ELSEVIER

Mechanisms of Development 66 (1997) 83–93

The *XHex* homeobox gene is expressed during development of the vascular endothelium: overexpression leads to an increase in vascular endothelial cell number

Craig S. Newman, Frank Chia, Paul A. Krieg*

Institute for Cellular and Molecular Biology and Department of Zoology, University of Texas at Austin, Austin, TX 78712, USA

Received 31 March 1997; received in revised form 12 June 1997; accepted 16 June 1997

Abstract

The *Hex/Prh* homeobox gene is expressed in a subset of adult blood cell types and may play a role in the differentiation of the myeloid and B-cell lineages. In a search for homeobox genes involved in cardiovascular development, we have independently isolated a *Xenopus laevis* cDNA which appears to be the amphibian orthologue of *Hex/Prh*. Based on high sequence similarity in a number of regions, particularly the critical homeobox, we have named this gene *XHex*. This developmentally regulated gene is first expressed in the dorsal endomesoderm of the gastrula stage embryo. This tissue goes on to contribute to the structures of the embryonic liver and *XHex* continues to be expressed in the liver throughout development. From the tailbud stage, *XHex* is expressed in vascular endothelial cells throughout the developing vascular network. Vascular expression of *XHex* is transient and commences slightly after expression of the receptor tyrosine kinase gene, *flk-1*, which is known to be essential for vascular development. This observation raises the possibility that *XHex* is one of the transcription factors that responds to the VEGF/Flk-1 signal transduction pathway leading to differentiation of vascular endothelial cells. *XHex* is unique amongst homeobox genes in displaying expression in the endothelial layer throughout the developing vasculature. Overexpression of *XHex* sequences in the frog embryo causes disruption to developing vascular structures and an increase in the number of vascular endothelial cells, suggesting a possible role in regulation of cell proliferation. © 1997 Elsevier Science Ireland Ltd.

Keywords: Xenopus; Homeodomain protein; Vasculogenesis; Liver; Thyroid gland

1. Introduction

While the first homeobox genes to be isolated were found to play essential roles in the specification of segmental identity or in the anterior/posterior patterning of the embryo, other homeobox genes appear to play a role in organogenesis. For example, homeobox genes are important for the development of the thyroid (Lazzaro et al., 1991; Guazzi et al., 1990, 1994), the heart (Komuro and Izumo, 1993; Lints et al., 1993) the spleen (Roberts et al., 1994; Dear et al., 1995) and the liver (Hentsch et al., 1996). Work in many developmental systems suggests that homeodomain proteins may be acting in the determination of cell fate as well as in other aspects of organogenesis. Other homeobox genes that may be involved in tissue determination are members of the *Hex/Prh* family. Homologues of the *Hex* gene have been isolated from mouse, human and chicken, and it has been suggested that *Hex* is involved in the differentiation of a subset of blood cells (Bedford et al., 1993; Crompton et al., 1992). Expressed primarily in B-cell and monomyeloid lineages, and also in pluripotent progenitor lines, *Hex* expression is absent from both the mature erythroid and T-cell lineages, suggesting that *Hex* expression in the precursor cell may influence hematopoietic differentiation.

Recently, a large amount of work has focused on determining the role of homeobox genes during cardiovascular development, especially on the mechanisms underlying specification and differentiation of myocardial tissue. In particular, a family of vertebrate homeobox genes related to the *Drosophila tinman* (*tin*) gene are found to be expressed from the earliest stages of heart development (Lints et al., 1993; Komuro and Izumo, 1993). At least three *tinman-*

* Corresponding author. Tel.: +1 512 4718785; fax: +1 512 4719651; e-mail pkrieg@mail.utexas.edu

0925-4773/97/$17.00
PII S0925-4773(97)00092-0

related genes, *Nkx2.3*, *Nkx2.5* and *Nkx2.7* are expressed in the heart forming region in zebrafish (Lee et al., 1996) and a new member of the family, *Nkx2.8* has recently been identified in developing heart tissues in chick and mouse (Boettger et al. 1997; Brand et al., 1997). In the case of *Nkx2.5*, ablation of gene function in the mouse leads to atypical cardiac development (Lyons et al., 1995). In experiments with *Xenopus* (Cleaver et al., 1996) and zebrafish (Chen and Fishman, 1996), overexpression of *Nkx2.3* or *Nkx2.5* leads to enlarged heart structures. Overall, these experiments suggest an important role for the *tinman*-related homeobox genes in embryonic cardiac development.

In contrast, little is currently known about the transcription factors involved in formation of the endocardial layer of the heart and development the embryonic vasculature. Most recent work in vascular development has focused on a signal transduction cascade that is essential for the specification of vascular tissue. Vascular endothelial growth factor (VEGF), a secreted protein, and its associated receptor tyrosine kinases Flk-1 and Flt-1 are key players in the formation of blood vessels. Specifically, both *flk-1* and *flt-1* transcripts are detected in pre-endothelial cells prior to final differentiation into vascular tissue and are thought to transduce the VEGF signal, leading to the formation of blood vessels. Ablation of either *VEGF* or *flk-1* gene function results in embryos severely lacking in vascular tissue,

1A

```
ATGCAGTACCAGCACCCCAGCTCCTCAGCTCTGGGGCTCAGTGTCCCCCTGTTCGCTCCC    60
 M  Q  Y  Q  H  P  S  S  S  A  L  G  L  S  V  P  L  F  A  P
ACCCCTCTGCAGCACCCAACTCCCTTCTACATTGATGATATCCTGGGAAGGAACAGCGCA   120
 T  P  L  Q  H  P  T  P  F  Y  I  D  D  I  L  G  R  N  S  A
TCTAATGGGACCCCAGCCTTACCCACCCCAACCCTGCCATCTCCCAACTCATCCTTCACC   180
 S  N  G  T  P  A  L  P  T  P  T  L  P  S  P  N  S  S  F  T
AGCCTGGTGGCTACTTACCGGACTCCCATCTATGAGCCTACTCCCATTCACCCTGCCTTC   240
 S  L  V  A  T  Y  R  T  P  I  Y  E  P  T  P  I  H  P  A  F
ACCCACCCAGGGGCAGCCCTAGCAGCTTCCTATGGAGCCAGCACCTATGCCTCCCCTCTG   300
 T  H  P  G  A  A  L  A  A  S  Y  G  A  S  T  Y  A  S  P  L
TACCCCTTCTCCAGGCCAGTCAGCGACTACACCCACGCCCTCATCCGACACGACTCTCTG   360
 Y  P  F  S  R  P  V  S  D  Y  T  H  A  L  I  R  H  D  S  L
GGCAAACCCCTGCTCTGGAGCCCCTTCATCCAGAGGCCTCTGCACAAGCGGAAAGGAGGA   420
 G  K  P  L  L  W  S  P  F  I  Q  R  P  L  H  K  R  K  G  G
CAAGTGAGGTTTTCCAACGATCAAACCATTGAGTTGGAGAAAAAATTCGAGACGCAAAAA   480
 Q  V  R  F  S  N  D  Q  T  I  E  L  E  K  K  F  E  T  Q  K
TATTTATCGCCCCCTGAGAGGAAGCGACTGGCTAAAATGCTGCAGCTCAGCGAGAGACAG   540
 Y  L  S  P  P  E  R  K  R  L  A  K  M  L  Q  L  S  E  R  Q
GTCAAAACCTGGTTCCAGAACAGAAGAGCCAAATGGAGGCGTCTCAAGCAGGAGAACCCA   600
 V  K  T  W  F  Q  N  R  R  A  K  W  R  R  L  K  Q  E  N  P
CAGGGAAATAAGAAAGACGAAACCGAAAGTCTAGAAAATATCTGCGAAGAGAGTCAGGAG   660
 Q  G  N  K  K  D  E  T  E  S  L  E  N  I  C  E  E  S  Q  E
AGGTGTTTGAGCGCCGAGCAGAAGAGCAGAGAGTCCTCCCTGGATGATCCCACCTCGTCG   720
 R  C  L  S  A  E  Q  K  S  R  E  S  S  L  D  D  P  T  S  S
CCCACCTCTCAAGGGAACCTGGATTCGGAGGTGTCTGACGATTCCGACCAGGAGGTGGAC   780
 P  T  S  Q  G  N  L  D  S  E  V  S  D  D  S  D  Q  E  V  D
ATTGAAGGCGACAAAGGATATTACAACTGTGCACATTAA  819
 I  E  G  D  K  G  Y  Y  N  C  A  H  *
```

