-158.

Nakamura, T., Largaespada, D.A., Shaughnessy Jr, J.D., Jenkins, N.A. and Copeland, N.G. (1996a) Cooperative activation of Hoxa and Pbx1-related genes in murine myeloid leukaemias. Nature Genetics 12, 149-153.

Nakano, T., Kodama, H. and Honjo, T. (1994) Generation of lymphohematopoietic cells from embryonic stem cells in culture. Science 265, 1098-101.

Narita, N., Heikinheimo, M., Bielinska, M., White, R.A. and Wilson, D.B. (1996) The gene for transcription factor GATA-6 resides on mouse chromosome 18 and is expressed in myocardium and vascular smooth muscle. Genomics 36.

Neuhaus, H., Rosen, V. and Thies, R.S. (1999) Heart specific expression of mouse BMP-10 a novel member of the TGF-beta superfamily. Mechanisms of Development 80, 181-184.

Newman, C.S. and Krieg, P.A. (1998) tinman-related genes expressed during heart development in Xenopus. Developmental Genetics 22, 230-8.

Nichols, K.E., Crispino, J.D., Poncz, M., White, J.G., Orkin, S.H., Maris, J.M. and Weiss, M.J. (2000) Familial dyserythropoietic anaemia and thrombocytopenia due to an inherited mutation in GATA1. Nature Genetics 24, 266-70.

Nisitani, S., Tsubata, T. and Honjo, T. (1994) Lineage marker-negative lymphocyte precursors derived from embryonic stem cells in vitro differentiate into mature lymphocytes in vivo. International Immunology 6, 906-16.

Nonchev, S., Maconochie, M., Vesque, C., Aparicio, S., Ariza-McNaughton, L., Manzanares, M., Maruthainar, K., Kuroiwa, A., Brenner, S. and Charnay P, K.R. (1996) The conserved role of Krox-20 in directing Hox gene expression during vertebrate hindbrain segmentation. Proc. Natl. Acad. Sci. U S A 93, 9339-45.

Olek, A., Oswald, J. and Walter, J. (1996) A modified and improved method for bisulphite based cytosine methylation analysis. Nucleic Acids Research 24, 5064-6.

Olson, E.N. and Srivastava, D. (1996) Molecular pathways controlling heart development. Science 272, 671-676.

Osborne, J., Hu, C., Hawley, C., Underwood, L.J., O'Brien, T.J. and Baker, V.V. (1998) Expression of HOXD10 gene in normal endometrium and endometrial adenocarcinoma. Journal of Social Gynecology Investigation. 5, 277-80.

Pandolfi, P.P., Roth, M.E., Karis, A., Leonard, M.W., Dzierzak, E., Grosveld, F.G., Engel, J.D. and Lindenbaum, M.H. (1995) Targeted disruption of the GATA3 gene causes severe abnormalities in the nervous system and in fetal liver haematopoiesis. Nature Genetics 11, 40-4.

Papalopulu, N., Lovell-Badge, R. and Krumlauf, R. (1991) The expression of murine Hox-2 genes is dependent on the differentiation pathway and displays a collinear sensitivity to retinoic acid in F9 cells and Xenopus embryos. Nucleic Acids Research 19, 5497-506.

Paro, R. and Hogness, D.S. (1991) The Polycomb protein shares a homologous domain with a heterochromatin-associated protein of Drosophila. Proc. Natl. Acad. Sci. U S A 88, 263-67.

Patel, N.H. (1994) The evolution of arthropod segmentation: insights from comparisons of gene expression patterns. Development Supplement, 201-7.

Pearce, J.J., Singh, P.B. and Gaunt, S.J. (1992) The mouse has a Polycomb-like chromobox gene. Development 114, 921-929.

Pendleton, J.W., Nagai, B.K., Murtha, M.T. and Ruddle, F.H. (1993) Expansion of the Hox gene family and the evolution of chordates. Proc. Natl. Acad. Sci. U S A 90, 6300-6304.

Pereira, F.A., Qiu, Y., Zhou, G., Tsai, M.J. and Tsai, S.Y. (1999) The orphan nuclear receptor COUP-TFII is required for angiogenesis and heart development. Genes Development 13, 1037-49.

Perkins, A., Kongsuwan, K., Visvader, J., Adams, J.M. and Cory, S. (1990) Homeobox gene expression plus autocrine growth factor production elicits myeloid leukemia. Proc. Natl. Acad. Sci. U S A 87, 8398-8402.

Perkins, A.C. and Cory, S. (1993) Conditional immortalization of mouse myelomonocytic, megakaryocytic and mast cell progenitors by the Hox-2.4 homeobox gene. EMBO J 12, 3835-46.

Peters, H., Doll, U. and Niessing, J. (1995) Differential expression of the chicken Pax-1 and Pax-9 gene: in situ hybridization and immunohistochemical analysis. Developmental Dynamics 203, 1-16.

Peters, H., Wilm, B., Sakai, N., Imai, K., Maas, R. and Balling, R. (1999) Pax1 and Pax9 synergistically regulate vertebral column development. Development 126, 5399-408.

Petersen, R., Sobel, S., Wang, C.T., Jaenisch, R. and Barklis, E. (1991) Cellular transcripts encoded at a locus which permits retrovirus expression in mouse embryonic cells. Gene 101, 177-83.

Pevny, L., Simon, M.C., Robertson, E., Klein, W.H., Tsai, S.F., D'Agati, V., Orkin, S.H. and Costantini, F. (1991) Erythroid differentiation in chimaeric mice blocked by a targeted mutation in the gene for transcription factor GATA-1. Nature 349, 257-60.

Piedra, M.E., Icardo, J.M., Albajar, M., Rodriguez-Rey, J.C. and Ros, M.A. (1998) Pitx2 participates in the late phase of the pathway controlling left-right asymmetry. Cell 94, 319-324.

Pinsonneault, J., Florence, B., Vaessin, H. and McGinnis, W. (1997) A model for extradenticle function as a switch that changes HOX proteins from repressors to activators. EMBO J 16, 2032-42.

Pollock, R.A., Jay, G. and Bieberich, C.J. (1992) Altering the boundaries of Hox3.1 expression: evidence for antipodal gene regulation. Cell 71, 911-23.

Popperl, H., Bienz, M., Studer, M., Chan, S.K., Aparicio, S., Brenner, S., Mann, R.S. and Krumlauf, R. (1995) Segmental expression of Hoxb-1 is controlled by a highly conserved autoregulatory loop dependent upon exd/pbx. Cell 81, 1031-42.

Popperl, H. and Featherstone, M.S. (1992) An autoregulatory element of the murine Hox-4.2 gene. EMBO J 11, 3637-80.

Porcher, C., Swat, W., Rockwell, K., Fujiwara, Y., Alt, F.W. and Orkin, S.H. (1996) The T cell leukemia oncoprotein SCL/tal-1 is essential for development of all hematopoietic lineages. Cell 86, 47-57.

Pragnell, I.B., Wright, E.G., Lorimore, S.A., Adam, J., Rosendaal, M., DeLamarter, J.F. and Freshney, M.E.L., Sproul A, Wilkie N. (1988) The effect of stem cell proliferation regulators demonstrated with an in vitro assay. Blood 72, 196-201.

