CHARACTERIZATION AND DEVELOPMENTAL ANALYSIS OF *PBX1* IN ZEBRAFISH

by

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Thesis submitted in fulfillment of the requirements
for the degree of
Doctor of Philosophy

September 2008

ACKNOWLEDGEMENT

I wish to thank my supervisors A/P Alexander Chong, Prof. Mohd. Nazalan and A/P Tengku Sifzizul for their guidance, encouragement and help throughout my study. I would also like to express my sincere gratitude to A/P Chan Woon Khiong for his valuable advice, suggestion and insights throughout my attachment in his lab. Without his support, my study would not have been completed.

My appreciation goes to Dr. Korzh for his kindness in giving me the Hedgehog pathways mutants. I am also very grateful for the insightful discussion I had with Cecilia.

A special word of thanks goes to my close friends Sue Ing and Pui Mun, who have spared no expense in making life as graduate student more enriching. My sincere thanks goes to my lab members Shubba, Renji, Hai Yan, Anusha, Wang Yue, Li Ling, Zhen Yang, Annete and Phaik Siew for their endless help and moral support.

On the personal level, I cannot sufficiently express my thanks to Hean Giap for his warmhearted concern and unswaying faith that he has shown me.

Last but not least, I would like to present this thesis to my parents for their continuous encouragement in my life.

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LIST OF ABBREVIATIONS

Ctrl	control
DBD	DNA-binding domain
DIC	differential interference contrast
DIG	Digoxigenin
E	gestational day
EGFP	Enhanced Green Fluorescent Proteins
FITC	Fluorescein isothiocyanate
GFP	green fluorescence protein
hpf	hours post-fertilization
ISH	*in situ* hybridization
MO	morpholino oligo
ORF	open reading frame
PBST	Phosphate Buffered Saline Tween-20
RT-PCR	reverse transcriptase polymerase chain reaction
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SSCT	Sodium chloride Sodium Citrate Tween-20
SSD	signal sensing domain
StdMO	standard morpholino oligo
TAD	trans-activating domain
WT	wild type

PENCIRIAN DAN ANALISIS FUNGSI *PBX1* DALAM PERKEMBANGAN IKAN ZEBRA

ABSTRAK

Gen *Pbx* ialah faktor transkripsi kelas homeodomain TALE yang memainkan pelbagai peranan yang penting dalam organogenesis. Sehingga kini, empat ahli *Pbx* telah ditemui dalam vertebrata. Objektif utama projek ini adalah untuk mengkaji dan menganalisis fungsi *pbx* dalam perkembangan embrio ikan zebra. Dengan mengunakan sistem model ikan zebra ini, diharapkan maklumat yang diperolehi boleh memudahkan permahaman organogenesis manusia.

Gen *pbx* ikan zebra, terutamanya *pbx1* telah diklon dan struktur gen telah dikaji dengan teliti. Ia mempunyai homologi yang tinggi dengan *Pbx1* haiwan mammalia dan dikategorikan dalam kelas yang sama dalam analisis phylogenetik. Protein yang dikodkan oleh *pbx1* mempunyai identiti '*DNA binding domain*' yang serupa seperti *Pbx1* haiwan mammalia. RT-PCR bagi organ ikan zebra dewasa menunjukkan kadar ekspresi yang tinggi dalam otak, pundi udara, otot dan limpa. Berikutan itu, analisis ekspresi *in vivo pbx1* semasa perkembangan embrio telah dilakukan dengan teknik '*in situ hyridization*'. Transkrip *pbx1* dijumpai pada tiga domain yang berasingan. Domain ekspresi yang paling ketara ialah pada sistem saraf pusat, sama seperti ekspresi *Pbx1* haiwan mammalia. Domain yang kedua pada '*pharyngeal arches*' posterior juga sama dengan ekspresi *pbx1* pada 'pharyngeal' dalam embrio tikus. Ekspresi ketiga pada pundi udara bermula dari 28 hpf dan kekal sehingga dewasa merupakan penanda paling awal yang dijumpai bagi pundi udara.

Tidak banyak yang diketahui setakat ini tentang pundi udara berbanding dengan organ lain, oleh itu, analisis fungsi *pbx1* yang seterusnya lebih difokus kepada pundi udara (homolog peparu haiwan mammalia).