B

```
                                                                  percent
                                                                  identity
XHex   KRKGGQVRFSNDQTIELEKKFETQKYLSPPERKRLAKMLQLSERQVKTWFQNRRAKWRRL
HEX    ------------------------------------------------------------ 100%
Hex    ----------V------------------------------------------------- 98%
Prh    ----------E----------------------L---------------------------- 97%
```

Fig. 1. Nucleotide and amino acid sequence of the *Xenopus Hex* gene. (A) *XHex* open reading frame and deduced amino acid sequence. Numbers refer to the cDNA sequence. The homeodomain is underlined and the TN domain is indicated by a dashed line. GenBank accession number for the *XHex* sequence is U94837. (B) A comparison of the XHex homeodomain with homeodomain sequences of other members of the Hex family. The unique pair of proline residues in the Hex/Prh proteins is underlined.

demonstrating the critical role of these genes in the formation of the cardiovascular system (Carmeliet et al., 1996; Ferrara et al., 1996; Shalaby et al., 1995). While this signal transduction system is likely to activate transcription factors required for vascular differentiation, to date, no homeobox gene has been found which is expressed in all endothelial cells and which may therefore play a role in vasculogenesis equivalent to that of the *tinman* family during cardiogenesis.

Here we report the cloning of *XHex*, a homeobox gene that probably represents the *Xenopus* homologue of the mouse *Hex* gene. *XHex* expression is regulated both spatially and temporally during early development and may play a role in the specification of several embryonic tissues including the liver and thyroid. Uniquely amongst homeobox genes, *XHex* is expressed in endothelial cells throughout the developing vascular system. In vascular tissues, it seems likely that *XHex* functions downstream of the VEGF/Flk-1 signaling pathway. Overexpression of XHex in the embryo, by injection of synthetic mRNA, leads to disruption of vascular structures and an increase in the number of vascular endothelial precursor cells.

2. Results

2.1. Isolation of the XHex clone

In a systematic screen for homeobox genes expressed in the amphibian cardiac anlage, a *Xenopus laevis* adult heart cDNA library (Ji et al., 1993) was probed with sequences containing the homeobox region of the *Hox 11*-related *Crobar* gene (Patterson, in prep.). Isolated recombinants were sequenced using a degenerate oligonucleotide primer complementary to the sequence coding for the third helix of the homeodomain. One clone displayed a high level of sequence similarity to the murine *Hex* gene. Further sequencing of the *XHex* cDNA revealed an 816 bp open reading frame encoding a 272 amino acid protein with a predicted molecular weight of 30.6 kDa (Fig. 1A). Over the entire length of the ORF, XHex is 75% identical to mouse *Hex* at the amino acid level. This is consistent with previous studies which indicate that homologous genes in *Xenopus* and mouse are approximately 70–75% identical (Tonissen and Krieg, 1993, 1994). Within the homeodomain, the mouse and frog proteins differ only at a single amino acid residue in helix 1, a conservative substitution of an isoleucine for a valine. XHex is 100% identical to the human HEX protein within the homeodomain, while the chicken homologue, Prh, differs at two sites (Fig. 1B). Analysis of the XHex protein sequence also reveals an abundance of proline residues (16% of all amino acids) in the 135 amino acid segment N-terminal to the homeodomain. This characteristic is conserved in both the mouse and chicken proteins, with prolines accounting for 21% and 20% of residues, respectively, in the equivalent region. Near the N-terminus of the protein is a ten amino acids sequence related to the TN-Domain found in the NK-2 family of homeobox genes (Harvey, 1996). A similar sequence is found in a number of other homeodomain proteins including engrailed, Hlx and goosecoid and is thought to act as a transcription repressor (Smith and Jaynes, 1996). Like the other Hex proteins, XHex contains a concentration of aspartate and glutamate residues in the C-terminal region which could possibly function as an acidic transcriptional activation domain (Ma and Ptashne, 1987).

2.2. Embryonic expression of XHex

The developmental profile of *XHex* expression was determined using RNase protection (Fig. 2). No expression of *XHex* is detectable in maternal RNA or in cleavage stage embryos. During gastrulation levels of *XHex* RNA rise rapidly, peaking at about stage 10.5. Following gastrulation the levels of *XHex* transcript exhibit a slight but reproducible decrease and then increase again during early tailbud stages. *XHex* continues to be expressed in the latest embryonic stages tested (stage 41).