Prelle, K., Wobus, A.M., Krebs, O., Blum, W.F. and Wolf, E. (2000) Overexpression of insulin-like growth factor-II in mouse embryonic stem cells promotes myogenic differentiation. Biochemical and Biophysical Research Communications 227, 631-8.

Prince, V. and Lumsden, A. (1994) Hoxa-2 expression in normal and transposed rhombomeres: independent regulation in the neural tube and neural crest. Development 120, 911-23.

Prince, V.E. and Rigby, P.W. (1991) Derivatives of Moloney murine sarcoma virus capable of being transcribed in embryonal carcinoma stem cells have gained a functional Sp1 binding site. Journal of Virology 65, 1803-11.

Puschel, A.W., Balling, R. and Gruss, P. (1991) Separate elements cause lineage restriction and specify boundaries of Hox-1.1 expression. Development 112, 279-287.

Qian, S., Capovilla, M. and Pirrotta, V. (1991) The bx region enhancer, a distant cis-control element of the Drosophila Ubx gene and its regulation by hunchback and other segmentation genes. EMBO J 10, 1415-1425.

Qian, S., Capovilla, M. and Pirrotta, V. (1993) Molecular mechanisms of pattern formation by the BRE enhancer of the Ubx gene. EMBO J 12, 3865-3877.

Qian, Y.Q., Otting, G., Billeter, M., Muller, M., Gehring, W. and Wuthrich, K. (1992) Nuclear magnetic resonance spectroscopy of a DNA complex with the uniformly 13C-labeled Antennapedia homeodomain and structure determination of the DNA-bound homeodomain. Journal of Molecular Biology 234, 1070-83.

Qian, Y.Q., Otting, G., Furukubo-Tokunaga, K., Affolter, M., Gehring, W.J. and Wuthrich, K. (1992) NMR structure determination reveals that the homeodomain is connected through a flexible linker to the main body in the Drosophila Antennapedia protein. Proc. Natl. Acad. Sci. U S A 89, 10738-42.

Rabin, M., Ferguson-Smith, A., Hart, C.P. and Ruddle, F.H. (1986) Cognate homeo-box loci mapped on homologous human and mouse chromosomes. Proc. Natl. Acad. Sci. U S A 83, 9104-8.

Ranganayakulu, G., Zhao, B., Dokidis, A., Molkentin, J.D., Olson, E.N. and Schulz, R.A. (1995) A series of mutations in the D-MEF2 transcription factor reveal multiple functions in larval and adult myogenesis in *Drosophila*. Developmental Biology 171, 169-181.

Raza-Egilmez, S.Z., Jani-Sait, S.N., Grossi, M., Higgins, M.J., Shows, T.B. and Aplan, P.D. (1998) NUP98-HOXD13 gene fusion in therapy-related acute myelogenous leukemia. Cancer Research 58, 4269-4273.

Regulski, M., Dessain, S., McGinnis, N. and McGinnis, W. (1991) High-affinity binding sites for the Deformed protein are required for the function of an autoregulatory enhancer of the Deformed gene. Gene Development 5, 278-86.

Reynolds, B.A. and Weiss, S. (1996) Clonal and population analyses demonstrate that an EGF-responsive mammalian embryonic CNS precursor is a stem cell. Developmental Biology 175, 1-13.

Ricchiuti, V., Zhang, J. and Apple, F.S. (1997) Cardiac troponin I and T alterations in hearts with severe left ventricular remodeling. Clinical Chemistry 43, 990-5.

Riley, P.R., Gertsenstein, M., Dawson, K. and Cross, J.C. (2000) Early exclusion of hand1-deficient cells from distinct regions of the left ventricular myocardium in chimeric mouse embryos. Developmental Biology 227, 156-68.

Robb, L. and C.G., B. (1996) The helix-loop-helix gene SCL: implicated in T-cell acute lymphoblastic leukaemia and in normal haematopoietic development. International Journal of biochemistry and molecular biology 28, 609-18.

Robb, L., Lyons, I., Li, R., Hartley, L., Kontgen, F., Harvey, R.P., Metcalf, D. and Begley, C.G. (1995) Absence of yolk sac hematopoiesis from mice with a targeted disruption of the scl gene. Immunity 92, 7075-9.

Robbins, J., Gulick, J., Sanchez, A., Howles, P. and Doetschman, T. (1990) Mouse embryonic stem cells express the cardiac myosin heavy chain genes during development in vitro. Journal of Biological Chemistry 265, 11905-9.

Robbins, S.E., Shu, W.P., Kirschenbaum, A., Levine, A.C., Miniati, D.N. and Liu, B.C. (1996) Bone extracellular matrix induces homeobox proteins independent of androgens: possible mechanism for androgen-independent growth in human prostate cancer cells. Prostate 29, 362-70.

Roberts, D.J., Johnson, R.L., Burke, A.C., Nelson, C.E., Morgan, B.A. and Tabin, C. (1995) Sonic hedgehog is an endodermal signal inducing Bmp-4 and Hox genes during induction and regionalization of the chick hindgut. Development 121, 3163-3174.

Robertson, E. (1987) Teratocarcinomas and embryonic stem cells a practical approach. IRL Press, Practical approach series, pp. 71-112.

Robertson, E.J. (1986) Pluripotential stem cell lines as a route into the mouse germ line. Trends in Genetics 8, 9-13.

Rohwedel, J., Horak, V., Hebrok, M., Fuchtbauer, E.M. and Wobus, A.M. (1995) M-twist expression inhibits mouse embryonic stem cell-derived myogenic differentiation in vitro. Experimental Cell Research 220, 92-100.

Rohwedel, J., Maltsev, V., Bober, E., Arnold, H.H., Hescheler, J. and Wobus, A.M. (1994) Muscle cell differentiation of embryonic stem cells reflects myogenesis in vivo: developmentally regulated expression of myogenic determination genes and functional expression of ionic currents. Developmental Biology 164, 87-101.

Rozovskaia, T., Feinstein, E., Mor, O., Foa, R., Blechman, J., Nakamura, T., Croce, C.M., Cimino, G. and Canaani, E. (2001) Upregulation of Meis1 and HoxA9 in acute lymphocytic leukemias with the t(4:11) abnormality. Oncogene 20, 874-878.

Russell, E.S. and Bernstein, S.E. (1966) Biology of the Laboratory mouse. McGraw-Hill, pp. 351-372.

Ryan, A.K., Blumberg, B., Rodriguez-Esteban, C., Yonei-Tamura, S., Tamura, K., Tsukui, T., de la Pena, J. and Sabbagh W, G.J., Choe S, Norris DP, Robertson EJ, Evans RM, Rosenfeld MG, Izpisua Belmonte JC. (1998) Pitx2 determines left-right asymmetry of internal organs in vertebrates. Nature 394, 545-551.

Saga, Y. (1998) Genetic rescue of segmentation defect in MesP2-deficient mice by MesP1 gene replacement. Mechanisms of Development 75, 53-66.