Fungsi *in vivo pbx1* dikaji dengan mengunakan teknik '*morpholino targeted gene knockdown*'. Kehilangan *pbx1* mengakibatkan ekspresi penanda 'hindbrain' dan tisu 'mesenchyme' pundi udara terjejas. Analisis terliti perkembangan pundi udara dengan menggunakan penanda *foxA3* menunjukkan *pbx1* adalah '*redundant*' semasa perkembangan awal tetapi penting dalam pertumbuhan selanjutnya. Tambahan pula, larva ikan zebra tanpa pundi udara tidak dapat berenang ataupun mencari makanan menyebabkan kematian pada 8 dpf. Selain itu, penglibatan *pbx1* dalam rangkaian Hedgehog semasa perkembangan pundi udara adalah sama seperti perkembangan peparu haiwan mammalia. *shh* didapati menjejaskan fungsi *pbx1* dalam pundi udara tetapi semua ahli Hedgehog diperlukan untuk menghilangkan fungsi *pbx1* sepenuhnya. Kesimpulannya, fungsi *pbx1* ikan zebra boleh dikatakan homolog kepada fungsi *Pbx1* haiwan mammalia dalam perkembangan beberapa organ yang telah dikaji dalam projek ini.

CHARACTERIZATION AND DEVELOPMENTAL ANALYSIS OF *PBX1* IN ZEBRAFISH

ABSTRACT

Pbx genes are TALE class homeodomain transcription factors. They play diverse and important roles in organogenesis. To date, four *Pbx* members have been identified in vertebrate. The objectives of this study were to characterize and analyze the developmental functions of zebrafish *pbx* members. It is hoped that the information gained from this model system will facilitate in the understanding of human organogenesis.

Zebrafish *pbx* genes, in particular *pbx1*, were cloned and characterized in this study. It displays strong homology to mammalian *Pbx1* and clustered together in the phylogenetic analysis. The encoded protein of *pbx1* exhibited similar identity of mammalian *Pbx1* in the DNA binding domain. RT-PCR of adult zebrafish organs showed high level expression in the brain, swim bladder, muscle and spleen. Following that, *in vivo* analyses of *pbx1* expression during embryonic development were performed by *in situ* hybridization. *pbx1* transcript was detected in three separate domains. The most significant area of expression is at the central nervous system, similar to mammalian *Pbx1*. The second domain is at the posterior pharyngeal arches, which is also observed in the pharyngeal expression during mouse embryonic development. The third expression in the swim bladder starts from 28 hpf and persists till adulthood which makes *pbx1* the earliest marker for swim bladder. In comparison to other organs, very little is known about swim bladder development, thus

subsequent functional studies were focused on swim bladder development, a homolog to mammalian lung.

Disruption of *pbx1* function *in vivo* using morpholino targeted gene knockdown, down regulated the expression of hindbrain markers and also swim bladder mesenchyme marker. Detailed analysis of the development of swim bladder using *foxA3* marker showed that *pbx1* is redundant during early swim bladder development but is essential in subsequent growth. In addition, the absence of swim bladder in the morphants had caused the lethality at 8 dpf due to their inability to swim, thus unable to feed themselves. Besides that, *pbx1* was shown to be involved in Hedgehog signaling pathways during swim bladder development similar to mammalian lung development. *shh* was found to sufficiently induce the effect of *pbx1* in the swim bladder but all of the Hedgehog signaling pathway members are needed to completely knockdown *pbx1*. Taken together, it seems likely that zebrafish *pbx1* functions are homologous to mammalian *Pbx1* in the development of these organs.

CHAPTER 1

INTRODUCTION

1.1 Development and organogenesis

Development is the emergence of organized structures from an initially very simple group of cells. Developmental processes can be distinguished into five processes, even though they may overlap with and influence one another considerably. After the fertilization of the egg to form a zygote, the first process known as cleavage occurs. The zygote undergoes rapid mitotic division with no significant growth, producing a cluster of cells, the same size as original zygote. After the cleavage has produced over 100 cells, the embryo becomes a blastula. This is when the pattern formation process takes place, where the overall embryo body plan is defined. The next stage in pattern formation is allocation of cells to three different germ layers namely endoderm, mesoderm and ectoderm. The cells of these germ layers later acquire different identities so that organized spatial patterns of cell differentiation emerge. The third important developmental process is the 'change in form' or morphogenesis. The characteristic and dramatic changes in form are most striking during gastrulation. Almost all embryos undergo gastrulation, during which the gut is formed and the main body plan emerges. Morphogenesis can also involve cell migration. The fourth developmental process is cell differentiation. Cells become structurally and functionally different from each other to form distinct cell types such as blood muscle or skin cells. Differentiation is a gradual process where cells go through several divisions in order to become fully differentiated. Lastly, the fifth process is growth and the increase in size. There is little growth during early embryonic development and the basic pattern is laid down on a small scale. However,

subsequent growth can be carried out in cell multiplication, increase in cell size and deposition of extracellular material such as bone and shell. These five developmental processes are neither independent of each other nor strictly sequential.