Hex genes have previously been isolated from chicken,

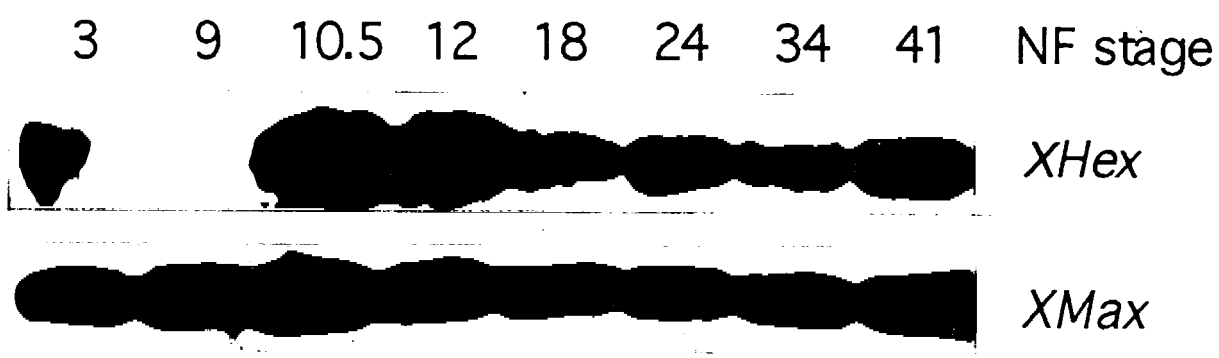
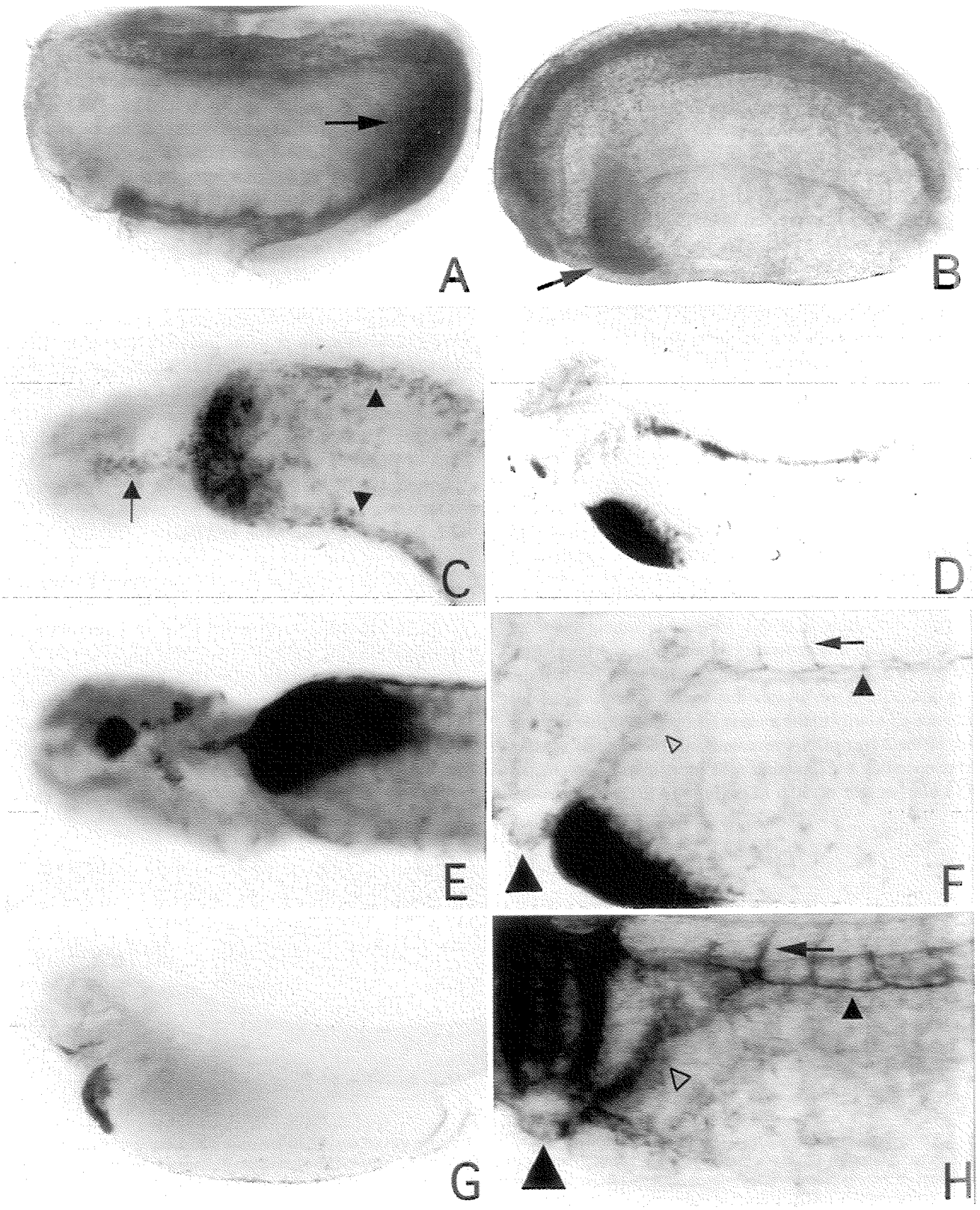


Fig. 2. Embryonic expression of *XHex*. Total RNA from the equivalent of five embryos was assayed by RNase protection using standard techniques. The *XHex* probe represents a 190 nt fragment from the 3′ end of the coding region. The probe for the *XMax* transcript, which is expressed at a constant level throughout early development, was included in the same tube to control for RNA recovery. The embryonic stages (Nieuwkoop and Faber, 1994) are indicated at the top of figure.

mouse and human (Bedford et al., 1993; Crompton et al., 1992). While these studies show that *Hex* genes are expressed in a large number of cell lines within the hematopoietic lineage and also in adult liver and lung, very little information is available on embryonic expression. To characterize expression of *XHex* during embryonic development, whole-mount in situ hybridization studies were performed (Figs. 3–5). In accordance with the RNase pro-



tection data, *XHex* transcripts are first detected during gastrulation. In the stage 10.5 embryo, *XHex* transcripts are localized to the endomesoderm of the dorsal side, but are absent from the overlying ectodermal cells (Fig. 3A). Sagittal sections through early gastrula embryos (st 10.5) confirm the presence of *XHex* transcripts in the dorsal endomesoderm (Fig. 4A). Sectioning of early neurula stages (st 16) demonstrates the presence of *XHex* positive cells in the endodermal layer of the anterior gut (Fig. 4B) and expression is maintained in these cells throughout neurulation. By the post-neurula stage (st 23) the *XHex* positive cells are found in the ventral, anterior gut (Fig. 3B), in a position consistent with the forming hepatic diverticulum (Hausen and Riebesell, 1991). *XHex* transcripts are present in the liver region at all stages examined (Figs. 3B–G, 4C and 5A and data not shown) and persists after hepatic differentiation has commenced, as assayed by expression of the liver markers fibrinogen and transferrin (data not shown).

In addition to the liver, *XHex* is expressed in precursors of the developing thyroid gland. Beginning at the tailbud stage, the ventral region of the head is intensely labeled (Fig. 3D, E, G) and anterior cross-sections of tailbud stage embryos show *XHex* positive tissue along the midline, ventral to the oral cavity. This is the region of the embryo that will give rise to the thyroid gland (Nieuwkoop and Faber, 1994). At the earliest stage of expression in this region (st 28), the anterior-most edge of staining is found in the pharyngeal floor itself (Fig. 4D) while staining in more posterior sections occurs in the tissue below the floor of the foregut (data not shown). As development proceeds, this group of *XHex* expressing cells becomes localized below the floor of the pharynx (Fig. 4E–F). By the late tailbud stage (st 37), the region of *XHex* expression extends posteriorly nearly to the level of the heart, consistent with the location of the thyroid at this stage (Nieuwkoop and Faber, 1994).

The other major domain of *XHex* expression in the embryo is in developing vascular tissues. During early tailbud stages (st 27), *XHex* expression can be seen on the ventral side of the embryo in bilateral stripes of cells flanking the forming blood islands (Fig. 3C). This is the region that will give rise to the vitelline veins. In order to investigate the relationship between *XHex* expressing cells and the blood cell precursors, we have also probed for sequences encoding *αT-globin*, the first globin to be expressed during *Xenopus* embryonic development (Patient et al., 1982). Cross-sections through an early tailbud embryo (st 30) stained for both *XHex* and *αT-globin* (Fig. 5C) show a close association between cells expressing these two markers. In addition, *XHex* expression is detected in developing endocardial tissues, which are visible as a thin line of cells projecting anteriorly from the forming liver (Fig. 3C). While expression of *XHex* soon decreases in the developing vitelline veins becoming undetectable by the mid tailbud stage (st 32), expression in the endocardial tissues intensifies, eventually marking the inner layer of cells surrounding the lumen of the heart (Fig. 3D–F). Sections at the level of the heart primordia of a tailbud stage embryo (st 30) reveal that the *XHex* positive cells of the endocardium are closely associated with the expressing cells in the developing liver (Fig. 5A). The surrounding myocardium is clearly negative and is never observed to express *XHex*. At the early tailbud stage, *XHex* expression is also detected in precursors of the posterior cardinal veins, which are visible as parallel stripes, ventral to the somite, on either side of the embryo (Figs. 3D and 5B). Slightly later in development, sprouting dorsally from this line of stained tissue are thin strands of *XHex* positive cells which appear to be developing intersomitic veins (Figs. 3F and 5D). A very similar pattern of expression is observed (Fig. 3H) in embryos probed for *X-msr* transcripts, which mark the developing vascular system (Devic et al., 1996). Also at this stage, *XHex* expression is seen in cross-sections through the head, where small foci of expressing cells can be detected near the brain and eye (data not shown). At late tailbud stages, when definitive vascular structures are forming, *XHex* transcripts are detected in many of the major blood vessels including the cardinal veins and the dorsal aorta (Fig. 5D, E) and in the paired ventral aortae (Fig. 5F). Double-stained whole-mount in situ hybridization using probes for *XHex* and *αT-globin* reveals that these vessels contain erythroid cells (Fig. 5D–E), indicating that they are functional vascular tissue. By the tadpole stage (st 39), *XHex* expression is limited to the liver and ventral head, having disappeared from the dorsal region of the embryo (Fig. 3G). We conclude therefore, that *XHex* is transiently expressed in the endothelial layer of developing vascular structures in the embryo prior to, and slightly after, the time of vascular differentiation.