Saga, Y., Hata, N., Koseki, H. and Taketo, M.M. (1997) Mesp2: a novel mouse gene expressed in the presegmented mesoderm and essential for segmentation initiation. Genes Development 11, 1827-1839.

Saldivar, J.R., Krull, C.E., Krumlauf, R., Ariza-McNaughton, L. and Bronner-Fraser, M. (1996) Rhombomere of origin determines autonomous versus environmentally regulated expression of Hoxa-3 in the avian embryo. Development 122, 895-904.

Salser, S.J. and Kenyon, C. (1994) Patterning *C. elegans:* homeotic cluster genes, cell fates and cell migrations. Trends in Genetics 10, 159-164.

Sanchez, A., Jones, W.K., Gulick, J., Doetschman, T. and Robbins, J. (1991) Myosin heavy chain gene expression in mouse embryoid bodies. An in vitro developmental study. Journal of Biological Chemistry 266, 22419-26.

Sanchez, M.J., Holmes, A., Miles, C. and Dzierzak, E. (1996) Characterization of the first definitive hematopoietic stem cells in the AGM and liver of the mouse embryo. Immunity 5, 513-25.

Sánchez-Herrero, E., Vernós, I., Marco, R. and Morata, G. (1985) Genetic organisation of the *Drosophila* Bithorax complex. Nature 313, 108-113.

Sander, K. (1976) Specification of the basic body pattern in insect embryogenesis. Advances in Insect Physiology 12, 125-238.

Sauvageau, G., Lansdorp, P.M., Eaves, C.J., Hogge, D.E., Dragowska, W.H., Reid, D.S., Largman, C. and Lawrence HJ, H.R. (1994) Differential expression of homeobox genes in functionally distinct CD34+ subpopulations of human bone marrow cells. Proc. Natl. Acad. Sci. U S A 91, 12223.

Sauvageau, G., Thorsteinsdottir, U., Eaves, C.J., Lawrence, H.J., Largman, C., Lansdorp, P.M. and Humphries, R. (1995) Overexpression of HOXB4 in hematopoietic cells causes the selective expansion of more primitive populations in vitro and in vivo. Genes Development 9, 1753-65.

Sauvageau, G., Thorsteinsdottir, U., Hough, M.R., Hugo, P., Lawrence, H.J., Largman, C. and Humphries, R.K. (1997) Overexpression of HOXB3 in hematopoietic cells causes defective lymphoid development and progressive myeloproliferation. Immunity 6, 13-22.

Schier, A.F. and Gehring, W.J. (1992) Direct homeodomain-DNA interaction in the autoregulation of the fushi tarazu gene. Nature 356, 804-7.

Schmitt, R.M., Bruyns, E. and Snodgrass, H.R. (1991) Hematopoietic development of embryonic stem cells in vitro: cytokine and receptor gene expression. Genes Development 5, 728-40.

Schneider-Maunoury, S., Topilko, P., Seitandou, T., Levi, G., Cohen-Tannoudji, M., Pournin, S., Babinet, C. and Charnay, P. (1993) Disruption of Krox-20 results in alteration of rhombomeres 3 and 5 in the developing hindbrain. Cell 75, 1199-214.

Schultheiss, T.M., Burch, J.B. and Lassar, A.B. (1997) A role for bone morphogenetic proteins in the induction of cardiac myogenesis. Genes Development 11, 451-462.

Schultheiss, T.M., Xydas, S. and Lassar, A.B. (1995) Induction of avian cardiac myogenesis by anterior endoderm. Development 121, 4203-4214.

Schüpbach, T. and Wieschaus, E. (1986) Germline autonomy of maternal-effect mutations altering the embryonic body pattern of *Drosophila*. Developmental Biology 113, 443-448.

Schwartz, R.J. and Olson, E.N. (1999) Building the heart piece by piece: modularity of cis-elements regulating Nkx2-5 transcription. Development 126, 4187-4192.

Sciavolino, P.J. and Abate-Shen, C. (1998) Molecular biology of prostate development and prostate cancer. Annual Medicine 30, 357-68.

Scott, E.W., Simon, M.C., Anastasi, J. and Singh, H. (1994) Requirement of transcription factor PU.1 in the development of multiple hematopoietic lineages. Science 265, 1573-7.

Scott, M.P. (1992) Vertebrate homeobox gene nomenclature. Cell 71, 551-553.

Scott, M.P., Tamkun, J.W. and Hartzell, G.W.r. (1989) The structure and function of the homeodomain. Biochim Biophys Acta. 989, 25-48.

Scott, M.P. and Weiner, A.J. (1984) Structural relationships among genes that control development: sequence homology between the Antennapedia, Ultrabithorax, and fushi tarazu loci of Drosophila. Proc. Natl. Acad. Sci. U S A 81, 4115-9.

Searcy, R.D. and Yutzey, K.E. (1998) Analysis of Hox gene expression during early avian heart development. Developmental Dynamics 213, 82-91.

Sekimoto, T., Yoshinobu, K., Yoshida, M., Kuratani, S., Fujimoto, S., Araki, M. and Tajima N, A.K., Yamamura K. (1998) Region-specific expression of murine Hox genes implies the Hox code-mediated patterning of the digestive tract. Genes to Cells 3, 51-64.

Selleri, L., Depew, M.J., Jacobs, Y., Chanda, S.K., Tsang, K.Y., Cheah, K.S. and Rubenstein JL, O.G.S., Cleary ML (2001) Requirement for Pbx1 in skeletal patterning and programming chondrocyte proliferation and differentiation. Development 128, 3543-57.

Shalaby, F., Rossant, J., Yamaguchi, T.P., Gertsenstein, M., Wu, X.F., Breitman, M.L. and AC., S. (1995) Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. Nature 376, 62-6.

Sham, M.H. (1993) The zinc finger gene *Krox20* regulates *HoxB2 (Hox2.8* during hindbrain segmentation. Cell 72, 183-96.

Sharp, P.A. (1999) RNAi and double-strand RNA. Genes Development 13, 139-42.

Shearn, A. (1989) The ash-1, ash-2 and trithorax genes of Drosophila melanogaster are functionally related. Genetics 121, 517-25.

Shen, W.F., Largman, C., Lowney, P., Corral, J.C., Detmer, K., Hauser, C.A., Simonitch, T.A. and Hack FM, L.H. (1989) Lineage-restricted expression of homeobox-containing genes in human hematopoietic cell lines. Proc. Natl. Acad. Sci. U S A 86, 8536-40.

Shen, W.F., Montgomery, J.C., Rozenfeld, S., Moskow, J.J., Lawrence, H.J., Buchberg, A.M. and Largman, C. (1997) AbdB-like Hox proteins stabilize DNA binding by the Meis1 homeodomain proteins. Molecular Cell Biology 17, 6448-6458.

Shen, W.F., Rozenfeld, S., Kwong, A., Kom ves, L.G., Lawrence, H.J. and Largman, C. (1999) HOXA9 forms triple complexes with PBX2 and MEIS1 in myeloid cells. Molecular Cell Biology 19, 3051-3061.