Organogenesis is the process by which the ectoderm, endoderm and mesoderm develop into specific organs and structures in the organism. This is a crucial phase of development that leads to a fully functioning organism which is capable of survival. The organ development involves similar processes as those in earlier development. The cellular mechanisms are similar too in some cases, but are much more complex.

The endoderm, one of the germ layers forms the epithelial lining of digestive tube and terminal part of the rectum. The lining cells of all glands in digestive tube including the liver and pancreas are formed by this layer. Besides that, it also forms the epithelium of auditory tube, tympanic cavity, trachea, bronchi, aveolar cells, urinary bladder, part of urethra, and also the internal layer of thyroid gland and thymus.

The mesoderm germ layer is an additional layer between the endoderm and ectoderm which make animal (triploblasty) more complex than cnidarians (diploblastis). Mesoderm formation leads to the formation of a coelom which allows organs formed inside a coelom to move freely and develop independently of the body wall. Organs formed by mesoderm are skeletal muscle, skeleton, skin dermis, eye lens, connective tissue, urogenital system, heart, lymph cells and spleen.

Ectoderm is the outermost of the germ layers. The ectoderm produces tissues within the epidermis and aids in the formation of neurons within the brain. It is also involved in the formation of melanocytes, hair and mammary glands.

There are a number of organisms that has been widely studied for the understanding of developmental processes. Each of these model organisms has its advantages and disadvantages. The chick embryo, for example, has long been studied as an example of vertebrate development because fertile eggs are easily available, the embryos can be manipulated microsurgically and it can be cultured outside the egg. However, little is known about chick's developmental genetics. Although we know a lot about the genetics of the mouse and the availability of genetic modification by transgenic techniques, it is more difficult to study as the development takes place entirely within the mother. Recently, zebrafish has emerged to be a popular model system, as it is easy to breed in large numbers, the embryos are transparent and it has great potential for genetic investigation (Wolpert *et al.,* 2006).

1.2 *Pbx* is a TALE class of homeodomain transcription factor

Transcription factor proteins are important for the transcription of genetic information from DNA to RNA. They bind to DNA and are involved in many important functions and biological roles such as basal transcription regulation, development, cell cycle control, response to intercellular signals and response to environment. Transcription factors consist of DNA-binding domain (DBD), trans-activating domain (TAD) and signal sensing domain (SSD). DBD binds to enhancer or promoter sequences adjacent to target genes to regulate transcription. TAD contains binding sites for other proteins such as transcription coregulator and SSD senses external signals and in response transmit these signals to the rest of the transcription complex resulting in up or down of gene expression (Stegmaier *et al.,* 2004).

Transcription factors are often classified based on the DNA binding domain. *Pbx* (Pre-B lymphoblastic leukemia transcription factor) protein is a member of TALE (three amino acid loop extension), in the class of Homeodomain and superclass of Helix-turn-helix group (Stegmaier *et al.,* 2004). *Pbx* is classified under the organization shown below:

- Superclass: Helix-turn-helix
    - Class: Homeodomain
        - Family: Homeodomain only
            - Subfamily: HOX
            - Subfamily: TALE
                - PBC (*Pbx* in vertebrates, *Exd* in *Drosophila*)
                - MEIS
                - TGIF
                - IRO
        - Family: POUdomain factors; includes Oct
        - Family: Homeodomain with LIM region
        - Family: homeodomain plus zinc finger motifs

The homeobox gene is about 180 base pairs long which encodes the homeodomain protein. Homeodomain proteins have been known since 1984 for their importance in development and initially identified in *Drosophila* as being responsible, when mutated, for homeotic transformation (conversion of a part of the body into the likeness of another) (Gehring, 1994). The structure of homeobox genes has been determined by NMR and X-ray crystallography and it consists of three α helices

which pack around a hybrophobic core (Gehring *et al.,* 1994). Sub-groups of homeobox genes were distinguished from the typical homeodomains by having more or fewer than 60 amino acids in the homeodomain (Bürglin, 1994). A particular subgroup that emerged with three extra amino acids between helix 1 and helix 2 has been named TALE. TALE homeodomain is much more conserved than typical homeodomains: positions 24-26 are always proline-tyrosine-proline. Another aspect that makes TALE homeodomain different from other homeodomain sub-familily is at residues 16 and 20. In typical homeodomain, residues 16 and 20 are leucine and phenylalanine or tyrosine, respectively. In TALE homeodomains, position 16 can be leucine, methionine, phenylalanine, even a cysteine, or serine and position 20 can be a phenylalanine, tryptophane, leucine or methionine. Residue 50 in the DNA-binding helix 3 of the TALE homeodomain is a small, non–polar residue. For the PBC class, it is a glycine and this residue suggest that there might not be a strong interaction with the DNA where additional specificity might be conferred by other parts of the protein, for example N-terminal region (Burglin *et al.,* 1997).