Fig. 3. Whole-mount in situ hybridization detection of *XHex* expression. With the exception of (A), anterior is to the left. (A) Lateral view of gastrula stage embryo stained for *XHex* showing expression in the embryonic endomesoderm (arrow). Dorsal is to the right. (B) Lateral view of a stage 23 embryo showing *XHex* expression in the anterior foregut (arrow), at the position of the hepatic diverticulum. (C) Ventral view of an early tailbud (st 27) embryo displaying *XHex* staining in the liver and in the vitelline vein precursors projecting posteriorly (arrowhead) and the pre-endocardial cells projecting anteriorly (arrow). (D) Lateral view of a stage 30 embryo. Strong *XHex* expression is apparent in the forming liver and in the ventral head. Note additional expression in the endocardium and the posterior cardinal vein. (E) Ventral view of a stage 32 embryo. Staining in cells anterior to the heart and in a region of the head is readily apparent. (F) A lateral view of the embryo shown in (E). Note expression in the forming vasculature including the endocardium (large arrowhead), posterior cardinal vein (small arrowhead), common cardinal vein (open arrowhead) and intersomitic veins (arrow). (G) A lateral view of stage 39 embryo demonstrating the transient nature of *XHex* staining in the vasculature. Expression in the liver and ventral head is still readily apparent. (H). Enlarged view of a tailbud stage embryo stained for *X-msr*, a marker of the forming vasculature. Compare with *XHex* staining in (F) and note that the same vascular structures are detected.

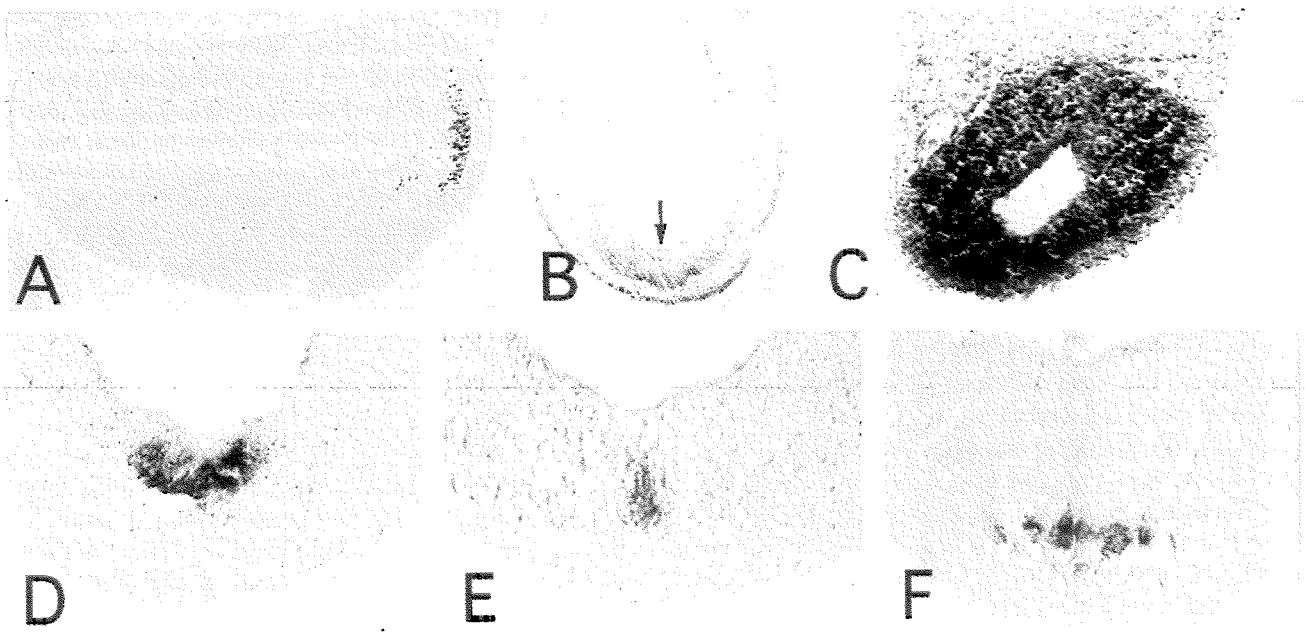


Fig. 4. Sections through embryos stained for *XHex* showing expression in the hepatic (AC) and thyroid (D–F) precursors. (A) and (B) are viewed at 100× magnification while (C) is 200×. All other sections are 400× magnification. (A) Sagittal section through an early gastrula stage embryo (st 11) showing *XHex* expression in the dorsal endomesoderm. Dorsal is to the right. (B) Cross-section through a neurula stage embryo. *XHex* expression can be seen in ventral endoderm (arrow). (C) Cross-section through a tailbud stage embryo (st 32) stained for *XHex*. Expression is concentrated in tissue surrounding the lumen of the hepatic diverticulum. (D) Cross-section through the pharyngeal cavity of an early tailbud stage embryo (st 32) showing *XHex* expression in the floor of the oral cavity. (E and F) Cross-sections through a late tailbud stage embryo (st 37) showing *XHex* transcripts in the anterior (E) and posterior (F) thyroid rudiment. The domain of *XHex* expression expands laterally in the more caudal sections.