Shepherd, J.C., McGinnis, W., Carrasco, A.E., De Robertis, E.M. and Gehring, W.J. (1984) Fly and frog homoeo domains show homologies with yeast mating type regulatory proteins. Nature 310, 70-1.

Shimell, M.J., Simon, J., Bender, W. and O'Connor, M.B. (1994) Enhancer point mutation results in a homeotic transformation in Drosophila. Science 264, 968-971.

Shin, C., Chang, H.H., Koh, K.S., Park, H.W. and Kim, M.H. (1998) Differential expression of the *Hoxa-9* gene during mouse development. Korean Journal of Genetics 20, 91-101.

Shivdasani, R.A., Fujiwara, Y., McDevitt, M.A. and Orkin, S.H. (1997) A lineage-selective knockout establishes the critical role of transcription factor GATA-1 in megakaryocyte growth and platelet development. EMBO J 16, 3965-73.

Shivdasani, R.A., Mayer, E.L. and Orkin, S.H. (1995) Absence of blood formation in mice lacking the T-cell leukaemia oncoprotein tal-1/SCL. Nature 373, 432-4.

Shivdasani, R.A. and Orkin, S.H. (1996) The transcriptional control of hematopoiesis. Blood 87, 4025-39.

Simeone, A., Acampora, D., Arcioni, L., Andrews, P.W., Boncinelli, E. and Mavilio, F. (1990) Sequential activation of HOX2 homeobox genes by retinoic acid in human embryonal carcinoma cells. Nature 346, 763-6.

Simeone, A., Acampora, D., Nigro, V., Faiella, A., D'Esposito, M., Stornaiuolo, A., Mavilio, F. and Boncinelli, E. (1991) Differential regulation by retinoic acid of the homeobox genes of the four HOX loci in human embryonal carcinoma cells. Mechanisms of Development 33, 215-27.

Simeone, A., Pannese, M., Acampora, D., D'Esposito, M. and Boncinelli, E. (1988) At least three human homeoboxes on chromosome 12 belong to the same transcription unit. Nucleic Acids Research 16, 5379-90.

Simon, J. (1995) Locking in stable states of gene expression: transcriptional control during Drosophila development. Current Opinions in Cellular Biology 7, 376-85.

Simon, M.C., Pevny, L., Wiles, M.V., Keller, G., Costantini, F. and Orkin, S.H. (1992) Rescue of erythroid development in gene targeted GATA-1- mouse embryonic stem cells. Nature Genetics 1, 92-8.

Smith, A.G. (1991) Culture and differentiation of embryonic stem cells. Journal of Tissue Culture Methods 13, 688-91.

Smith, A.G., Heath, J.K., Donaldson, D.D., Wong, G.G., Moreau, J., Stahl, M. and Rogers, D. (1988) Inhibition of pluripotential embryonic stem cell differentiation by purified polypeptides. Nature 336, 688-90.

Smith, A.G., Nichols, J., Robertson, M. and Rathjen, P.D. (1992) Differentiation inhibiting activity (DIA/LIF) and mouse development. Developmental Biology 151, 339-51.

Sommer, R.J. and Tautz, D. (1993) Involvement of an orthologue of the Drosophila pair-rule gene hairy in segment formation of the short germ-band embryo of Tribolium (Coleoptera). Nature 361, 448-450.

Srebrow, A., Friedmann, Y., Ravanpay, A., Daniel, C.W. and Bissell, M.J. (1998) Expression of Hoxa-1 and Hoxb-7 is regulated by extracellular matrix-dependent signals in mammary epithelial cells. Journal of Cellular Biochemistry 69, 377-91.

Srivastava, D., Cserjesi, P. and Olson, E.N. (1995) A subclass of bHLH proteins required for cardiac morphogenesis. Science 270, 1995-1999.

Srivastava, D., Thomas, T., Lin, Q., Kirby, M.L., Brown, D. and Olson, E.N. (1997) Regulation of cardiac mesodermal and neural crest development by the bHLH transcription factor, dHAND. Nature Genetics 16, 154-160.

Stevenson, A.J., Clarke, D., Meredith, D.M., Kinsey, S.E., Whitehouse, A. and Bonifer, C. (2000) Herpesvirus saimiri-based gene delivery vectors maintain heterologous expression throughout mouse embryonic stem cell differentiation in vitro. Gene Therapy 7, 464-71.

Strachan, T. and Read, A.P. (1996) Human Molecular Genetics. BIOS Scientific Publishers Ltd.

Strubing, C., Ahnert-Hilger, G., Shan, J., Wiedenmann, B., Hescheler, J. and Wobus, A.M. (1995) Differentiation of pluripotent embryonic stem cells into the neuronal lineage in vitro gives rise to mature inhibitory and excitatory neurons. Mechanisms of Development 53, 275-87.

Struhl, G. (1981) A gene required for correct initiation of segmental determination in *Drosophila*. Nature 293, 36-41.

Struhl, G. (1982) Genes controlling segmental specification in the Drosophila thorax. Proc. Natl. Acad. Sci. U S A 79, 7380-4.

Struhl, G. and Akam, M. (1985) Altered distributions of Ultrabithorax transcripts in extra sex combs mutant embryos of Drosophila. EMBO J 4, 3259-64.

Struhl, G. and White, R.A. (1985) Regulation of the Ultrabithorax gene of Drosophila by other bithorax complex genes. Cell 43, 507-519.

Suemori, H., Takahashi, N. and Noguchi, S. (1995) Hoxc-9 mutant mice show anterior transformation of the vertebrae and malformation of the sternum and ribs. Mechanisms of Development 51, 265-73.

Sui, G., Soohoo, C., Affar el, B., Gay, F., Shi, Y., Forrester, W.C. and Shi, Y. (2002) A DNA vector-based RNAi technology to suppress gene expression in mammalian cells. Proc. Natl. Acad. Sci. U S A 99, 5515-20.

Swiatek, P.J. and Gridley, T. (1993) Perinatal lethality and defects in hindbrain development in mice homozygous for a targeted mutation of the zinc finger gene Krox20. Genes Development 7, 2071-84.

Takeshita, K., Bollekens, J.A., Hijiya, N., Ratajczak, M., Ruddle, F.H. and Gewirtz, A.M. (1993) A homeobox gene of the Antennapedia class is required for human adult erythropoiesis. Proc. Natl. Acad. Sci. U S A 90, 3535-8.

Tamkun, J.W., Deuring, R., Scott, M.P., Kissinger, M., Pattatucci, A.M., Kaufman, T.C. and Kennison, J.A. (1992) brahma: a regulator of Drosophila homeotic genes structurally related to the yeast transcriptional activator SNF2/SWI2. Cell 68, 561-72.

Tavassoli, M. (1991) Embryonic and fetal hemopoiesis: an overview. Blood cells 17, 269-81.

Taylor, H.S., Vanden Heuvel, G.B. and Igarashi, P. (1997) A conserved Hox axis in the mouse and human female reproductive system: late establishment and persistent adult expression of the Hoxa cluster genes. Biological Reproduction 57, 1338-45.