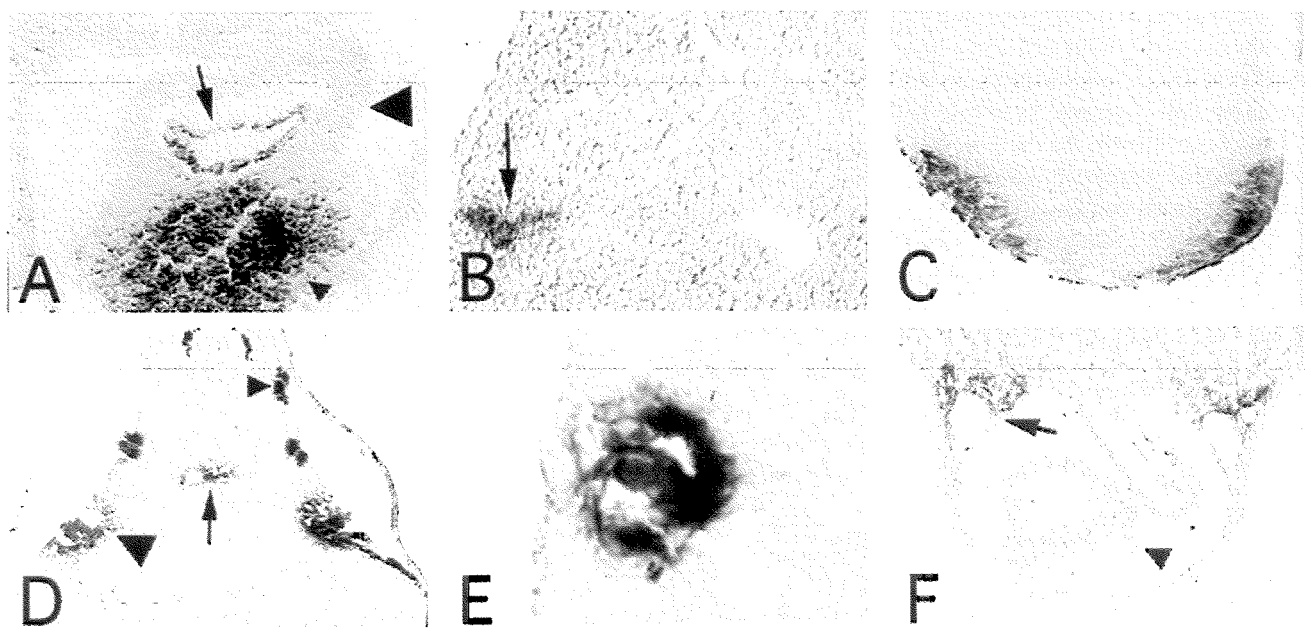


Fig. 5. Sections through embryos stained for *XHex* showing expression in the forming vasculature. All sections are viewed at 400× magnification except (C) and (D) which are 200×. Ventral is down in all sections. (A) Section through a tailbud stage embryo (st 32) at the level of the heart showing *XHex* expression in the endocarium (arrow) but not in the myocardium (large arrowhead). *XHex* transcripts are abundant in the hepatic precursors (small arrowhead). (B) Cross-section through an early tailbud stage embryo (st 28) showing *XHex* expression in the precursors to the posterior cardinal vein (arrow). (C) Cross-section through a stage 30 embryo double stained for both *XHex* (purple) and *globin* (turquoise) at the level of the anterior ventral blood island. *XHex* expressing cells are visible coalescing around the *globin* positive cells. (D) *XHex* (purple) and *globin* (turquoise) expression in a stage 34 embryo. *XHex* marks the forming vascular endothelium of the posterior cardinal vein (large arrowhead), dorsal aorta (arrow) and intersomitic veins (small arrowhead). (E) Enlarged view of posterior cardinal veins in the stage 34 embryo shown in (D) (F) Cross-section through a late tailbud stage embryo (st 37) in the anterior cardiac region showing *XHex* in the ventral aortae (arrow) but not in the myocardium (arrowhead).

Fig. 6. Overexpression of *XHex* mRNA results in an increase in the number of vascular endothelial precursor cells. Embryos were injected on the left side at the 8–16 cell stage. All embryos are stained with the *X-msr* vascular marker (Devic et al., 1996). (A) Uninjected side of an early tailbud stage embryo (st 31). (B) Injected side of the embryo pictured in (A). Note the increase in *X-msr* staining in the posterior of the embryo. (C) and (D) Close-up views of the embryos shown in (A) and (B) respectively. (E) Caudal region of the uninjected side of a mid-tailbud embryo (st 36), at the time of intersomitic vessel formation. (F) The injected side of the embryo presented in panel (E) again showing an increase in *X-msr* staining cells.

2.3. Overexpression of XHex sequences

To further examine the role of the *XHex* gene during vascular development, synthetic messenger RNA was injected into one side of an early blastula embryo. After growing to the tailbud stage, injected embryos were assayed for alterations to the vasculature by whole-mount in situ hybridization using a probe for the vascular marker *X-msr* (Devic et al., 1996). The uninjected side of each embryo serves as a sensitive control for vascular structure. These experiments indicate that overexpression of *XHex* mRNA results in abnormalities in vascular structures on the injected side (Fig. 6). These alterations are most conspicuous in the developing cardinal veins, which show a disorganization of vascular structure and an increased number of vascular precursor cells, relative to controls. This increased cell number is also observed in the developing intersegmental veins which are attached to the posterior cardinal vein (data not shown). Disruptions of the vascular pattern are observed in 50% (23/46) of *XHex* injected embryos, but only 11% (11/54) of control injected embryos. The severe vascular phenotype (Fig. 6) is observed in 11% (5/46) of *XHex* injected embryos but never in control injected embryos. While the proportion of the severe phenotype is relatively low, this is not really surprising, since only a very small percentage of the injected *XHex* mRNA is likely to remain in the embryo

at the time of vascular development (Vize et al., 1991). It is important to note that we have never observed ectopic expression of vascular marker sequences in *XHex* injected embryos.

3. Discussion

3.1. XHex expression the developing liver

In a search for novel homeobox genes that may play a role in cardiovascular development we have isolated a cDNA encoding XHex, the *Xenopus* homologue of chicken Prh and mouse Hex. When examined by whole mount in situ hybridization, the earliest site of *XHex* expression is the dorsal endomesoderm of the gastrula. This region of the embryo ultimately contributes to the structures of the foregut, including the liver (Bouwmeester et al., 1996). While a large number of genes are known to be expressed in the neighboring involuting prechordal mesoderm, *XHex* is one of only a few genes expressed in developing endodermal tissues. Interestingly, the expression pattern of the *cerherus (cer)* gene, which encodes a novel secreted protein, (Bouwmeester et al., 1996) appears to overlap that of *XHex*. However, while *cer* is only transiently expressed in these cells, *XHex* expression is maintained in the liver lineage throughout development.

The first overt sign of liver organogenesis is the formation of the liver diverticulum which forms as an outpocketing of the ventral foregut during neurulation. Experiments performed primarily in chick have shown that hepatic precursors are specified prior to this time perhaps by signals arising from the cardiac precursor cells (Le Douarin, 1975). Consistent with this model, cardiac precursors in the amphibian are specified during the early stages of gastrulation, through interactions with the dorsal lip of the blastopore (Sater and Jacobson, 1989; Sater and Jacobson, 1990). These newly-specified cardiac progenitor cells may in turn signal adjacent endodermal cells to develop into liver. Therefore. the appearance of *XHex* transcripts during mid-gastrulation could represent an immediate response to inductive signals emanating from the nearby cardiac precursor cells.

The mouse and chicken *Hex* genes have been detected in a range of hematopoietic cell lines (Bedford et al., 1993; Crompton et al., 1992) and are expressed particularly strongly in the B-cell lineage. As in other organisms. the *Xenopus* liver is a site of hemopoiesis, however, in frogs, this does not occur until the late tadpole stage of development. much later than any of the embryos examined in this study. It is therefore unlikely that the observed expression of *XHex* in the early liver is due to the presence of blood precursor cells. In support of this argument, *XHex* expression is clearly seen in the endodermal layer of the neurula stage embryo (Fig. 4B), while blood precursor cells are known to originate from the mesodermal germ layer. Furthermore, organ transplant experiments have demonstrated that B-cell precursors do not colonize the embryonic liver until stage 39 (Hadji-Azimi et al., 1982). Thus, it seems unlikely that the *XHex* expressing cells observed in the early liver anlage are fated to become blood cells.

3.2. XHex expression in the developing thyroid gland

A previous anatomical description of the formation of the thyroid gland (Nieuwkoop and Faber, 1994) states that the thyroid anlage first appears as a medial depression in the floor of the oral cavity at the tailbud stage (st 33). The endodermal epithelium thickens and forms a caudal-directed finger-like projection which is the developing thyroid gland. Cross-sections of in situ hybridized embryos reveal that *XHex* is expressed in these tissues at the floor of the oral cavity commencing at the early tailbud stage (Fig. 4D). prior to the first signs of overt morphological differentiation, thereby suggesting a role for *XHex* in the formation of the thyroid. Another homeobox gene, *TTF-1*, is also expressed in the predifferentiation thyroid in rat (Lazzaro et al., 1991), as is the mammalian paired box gene, *Pax8* (Plachov et al., 1990; Poleev et al., 1992). Considering the known ability of homeodomain proteins to form multimers (Wilson et al., 1995), these proteins could potentially interact to bring about differentiation of the thyroid.

3.3. XHex expression in the developing vascular system

Perhaps the most striking observation resulting from the embryonic expression studies is the presence of *XHex* transcripts in the developing vascular endothelium. While two other homeobox genes, *Gax* and *Hoxa*-2 (formerly *Hox 1.11*) are expressed in vascular tissues, both are expressed in the layer of vascular smooth muscle cells (VSMC) that surround the vascular endothelium (Gorski et al., 1993; Patel et al., 1992). *Gax* is expressed in the VSMC of the adult rat aorta and is believed to be involved in the regulation of the cell cycle (Gorski et al., 1993). *Hoxa-2* is expressed in a number of embryonic tissues, including the VSMC layer of vessels leading from the heart and in the adult aorta. Neither gene, however, is transcribed in the endothelial layer of cells. In addition. another homeobox gene, *Msx-1*, is expressed in a variety of tissues including the endocardium of the heart (Roberts et al., 1989). However, endocardial expression of *Msx-1* is limited to a subset of cells that are destined to delaminate from the endothelial layer to form the endocardial septa and cushions. Thus, the *XHex* homeobox gene appears to be unique in exhibiting widespread expression in the endothelial layer of the cardiovascular system.

Our experiments demonstrate that *XHex* is expressed in a number of embryonic blood vessels including the vitelline and posterior cardinal veins, the dorsal aorta and the endocardium of the heart. We also observe *XHex* expression in the developing intersomitic veins (Figs. 3F and Fig. 5D).

Interestingly, while *XHex* expression is maintained in hepatic tissue throughout embryogenesis, transcripts are only transiently present in the endothelial precursor cells. This transient expression suggests that *XHex* is unlikely to be playing a role in the maintenance of the differentiated phenotype of the vascular cells, but may be involved in the differentiation process itself. One protein known to be essential for vascular development is the receptor tyrosine kinase Flk-1, (Millauer et al., 1993; Yamaguchi et al., 1993), which is a high affinity receptor for VEGF. Significantly, comparison of the expression patterns of the *XHex* and *Xenopus flk-1* genes reveals a striking spatial similarity (Cleaver et al., 1997). However, it is clear that *XHex* transcription commences several hours after the onset of *XFlk* expression in vascular endothelial tissues. This raises the intriguing possibility that *XHex* is a downstream target of the *VEGF/Flk-1* signal transduction pathway. Overexpression of the *XHex* gene results in disruption of vascular structures and an increase in the number of pre-endothelial cells in the embryo. The observed phenotype is similar to, but less dramatic than that seen when VEGF, a known vascular endothelial cell mitogen (Leung et al., 1989), is overexpressed (Cleaver et al., 1997). We also note that, although lineage tracing indicates that a significant proportion of the cells on the injected side of the embryo receive mRNA, no ectopic prevascular cells are ever observed following *XHex* overexpression. In all cases, only existing vessels are atypical, generally appearing thicker and disorganized compared with the control side of the embryo. This result suggests that *XHex* alone is not capable of specifying an endothelial cell fate, but may play a role in cellular proliferation.

The expression of mammalian and avian *Hex* in a subset of the adult blood cells raises the question of whether *XHex* is playing the same role in the adult frog. Contributions to the blood come from two mesodermal tissue in the embryo, the ventral blood island (VBI) and the dorso-lateral plate (DLP). The VBI contributes to the erythroid and leukocyte populations in both the embryo and the adult, while the DLP produces only late larval and adult blood cells (Maeno et al., 1985). In *Xenopus* transcripts of the GATA-2 and 3 genes serve as markers for both the VBI and DLP mesoderm prior to the differentiation of the blood (Bertwistle et al., 1996). Comparing the GATA and *XHex* expression patterns, the only region of overlap occurs in the DLP, although GATA expression slightly precedes *XHex* transcription. Despite this apparent coincidence of expression, it is unclear whether *XHex* expression occurs in the same blood precursor cells as GATA or in the posterior cardinal vein which is forming nearby. Certainly, the major domains of *XHex* expression in the developing embryonic vasculature are not consistent with hematopoietic tissues. Overall therefore, studies of embryonic expression of *XHex*, reveal little evidence to support a role in differentiation of the early hematopoietic lineage. We note however, that a survey of adult tissues indicates that the spleen expresses *XHex* at high levels (data not shown). Since the spleen is a hematopoietic organ rich in B-cells, this observation is consistent with *XHex* expression in differentiating blood cells in the adult. Recently, Asahara et al. (1997) have reported the isolation of putative endothelial progenitor cells in circulating peripheral blood. It seems possible that as pre-endothelial cells, this group of precursors may represent a subset of *HEX* expressing cells found in blood.

In addition to its apparent role during embryonic neovascularization, *Hex* may also be involved in other aspects of vascular growth and repair that are of medical significance. For example, it is known that secretion of VEGF by tumor cells leads to increased vascularization of tumors, presumably via the *Flk-1* signaling pathway (Ferrara et al., 1992). If *Hex* is indeed a target of the *VEGF/Flk-1* signal transduction pathway, we predict that *Hex* expression will be upregulated during tumor angiogenesis. It is therefore possible that initiation of *Hex* transcription is an early step in the pathway leading to vascularization of tumors. Similarly, the VEGF/Flk-1 pathway is known to be activated after damage to the endothelial walls of blood vessels after angioplasty. Previous work by Weir et al. (1995) has shown that expression of homeobox genes is altered by denudation of the endothelium. In this case, *Gax* is down-regulated in VSMC in response to vascular damage caused by balloon angioplasty. Similarly, it is possible that *Hex* expression will be activated in the remaining endothelial cells of the damaged vessels, serving as a positive indicator of injury repair. In conclusion, we believe that understanding the biological function of the *Hex* genes will be important, not only for detailing the series of events leading to development of the embryonic hepatic and vascular systems, but also for understanding medical conditions involving vascular growth and repair.

4. Experimental procedures

4.1. Isolation of XHex sequences

Approximately 10^6 plaques of a *Xenopus laevis* adult heart cDNA library (Ji et al., 1993) were plated and screened at low stringency (5 × SSC/0.1% SDS at room temperature for 30 min) with ^{32}P-labeled DNA probe containing the homeobox of the *Crobar* gene (Patterson, in prep.). Of the recombinants detected under these low stringency conditions, a single clone contained *Hex* related sequences when sequenced using the homeobox degenerate primer 5′-AACCANGTYTTNACYTG-3′. Sequencing of this clone yielded the entire *XHex* open reading frame.