Thorsteinsdottir, U., Kroon, E., Jerome, L., Blasi, F. and Sauvageau, G. (2001) Defining roles for HOX and MEIS1 genes in induction of acute myeloid leukemia. Molecular Cell Biology 21, 224-34.

Thorsteinsdottir, U., Krosl, J., Kroon, E., Haman, A., Hoang, T. and Sauvageau, G. (1999) The oncoprotein E2A-Pbx1a collaborates with Hoxa9 to acutely transform primary bone marrow cells. Molecular Cell Biology 19, 6355-66.

Thorsteinsdottir, U., Mamo, A., Kroon, E., Jerome, L., Bijl, J., Lawrence, H.J., Humphries, K. and Sauvageau, G. (2002) Overexpression of the myeloid leukemia-associated Hoxa9 gene in bone marrow cells induces stem cell expansion. Blood 99, 121-9.

Thorsteinsdottir, U., Sauvageau, G., Hough, M.R., Dragowska, W., Lansdorp, P.M., Lawrence, H.J., Largman, C. and Humphries, R.K. (1997) Overexpression of HOXA10 in murine hematopoietic cells perturbs both myeloid and lymphoid differentiation and leads to acute myeloid leukemia. Molecular Cell Biology 17, 495-505.

Tiberio, C., Barba, P., Magli, M.C., Arvelo, F., Le Chevalier, T., Poupon, M.F. and Cillo, C. (1994) HOX gene expression in human small-cell lung cancers xenografted into nude mice. International Journal of Cancer 58, 608-15.

Ting, C.N., Olson, M.C., Barton, K.P. and Leiden, J.M. (1996) Transcription factor GATA-3 is required for development of the T-cell lineage. Nature 384, 474-8.

Tsai, F.Y., Keller, G., Kuo, F.C., Weiss, M., Chen, J., Rosenblatt, M., Alt, F.W. and Orkin, S.H. (1994) An early haematopoietic defect in mice lacking the transcription factor GATA-2. Nature 371, 221-6.

Tsukiyama, T. and Niwa, O. (1992) Isolation of high affinity cellular targets of the embryonal LTR binding protein, an undifferentiated embryonal carcinoma cell-specific repressor of Moloney leukemia virus. Nucleic Acids Research 20, 1477-82.

van der Hoeven, F., Zakany, J. and Duboule, D. (1996) Gene transpositions in the HoxD complex reveal a hierarchy of regulatory controls. Cell 85, 1025-35.

van der Lugt, N.M., Domen, J., Linders, K., van Roon, M., Robanus-Maandag, E., te Riele, H., van der Valk, M. and Deschamps J, S.M., van Lohuizen M, et al. (1994) Posterior transformation, neurological abnormalities, and severe hematopoietic defects in mice with a targeted deletion of the bmi-1 proto-oncogene. Genes Development 8, 757-769.

van Dijk, M.A. and Murre, C. (1994) extradenticle raises the DNA binding specificity of homeotic selector gene products. Cell 78, 617-24.

Van Parijs, L., Refaeli, Y., Abbas, A.K. and Baltimore, D. (1999) Autoimmunity as a consequence of retrovirus-mediated expression of C-FLIP in lymphocytes. Immunity 11, 763-70.

Vider, B.Z., Zimber, A., Hirsch, D., Estlein, D., Chastre, E., Prevot, S., Gespach, C., Yaniv, A. and Gazit, A. (1997) Human colorectal carcinogenesis is associated with deregulation of homeobox gene expression. Biochemical and Biophysical Research Communications 232, 742-8.

Vieille-Grosjean, I. and Huber, P. (1995) Transcription factor GATA-1 regulates human HOXB2 gene expression in erythroid cells. Journal of Biological Chemistry 270, 4544-50.

Vieille-Grosjean, I., Hunt, P., Gulisano, M., Boncinelli, E. and Thorogood, P. (1997) Branchial HOX gene expression and human craniofacial development. Developmental Biology 183, 49-60.

Vieille-Grosjean, I., Roullot, V. and Courtois, G. (1992) Lineage and stage specific expression of HOX 1 genes in the human hematopoietic system. Biochemical and Biophysical Research Communications 183, 1124-30.

Vyas, P., Ault, K., Jackson, C.W., Orkin, S.H. and Shivdasani, R.A. (1999) Consequences of GATA-1 deficiency in megakaryocytes and platelets. Blood 93, 2867-75.

Wakimoto, B. and Kaufman, T.C. (1981) Analysis of larval segmentation in lethal genotypes associated with the antennapedia gene complex in Drosophila melanogaster. Developmental Biology 81, 51-64.

Walsh, C.P., Chaillet, J.R. and Bestor, T.H. (1998) Transcription of IAP endogenous retroviruses is constrained by cytosine methylation. Nature Genetics 20, 116-7.

Wang, G.F., Nikovits, W., Schleinitz, M. and Stockdale, F.E. (1996) Atrial chamber-specific expression of the slow myosin heavy chain 3 gene in the embryonic heart. Journal of Biological Chemistry 271, 19836-19845.

Wang, R., Clark, R. and Bautch, V.L. (1992) Embryonic stem cell-derived cystic embryoid bodies form vascular channels: an in vitro model of blood vessel development. Development 114, 303-16.

Wartenberg, M., Gunther, J., Hescheler, J. and Sauer, H. (1998) The embryoid body as a novel in vitro assay system for antiangiogenic agents. Lab Investigations 78, 1301-14.

Watanabe, Y., Duprez, D., Monsoro-Burq, A.H., Vincent, C. and Le Douarin, N.M. (1998) Two domains in vertebral development: antagonistic regulation by SHH and BMP4 proteins. Development 125, 2631-9.

Wedeen, C., Harding, K. and Levine, M. (1986) Spatial regulation of Antennapedia and bithorax gene expression by the Polycomb locus in Drosophila. Cell 44, 739-48.

Weiss, S., Dunne, C., Hewson, J., Wohl, C., Wheatley, M., Peterson, A.C. and Reynolds, B.A. (1996) Multipotent CNS stem cells are present in the adult mammalian spinal cord and ventricular neuroaxis. Journal of Neuroscience 16, 7599-609.

White, R.A.H. and Lehmann, R. (1986) A gap gene, *hunchback,* regulates the spatial expression of *Ultrabithorax.* Cell 47, 311-321.

Wiles, M.V. and Keller, G. (1991) Multiple hematopoietic lineages develop from embryonic stem (ES) cells in culture. Development 111, 259-67.

Wilkinson, D.G. (1992) In situ Hybridisation A Practical Approach. IRL Press at Oxford University Press, London.

Wilkinson, D.G., Bhatt, S., Cook, M., Boncinelli, E. and Krumlauf, R. (1989) Segmental expression of Hox-2 homoeobox-containing genes in the developing mouse hindbrain. Nature 341, 405-9.

Williams, B.R. (1997) Role of the double-stranded RNA-activated protein kinase (PKR) in cell regulation. Biochemical Soc. Trans. 25, 509-13.

Williams, R.L., Hilton, D.J., Pease, S., Willson, T.A., Stewart, C.L., Gearing, D.P., Wagner, E.F., Metcalf, D. and Nicola NA, G.N. (1988) Myeloid leukaemia inhibitory factor maintains the developmental potential of embryonic stem cells. Nature 336, 684-7.