4.2. Whole-mount in situ hybridization and sectioning

Digoxygenin-labeled antisense RNA probes were prepared using the standard protocol (Boehringer Mannheim). *XHex* template was linearized using Not I and transcribed using T7 RNA polymerase to yield a 2.2 kb product. *X-msr* probe was prepared with T7 RNA polymerase after linear-

ization with Bam HI. *αT-globin* probe was transcribed with SP6 RNA polymerase from template linearized with Eco RI. Whole-mount in situ hybridization was carried out as described by Harland (1991) except that CHAPS was omitted at all steps. Embryos were then rinsed in PBS, dehydrated in EtOH (3 × 10 min), incubated in xylene (2 × 10 min). incubated in paraplast (1 × 10 min in 1:1 xylene/paraplast; 1 × 30 min, 1 × overnight in 100% paraplast) and embedded in paraplast. Sections (10 μm) were cut, mounted in Permount and observed by differential interference optics. All embryos are staged according to Nieuwkoop and Faber (1994).

4.3. RNase protection and RT-PCR analysis

Embryonic RNA was isolated by homogenizing embryos in 50 mM Tris-Cl (pH 7.5), 50 mM NaCl, 10 mM EDTA, and 0.59% SDS. Proteinase K was added to 0.24 mg/ml and after 1 h at 37°C, the homogenate was phenol/chloroform extracted and isopropanol precipitated. A further purification was achieved by resuspending the RNA and precipitating with an equal volume of 8 M LiCl. RNA from the equivalent of five embryos was then subjected to RNase protection analysis as described by Krieg and Melton (1987) using an antisense probe containing the final 190 nt of the *XHex* coding region. As a control, the *XHex* protection reaction contained a probe for the *Xmax* sequence which is expressed at constant levels throughout early development (Tonissen and Krieg, 1994).

4.4. Overexpression of XHex mRNA

The *XHex* open reading frame was cloned into both the pT7TS (Cleaver et al., 1996) and pCS2 + (Turner and Weintraub, 1994) transcription vectors and synthetic messenger RNA was prepared using the T7 or SP6 mMessage mMachine Kits (Ambion Inc.). The resultant capped RNA was phenol/chloroform extracted and separated from the unincorporated cap analog on a Sephadex G-50 Nick column (Pharmacia Biotech). The mRNA was stored at −20°C as an ethanol precipitate. Approximately 250 pg of *XHex* mRNA, mixed with tetramethyl rhodamine dextran (Molecular Probes) as a lineage tracer, was injected into the ventral equatorial region of the 8–16 cell embryo. Embryos displaying lineage tracer in the flank region, where the posterior cardinal veins are developing, were then assayed for perturbation of the vasculature at the mid-tailbud stage using whole mount in situ hybridization and the *X-msr* vascular marker (Devic et al., 1996). In all cases. disruption to vascular structures was assessed by comparing the injected with the uninjected side of the embryo.

Acknowledgements

The authors wish to thank Peter Vize and Ondine Cleaver for discussion and critical reading of the manuscript. Thanks to Dan Schoenberg for the *Xenopus* transferrin and fibrinogen probes, to Margaret Saha for providing an independently isolated clone containing *X-msr* sequences and to Roger Patient for the *Xenopus αT-globin* probe. This work was supported by NIH grant number HL 52746 to PAK.

References

Asahara, T., Murohara, T., Sullivan, A., Silver, M., van der Zee, R., Li, T., Witzenbichler, B., Schatteman, G. and Isner, J.M. (1997) Isolation of putative progenitor endothelial cells for angiogenesis. Science 275, 964–967.

Bedford, F.K., Ashworth, A., Enver, T. and Wiedemann. L.M. (1993) *HEX*: a novel homeobox gene expressed during haematopoiesis and conserved between mouse and human. Nucleic Acids Res. 21, 1245–1249.

Bertwistle, D., Walmsley, M.E., Read, E.M., Pizzey, J.A. and Patient, R.K. (1996) GATA factors and the origins of adult and embryonic blood in *Xenopus*: responses to retinoic acid. Mech. Dev. 57, 199–214.

Boettger, T., Stein, S. and Kessel, T. (1997) The chicken NKX2-8 homeobox gene: A novel member of the NK-2 gene family. Dev. Genes Evol. 207, 65–70.

Brand, T., Andree, B., Schneider, A. and Arnold, H-H. (1997) Chicken Nkx2.8, a novel homeobox gene expressed during early heart and foregut development. Mech. Dev. 64, 53–59.

Bouwmeester, T., Kim, S.-H., Sasai, Y., Lu, B. and De Robertis, E.M. (1996) Cerberus is a head-inducing secreted factor expressed in the anterior endoderm of Spemann's organizer. Nature 382, 595–601.

Carmeliet, P., Ferreira, V., Breier, G., Pollefeyt. S., Kieckens, L., Gertsenstein, M., Fahrig, M., Vandenhoeck, A., Harpal, K., Eberhardt, C., Declercq, C., Pawling, J., Moons, L., Collen, D., Risau, W. and Nagy, A. (1996) Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. Nature 380, 435–439.

Chen, J.-N. and Fishman, M.C. (1996) Zebrafish *tinman* homolog demarcates the heart field and initiates myocardial differentiation. Development 122, 3809–3816.

Cleaver, O.B., Patterson, K.D. and Krieg, P.A. (1996) Overexpression of the *tinman* related genes *XNkx-2.5* and *XNkx-2.3* in *Xenopus* embryos results in myocardial hyperplasia. Development 122, 3549–3556.

Cleaver, O.B., Tonissen, K.F., Saha, M. and Krieg, P. (1997) Neovascularization of the *Xenopus* embryo. Dev. Dyn., in press.

Crompton, M.R., Bartlett, T.J., MacGregor, A.D., Manfioletti, G., Buratti, E.. Giancotti, V. and Goodwin, G.H. (1992) Identification of a novel vertebrate homeobox gene expressed in haematopoietic cells. Nucleic Acids Res. 20, 5661–5667.

Dear, T.N., Colledge, W.H., Carlton, M.B., Lavenir, I., Larson, T., Smith, A.J., Warren, A.J., Evans, M.J., Sofroniew, M.V. and Rabbitts, T.H. (1995) The Hox11 gene is essential for cell survival during spleen development. Development 121, 2909–2915.

Devic, E., Paquereau, L., Vervier, P., Knibiehler, B. and Audigier, Y. (1996) Expression of a new G protein-coupled receptor X-msr is associated with an endothelial lineage in *Xenopus laevis*. Mech. Dev. 59, 129–140.

Ferrara, N., Houck, K., Jakeman, L. and Leung, D.W. (1992) Molecular and biological properties of the vascular endothelial growth factor family of proteins. Endocr. Rev. 13, 18–32.

Ferrara, N., Carver-Moore, K., Chen, H., Dowd, M., Lu, L., O'Shea, K.S., Powell-Braxton, L., Hillan, K.J. and Moore, M.W. (1996) Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. Nature 380, 439–442.

Gorski, D.H., LePage, D.F., Patel, C.V., Copeland, N.G., Jenkins, N.A. and Walsh, K. (1993) Molecular cloning of a diverged homeobox gene

that is rapidly down-regulated during the G_0/G_1 transition in vascular smooth muscle cells. Mol. Cell. Biol. 13, 3722–3733.

Guazzi, S., Lonigro, R., Pintonello, L., Boncinelli, E., Di Lauro, R. and Mavilio, F. (1994) The thyroid transcription factor-1 gene is a candidate target for regulation by Hox proteins. EMBO J. 13, 3339–3347.

Guazzi, S., Price, M., De Felice, M., Damante, G.. Mattei, M.-G. and Di Lauro, R. (1990) Thyroid nuclear factor 1 (TTF-1) contains a homeodomain and displays a novel DNA binding specificity. EMBO J. 9, 3631–3639.

Hadji-Azimi, T., Schwager, J. and Thiebaud, C. (1982) B-lymphocyte differentiation in *Xenopus laevis* larvae. Dev. Biol. 90, 253–258.

Harland, R. (1991) In situ hybridization: An improved whole mount method for *Xenopus* embryos. Meth. Cell Biol. 36, 685–695.

Harvey, R.P. (1996) NK-2 homeobox genes and heart development. Dev. Biol. 178, 203–216.

Hausen, P. and Riebesell, M., (1991) The Early Development of *Xenopus laevis*. Springer-Verlag, New York.

Hentsch, B., Lyons, I., Li, R., Hartley, L., Lints, T.J., Adams, J.M. and Harvey, R.P. (1996) *Hlx* homeobox gene is essential for an inductive tissue interaction that drives expansion of embryonic liver and gut. Genes Dev. 10, 70–79.

Ji, H., Sandberg, K., Zhang, Y. and Catt, K.J. (1993) Molecular cloning, sequencing and functional expression of an amphibian angiotensin II receptor. Biochem. Biophys. Res. Commun. 194, 756–762.

Komuro, I. and Izumo, S. (1993) *Csx*: A murine homeobox-containing gene specifically expressed in the developing heart. Proc. Natl. Acad. Sci. USA 90, 8145–8149.

Krieg, P.A. and Melton, D.A. (1987) In vitro RNA synthesis with SP6 RNA polymerase. Meth. Enzymol. 155, 397–415.

Lazzaro, D., Price, M., De Felice, M. and Di Lauro, R. (1991) The transcription factor *TTF*-1 is expressed at the onset of thyroid and lung morphogenesis and in restricted regions of the foetal brain. Development 113, 1093–1104.

Le Douarin, N.M. (1975) An experimental analysis of liver development. Med. Biol. 53, 427–455.

Lee, K.-H., Xu, Q. and Breitbart, R.E. (1996) A new *tinman-related* gene, *nkx2.7*, anticipates the expression of *nkx2.5* and *nkx2.3* in zebrafish heart and pharyngeal endoderm. Dev. Biol. 180, 722–731.

Leung, D.W., Cachianes, G., Kuang, W., Goeddel, D.V. and Ferrara, N. (1989) Vascular endothelial growth factor is a secreted angiogenic mitogen. Science 246, 1306–1309.

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.

Lyons, I., Parsons, L.M., Hartley, L., Li, R., Andrews. J.E., Robb, L. and Harvey, R.P. (1995) Myogenic and morphogenetic defects in the heart tubes of murine embryos lacking the homeobox gene *Nkx-2.5*. Genes Dev. 9, 1654–1666.

Ma, J. and Ptashne, M. (1987) A new class of yeast transcriptional activators. Cell 51, 113–119.

Maeno, M., Tochinai, S. and Katagiri, CH. (1985) Differential participation of ventral and dorsolateral mesoderms in the hemopoiesis of *Xenopus*, as revealed in diploid-triploid or interspecific chimeras. Dev. Biol. 110, 503–508.

Millauer, B., Vizigmann, Voos, S., Schnurch, H., Martinez, R., Moller, N.P., Risau, W. and Ullrich, A. (1993) High affinity VEGF binding and developmental expression suggest Flk-l as a major regulator of vasculogenesis and angiogenesis. Cell 72, 835–846.

Nieuwkoop, P.D. and Faber, J. (1994) Normal table of *Xenopus laevis* (Daudin), 2nd edn. Garland Publishing Inc., New York.

Patel, C.V., Gorski, D.H., LePage, D.F., Lincecum, J. and Walsh, K. (1992) Molecular cloning of a homeobox transcription factor from adult aortic smooth muscle. J. Biol. Chem. 267, 26085–26090.

Patient, R.K., Banville, D.L., Brewer, A.C., Elkington, J.A., Greaves, D.R., Lloyd, M.M. and Williams, J.G. (1982) The organization of the tadpole and adult alpha-globin genes of *Xenopus laevis*. Nucleic Acids Res. 10, 7935–7945.

Plachov, D., Chowdhury, H., Walther, C., Simon, D., Guenet, J.-L. and Gruss, P. (1990) Pax8, a murine paried box gene expressed in the developing excretory system and thyroid gland. Development 110, 643–651.

Poleev, A., Fickenscher, H., Mundlos, S., Winterpacht, A., Zabel, B., Fidler, A., Gruss. P. and Plachov, D. (1992) PAX8 a human paired box gene: isolation and expression in developing thyroid, kidney and Wilms' tumors. Development 116, 611–623.

Roberts, B., Sassoon, D., Jacq, B., Gerhing, W. and Buckingham, M. (1989) Hox-7, a mouse homeobox gene with a novel pattern of expression during embryogenesis. EMBO J. 8. 91–100.

Roberts, C.W.M., Shutter, J.R. and Korsmeyer, S.J. (1994) Hox 11 controls the genesis of the spleen. Nature 368, 747–749.

Sater, A.K. and Jacobson, A.G. (1989) The specification of heart mesoderm occurs during gastrulation in *Xenopus laevis*. Development 105, 821–830.

Sater, A.K. and Jacobson, A.G. (1990) The role of the dorsal lip the induction of heart mesoderm in *Xenopus laevis*. Development 108, 461–470.

Shalaby, F., Rossant, J., Yamaguchi, T.P., Gertenstein, M., Wu, X-F., Breitman, M.L. and Schuh, A.C. (1995) Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. Nature 376, 62–66.

Smith, S.T. and Jaynes, J.B. (1996) A conserved region of engrailed, shared among all en-, Nk1-, Nk2- and msh-class homeoproteins. mediates active transcriptional repression in vivo. Development 122, 3141–3150.

Tonissen, K.F. and Krieg, P.A. (1993) Two neural-cell adhesion molecules (NCAM) encoding genes in *Xenopus laevis* are expressed during development and in adult tissues. Gene 127, 243–247.

Tonissen, K.F. and Krieg, P.A. (1994) Analysis of a variant *Max* sequence expressed in *Xenopus laevis*. Oncogene 9, 33–38.

Turner, D.L. and Weintraub, H. (1994) Expression of achaete-scute homolog 3 in Xenopus embryos converts ectodermal cells to a neural fate. Genes Dev. 8, 1434–1447.

Vize, P.D., Hemmati-Brivanlou, A., Harland. R.M. and Melton, D.A. (1991) Assays for gene function in developing *Xenopus* embryos. Methods Cell Biol. 36, 367–387.

Weir, L. Chen, D., Pastore, C., Isner, J.M. and Walsh, K. (1995) Expression of *gax*, a growth arrest homeobox gene, is rapidly down-regulated in the rat carotid artery during the proliferative response to balloon injury. J. Biol. Chem. 270, 5457–5461.

Wilson, D.S., Guenther, B., Desplan, C. and Kuriyan, J. (1995) High resolution crystal structure of a Paired (Pax) cooperative homeodomain dimer on DNA. Cell 82, 709–719.

Yamaguchi, T.B., Dumont, D.J., Conlon, R.A., Breitman, M.L. and Rossant, J. (1993) flk-1, an flt-related receptor tyrosine kinase is an early marker for endothelial cell precursors. Development 118, 488–498.