Wirz, J., Fessler, L. and Gehring, W. (1986) Localization of the *Antennapedial* protein *Drosophila* embryos and imaginal discs. EMBO J 5, 3327-3334.

Wobus, A.M., Kaomei, G., Shan, J., Wellner, M.C., Rohwedel, J., Ji Guanju, Fleischmann, B., Katus, H.A. and Hescheler J, F.W. (1997) Retinoic acid accelerates embryonic stem cell-derived cardiac differentiation and enhances development of ventricular cardiomyocytes. Journal of Molecular Cell Cardiology 29, 1525-39.

Wobus, A.M., Wallukat, G. and Hescheler, J. (1991) Pluripotent mouse embryonic stem cells are able to differentiate into cardiomyocytes expressing chronotropic responses to adrenergic and cholinergic agents and Ca2+ channel blockers. Differentiation 48, 173-82.

Wolff, C., Sommer, R., Schroder, R., Glaser, G. and Tautz, D. (1995) Conserved and divergent expression aspects of the Drosophila segmentation gene hunchback in the short germ band embryo of the flour beetle Tribolium. Development 121, 4227-4236.

Wolgemuth, D.J., Behringer, R.R., Mostoller, M.P., Brinster, R.L. and Palmiter, R.D. (1989) Transgenic mice overexpressing the mouse homoeobox-containing gene Hox-1.4 exhibit abnormal gut development. Nature 337, 464-7.

Wong, M.W., Pisegna, M., Lu, M.F., Leibham, D. and Perry, M. (1994) Activation of Xenopus MyoD transcription by members of the MEF2 protein family. Developmental Biology 166, 683.

Wong, P.M., Chung, S.W., Chui, D.H. and Eaves, C.J. (1986) Properties of the earliest clonogenic hemopoietic precursors to appear in the developing murine yolk sac. Proc. Natl. Acad. Sci. U S A 83, 3851-4.

Yamada, G., Kioussi, C., Schubert, F.R., Eto, Y., Chowdhury, K., Pituello, F. and Gruss, P. (1994) Regulated expression of Brachyury(T), Nkx1.1 and Pax genes in embryoid bodies. Biochemical and Biophysical Research Communications 199, 552-563.

Yamagishi, H., Yamagishi, C., Nakagawa, O., Harvey, R.P., Olson, E.N. and Srivastava, D. (2001) The combinatorial activities of Nkx2.5 and dHAND are essential for cardiac ventricle formation. Developmental Biology 239, 190-203.

Yaron, Y., McAdara, J.K., Lynch, M., Hughes, E. and Gasson, J.C. (2001) Identification of novel functional regions important for the activity of HOXB7 in mammalian cells. Journal of Immunology 166, 5058-67.

Yelon, D. and Stainier, D.Y. (1999) Patterning during organogenesis: genetic analysis of cardiac chamber formation. Cell and Developmental Biology 10, 93-98.

Yoder, J.A., Soman, N.S., Verdine, G.L. and Bestor, T.H. (1997) DNA (cytosine-5)-methyltransferases in mouse cells and tissues. Studies with a mechanism-based probe. Journal of Molecular Biology 270, 385-95.

Yokouchi, Y., Sakiyama, J. and Kuroiwa, A. (1995) Coordinated expression of Abd-B subfamily genes of the HoxA cluster in the developing digestive tract of chick embryo. Developmental Biology 169, 76-89.

Yutzey, K.E., Rhee, J.T. and Bader, D. (1994) Expression of the atrial-specific myosin heavy chain AMHC1 and the establishment of anteroposterior polarity in the developing chicken heart. Development 120, 871-83.

Zadeh, B.J., Gonzalez-Sanchez, A., Fischman, D.A. and Bader, D.M. (1986) Myosin heavy chain expression in embryonic cardiac cell cultures. Developmental Biology 115, 204-14.

Zamore, P.D., Tuschl, T., Sharp, P.A. and Bartel, D.P. (2000) RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. Cell 101, 25-33.

Zhang, C.C., Muller, J., Hoch, M., Jackle, H. and Bienz, M. (1991) Target sequences for hunchback in a control region conferring Ultrabithorax expression boundaries. Development 113, 1171-79.

Zhang, F., Popperl, H., Morrison, A., Kovacs, E.N., Prideaux, V., Schwarz, L., Krumlauf, R., Rossant, J. and MS., F. (1997) Elements both 5' and 3' to the murine Hoxd4 gene establish anterior borders of expression in mesoderm and neurectoderm. Mechanisms of Development 67, 49-58.

Zimmermann, F. and Rich, I.N. (1997) Mammalian homeobox B6 expression can be correlated with erythropoietin production sites and erythropoiesis during development, but not with hematopoietic or nonhematopoietic stem cell populations. Blood 89, 2723-35.

Materials and methods appendix A

All chemicals used were supplied by Sigma, Poole, Dorset, England unless stated otherwise.

A.1 Blocking Solution

1% (w/v) hybridisation blocking reagent (supplied by Roche, Lewes, East Sussex, England)

Dissolved in 1x maleic acid buffer (see appendix A1.8)

A.2 Denaturing Solution

0.5M NaOH

1.5M NaCl

Made up in ddH_2O

A.3 Detection Buffer

100mM Tris-HCl, pH 9.5

100mM NaCl

50mM $MgCl_2$

A.4 Diethyl Pyrocarbonate (DEPC) water

0.1% (v/v) DEPC in ddH_2O

Left in fume hood for twelve hours,

and autoclaved

A.5 2x HEPES buffered-saline

50mM HEPES

280mM NaCl

1.5mM Na_2HPO_4

10mM KCl

A.6 *In situ* hybridisation solution

50% (v/v) formamide

1% (v/v) SDS

2xSSC, pH 4.5

50µg/ml heparin

50µg/ml yeast RNA

A.7 *In situ* hybridisation wash solution 1

50% (v/v) formamide

5xSSC, pH 4.5

(see appendix A.17)

1% (v/v) SDS

A.8 *In situ* hybridisation wash solution 2

50% (v/v) formamide

2xSSC, pH 4.5 (see appendix A.17)

A.9 LB Agar (1 litre)

35g Lennox L agar

(Sigma, Poole, Dorset, England #L-2897)

A.10 LB Broth (1 litre)

20g Lennox L broth

(Sigma, Poole, Dorset, England #L-3022)

A.11 Maleic acid Buffer 10x

100mM maleic acid

150mM NaCl

Adjust to pH 7.5 using 1M NaOH

A.12 Neutralisation Solution

0.5M Tris-HCl adjust to pH 7.4

3M NaCl

Made up in ddH_2O

A.13 Noble agar

0.6 or 1.2% (w/v) noble agar in ddH_2O

A.14 NTMT

100mM Tris-base

50mM $MgCl_2$

100mM NaCl

0.1% Tween 20

A.15 Phosphate Buffered Saline (PBS)

Sigma, Poole, Dorset,

England tablets containing:

0.01M phosphate buffer,

0.0027M KCl,

A.16 Phosphate Buffered Saline + Tween (PBT)

PBS + 10% (v/v) Tween 20

A.17 Sodium chloride/sodium citrate (SSC) 20x

3M NaCl

0.3M $Na_3citrate.2H_2O$

Adjust to pH 7.0 with 1M HCl

A.18 TAE buffer (50x) – 1 litre

242g Tris-base

37.2g $Na_2EDTA.H_2O$

57.1ml glacial acetic acid

A.19 TBST 1x

0.14M NaCl

2.7M KCl

25mM Tris-HCl, pH 7.5

0.1% Tween 20

A.20 TE Buffer

10mM Tris-HCl pH 7.5

1mM EDTA, pH 8.0

A.21 6x TOPO Cloning 'Stop' solution

0.3M NaCl

0.06M $MgCl_2$

A.22 Wash Solution 1

2x SSC (se appendix A.17)

0.1% SDS

A.23 Wash Solution 2

0.5x SSC (see appendix A.17)

0.1% SDS

A.24 Wholemount hybridisation solution

50% (v/v) formamide

5xSSC, pH 4.5

(see appendix A.17)

1% (v/v) SDS

50µg/ml heparin

50µg/ml yeast RNA

A.25 Wholemount post hybridisation wash solution 1

50% (v/v) formamide

5xSSC, pH 4.5

(see appendix A.17)

1% (v/v) SDS

A.26 Wholemount post hybridisation wash solution 2

0.5M NaCl

0.01M Tris-HCl, pH 7.5

0.1% Tween 20

A.27 Wholemount post hybridisation wash solution 3

50% (v/v) formamide

2xSSC, pH 4.5

(see appendix A.17)

Sequence Appendix B
All accession numbers used in this thesis are from the Genbank database

B.1 Mus Musculus homeobox A9 NM_010456

```
1201 acatgaaatc tgcagtttca taatttcggc gggtcgggct gggccggcca ggcgcgggct

        Hoxa-9 For PCR primer
        acaatgg ccaccaccgg ggc
1261 actgcaatgg ccaccaccgg ggccctgggc aactactatg tggactcctt cctgctgggc

1321 gccgacgctg ctgatgagct gggtgcggga cgctacgctc cagggaccct gggtcaaccc

1381 ccaaggcagg cggcagctct ggccgaacac cccgacttca gtccttgcag cttccagtcc

1441 aaggcggcgg tgtttggtgc ctcgtggaac ccagtgcacg cggcgggcgc caatgcggtg

1501 cctgctgcag tgtatcatca ccaccaccac ccctacgtgc atccccaggc gcccgtggcg

                            Deletion in alternatively spliced Hoxa-9

1561 gcggcggcgc cggacggcag gtatatgcgc tcctggctgg aacccacgcc cggtgcgctc

1621 tccttcgcgg gcttaccctc cagccggcct tatggcatta aacctgaacc gctctcggcc

1681 agaaggggtg actgtcccac gcttgacact cacactttgt ccctgactga ctatgcttgt

1741 ggttctcctc cagttgatag agaaaaacaa cccagcgaag gcgccttctc cgaaaacaat

                                        Cla 1

1801 gccgagaatg agagcggcgg agacaagccc cccatcgatc ccaataaccc ggctgccaac

                                             c
1861 tggctacatg ctcgctccac tcggaagaag cgatgcccct acacaaaaca ccagacgctg


1971 gaactggaga aggagtttct gtttaacatg tacctcacac gggaccgcag gtacgaggtg

1981 gcccggctgc tcaacctcac cgaaaggcag gtcaagatct ggttccagaa ccgcaggatg

                        Hoxa-9 Rev PCR primer
                        gtt cctggctcgt tttctgc
2041 aaaatgaaga aaatcaacaa ggaccgagca aaagacgagt gagcctttta ggggctcatc

2101 taaaaagaga gcaagctaga aagaaaaaga aaggactgtc cgtctccctc tgtctcctct
```

B.2 pcDNA1.3/CT-GFP-TOPO

751 acaactccgc cccattgacg caaatgggcg gtaggcgtgt acggtgggag gtctatataa

T7 Forward
taatacga

811 gcagagctct ctggctaact agagaaccca ctgcttactg gcttatcgaa attaatacga

sequencing primer
ctcactatag gg

871 ctcactatag ggagacccaa gctggctagt taagcttggt accgagctcg gatccactag

EcoRV

931 tccagtgtgg tggaattgcc ct **Hoxa-9 PCR insert** aagggcaatt ctgcagatat

974 agcacagtgg cggccgctcg agtctagaat ggctagcaaa ggagaagaac ttttcttcac

1034 tggagttgtc ccaattcttg ttgaattaga tggtgatgtt aatgggcaca aattttctgt

GFP Reverse sequencing primer
cgatgtat gcctttcgaa tggg

1094 cagtggagag ggtgaaggtg atgctacata cggaaagctt acccttaaat ttatttgcac

1054 tactgga

B.3 pBluescript II KS+

T7 For sequencing primer
ta atacgactca ctatagggc

598 ttgtaaaacg acggccagtg agcgcgcgta atacgactca ctatagggcg aattggagct ccaccgcggt

SmaI Eco RI Hind III

568 ggcggccgct ctagaactag tggatcccc gggctgcagg aattcgatat caagcttatc gataccgtcg

T3 Rev sequencing primer
gggaaa tcactcccaa ttaa

628 acctcgaggg ggggcccggt acccagcttt tgttcccttt agtgagggtt aattgcgcgc ttggcgtaat

688 catggtcata gctgtttcc

B.4 Mus Musculus ventricular myosin light chain (vMLC) NM_010861

```
   1 cctcgaactc tccagaggtg gcaactggcc tcagacacca tggcaccttt gtttgccaag

  61 aagcggatag aaggcgggac gtccaacgtg ttctccatgt ttgagcagac ccagatccag

                                                 vMLC FOR PCR primer
                                                 cggcttcat cgacaagaat
 121 gagttcaagg aagccttcac aatcatggac cagaacagag acggcttcat cgacaagaat

     g
 181 gacctaaggg acacatttgc tgccctagga cgagtgaacg tgaaaaatga agagatcgat

 241 gaaatgatca aagaggctcc aggtccaatt aacttcaccg tgttcctcac gatgtttggg

 301 gagaaactta aaggggctga tcctgaagag accattctca acgcattcaa ggtgtttgat

 361 cccgagggca aagggtcact gaaggctgac tatgtccggg agatgctgac cacacaagca

 421 gagaggttct ccaaagagga gatcgaccag atgttcgcag cctttccccc tgacgttacc

 481 ggcaatcttg attataagaa tttggtccac atcattaccc acggagaaga gaaggactga

 541 gccctgaacc acagcctcag gtgacccaca gcccactctc catcccaggg ctgtgcgcaa

         vMLC REV PCR primer
        ttgtcct tcagaaccga gac
 601 ataaacagga agtcttggct ctg
```

B.5 LX-GFP retroviral vector – a gift from Dr. L Fairbarin, University of Manchester

```
1301  ACCTTCTGCT CTGCAGAATG GCCAACCTTT AACGTCGGAT GGCCGCGAGA

1351  CGGCACCTTT AACCGAGACC TCATCACCCA GGTTAAGATC AAGGTCTTTT

1401  CACCTGGCCC GCATGGACAC CCAGACCAGG TCCCCTACAT CGTGACCTGG

                                      5' sequencing primer
                                      GTCAAGCCCT TTGTACACCC
1451  GAAGCCTTGG CTTTTGACCC CCCTCCCTGG GTCAAGCCCT TTGTACACCC

1501  TAAGCCTCCG CCTCCTCTTC CTCCATCCGC CCCGTCTCTC CCCCTTGAAC

1551  CTCCTCGTTC GACCCCGCCT CGATCCTCCC TTTATCCAGC CCTCACTCCT

                                      BamH1 site
                                          |
1601  TCTCTAGGCG CCGGAATTAG CTTATCGATA GGGG|GATCCG CCCCTCTCCC

                                       3' Sequencing Primer
                                      CGCTTGGAAT AAGGCCGGTG
1651  TCCCCCCCCC CTAACGTTAC TGGCCGAAGC CGCTTGGAAT AAGGCCGGTG

1701  TGCGTTTGTC TATATGTTAT TTTCCACCAT ATTGCCGTCT TTTGGCAATG

1751  TGAGGGCCCG GAAACCTGGC CCTGTCTTCT TGACGAGCAT TCCTAGGGGT

1801  CTTTCCCCTC TCGCCAAAGG AATGCAAGGT CTGTTGAATG TCGTGAAGGA

1851  AGCAGTTCCT CTGGAAGCTT CTTGAAGACA AACAACGTCT GTAGCGACCC

1901  TTTGCAGGCA GCGGAACCCC CCACCTGGCG ACAGGTGCCT CTGCGGCCAA

1951  AAGCCACGTG TATAAGATAC ACCTGCAAAG GCGGCACAAC CCCAGTGCCA
```

B.6 Mus Musculus homeobox C9 - gi: 6680254

IMAGE cDNA clone similar to Hoxc-9 – gi: 4059801

```
Hoxc-9      gttcatacaataatcttatgtatgtaaaaccctgttacgatgtcggcgacggggcccatc 369
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
IMAGE       gttcatacaataatcttatgtatgtaaaaccctgttacgatgtcggcgacggggcccatc 60

Hoxc-9      agtaactattacgtggactcgctcatctctcacgacaatgaagacctcctagcgtccagg 429
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
IMAGE       agtaactattacgtggactcgctcatctctcacgacaatgaagacctcctagcgtccagg 120

Hoxc-9      tttccggccaccggggctcaccctgccgccgccagacccagcggcttggtgccggactgt 489
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
IMAGE       tttccggccaccggggctcaccctgccgccgccagacccagcggcttggtgccggactgt 180

Hoxc-9      agcgattttccgtcctgtagcttcgcgcccaagccggctgtattcagtacgtcgtgggcg 549
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
IMAGE       agcgattttccgtcctgtagcttcgcgcccaagccggctgtattcagtacgtcgtgggcg 240

Hoxc-9      ccggtgccctcgcagtcgtctgtggtctatcacccttacggcccccagccccacctcggc 609
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
IMAGE       ccggtgccctcgcagtcgtctgtggtctatcacccttacggcccccagccccacctcggc 300

Hoxc-9      gccgacacgcgctacatgcggacttggctcgagccgctgtccggcgccgtctccttcccc 669
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
IMAGE       gccgacacgcgctacatgcggacttggctcgagccgctgtccggcgccgtctccttcccc 360

Hoxc-9      agcttcccggccgggggccgtcactacgccctcaagcccgacgcctacccggggcgccgc 729
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
IMAGE       agcttcccggccgggggccgtcactacgccctcaagcccgacgcctacccggggcgccgc 420

Hoxc-9      gccgactgcggcccgggcgacggccgcagctacccggactacatgtac-ggctcgcccgg 788
            |||||||||||||||||||||| |||||||||||||||||||||||| |||||| ||||
IMAGE       gccgactgcggcccgggcgacggncgcagctacccggactacatgtacnggctcg-ccgg 479

Hoxc-9      ggaactgcgcgaccgcgccccgcagacgctgccctcgcccgaggcggacgcgctggccgg 848
            || |||||||||||||| ||||||||||||||||||||||||||||||||||||||||||
IMAGE       gggactgcgcgaccgcg-cccgcagacgctgccctcgcccgaggcggacgcgctggccgg 538

Hoxc-9      cagcaagcacaaagaggagaaggccgacctggaccctagcaaccccgtggccaactggat 908
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
IMAGE       cagcaagcacaaagaggagaaggccgacctggaccctagcaaccccgtggccaactggat 598

Hoxc-9      ccacgcccgttccacaaggaagaagcgctgcccctacaccaagtaccagacgctggaact 968
            |||||| |||| ||||||||||||||||||||||||||||||||||||||||||||||||
IMAGE       ccacgcncgttncacaaggaagaagcgctgcccctacaccaagtaccagacgctggaact 658

Hoxc-9      ggagaaggagtttctcttcaatatgtatttaaccagggaccgtcggtacgaggtggcccg 1028
            ||||||||||||||||||  ||||||||||||||||||||    ||||||||||||||
IMAGE       ggagaaggagtttctctttcatatgtatttaaccagggacccgtcgtacgaggtggcccg 718

Hoxc-9      tgttctcaatctcactgagcggcaggtcaaaatctggtttcagaaccggaggatgaagat 1088
            |  ||||||||||||||||||||||||||||||||||| |||||| |||  ||||| ||||
IMAGE       ttgtctcaatctcactgagcggcaggtcaaaatctgggttcagacccgagggatg-agat 777
```

B.7 Mus musculus mRNA for Hoxa-9 – gi: 3080547
IMAGE cDNA clone similar to Hoxc-9 – gi: 4059801

```
IMAGE     588  gccaactggatccacgcncgttncacaaggaagaagcgctgcccctacaccaagtaccag 647
               ||||||||| | || || || | |||  |||||||||| ||||| ||||| ||  |||||
Hoxa-9    1855 gccaactggctacatgctcgctccactcggaagaagcgatgcccttacacaaaacaccag 1914

IMAGE     648  acgctggaactggagaaggagtttctctttcatatgtatttaaccagggacccgtcgtac 707
               |||||||||||||||||||||||||| ||| | |||||  | ||  ||||||    ||||
Hoxa-9    1915 acgctggaactggagaaggagtttctgtttaacatgtacctcacacgggaccgcaggtac 1974

IMAGE     708  gaggtggcccgttgtctcaatctcactgagcggcaggtcaaaatctgggttcagacccga 767
               ||||||||||    ||||| ||||| ||  |||||||||| |||||| | |||| |||
Hoxa-9    1975 gaggtggcccggctgctcaacctcaccgaaaggcaggtcaagatctggttccagaaccgc 2034

IMAGE     768  gggatga 774
               |||||||
Hoxa-9    2035 aggatga 2041
```