1.3 Identification of *Pbx* and its gene structure

Pbx1 was originally identified because of its involvement in t(1;19) chromosomal translocation in pre-B-cell acute lymphoblastic leukemias (Kamps *et al.,* 1990; Nourse *et al.,* 1990). This translocation results in the formation of a fusion transcript that codes for E2A-Pbx1 chimeric protein. The C-terminal region of the E2A, which contains the helix-loop-helix (bHLH) DNA-binding and dimerization motifs, is replaced with *Pbx1*. The highly related *Pbx2* and *Pbx3* genes were later identified on the basis of sequence conservation with *Pbx1* (Monica *et al.,* 1991). Human *Pbx2* and *Pbx3* proteins are 92% and 94% identical to *Pbx1* over a large

region of 266 amino acid within and flanking homeodomains. The fourth member, *Pbx4* was later isolated in mouse and human (Wagner *et al.,* 2001) and in zebrafish *pbx4/lazarus* (Popperl *et al.,* 2000; Vlachakis *et al.,* 2000). However, mammalian *Pbx4* is not closely related to *pbx4/lazarus* and exhibits different expression pattern as it may represent diverged orthologues of *pbx4*/*lazarus* (Popperl *et al.,* 2000; Vlachakis *et al.,* 2000). Other *Pbx* orthologs were characterized in *Caenorhabditis elegans* (*Ceh-20*) and *Drosophila* (*Exd*) (Burglin and Ruvkun 1992; Rauskolb *et al.,* 1993). In *Drosophila*, *Exd* acts as a co-factor to direct homeotic selector proteins to target genes (Rauskolb and Wieschaus 1994; Wilson and Desplan, 1995).

Pbx proteins consist of two DBD which are PBC domain and Homeodomain. The PBC domain is a large bipartite domain upstream of the homeodomain (Bürglin *et al.,* 1992). It is situated directly adjacent to the homeodomain, in fact, the 40 amino acids in the PBC / homeodomain junction are identical between *ceh-20* and *Pbx2*, making this the most conserved region. One possible function for this region could be to provide additional DNA contacts, since the 5' end of the homeodomain has been shown to make contacts in the minor groove of the DNA (Kissinger *et al.,* 1990; Wolberger *et al.,* 1991).

Pbx proteins share remarkable sequence identity within and flanking their DNA-binding homeodomains. The extensive homology suggests that the Pbx proteins may share functional similarities which are mediated by protein motifs within the conserved regions. The limited divergence may show that the Pbx proteins bind to a common DNA sequence but with different affinities for the target DNA sequence due to slight structural differences in the amino acids (Bürglin, 1992).

Although, Pbx proteins has remarkably similar and conserve domains, they are distinguished by divergence at their C and N termini. C terminal diversity is resulted

from coding differences and also posttranscriptional mechanism due to differential splicing. Various isoforms of Pbx proteins arise from differential splicing of *Pbx* transcripts to yield high molecular weight (mw) (Pbx1a, Pbx2, Pbx3a, and Pbx4) and low mw (Pbx1b, Pbx3b) forms (Monica *et al.,* 1991; Waskiewicz *et al.,* 2002). This differential splicing is a feature of many homeobox transcripts that has unknown functional consequences on the resultant proteins. Since spliced variants of *Pbx2* and *Pbx4* were not detected, it is predicted that the longer form of isoforms have more specific functional roles while the shorter isoforms may have more prevalent function (Bürglin, 1997).

1.4 Interaction and mechanism of Pbx proteins

Pbx has the ability to modulate the DNA-binding activity of the Hox proteins (Van Dijk *et al.,* 1995). The cooperative DNA binding of HOX and Pbx proteins result in heterodimeric complexes that show increased DNA-binding affinity and specificity (Goutte and Johnson, 1993). In addition, *Pbx* also modulates the mammalian Engrailed-2 (*En-2*), but the Pbx/En-2 complexes has different binding sites compared to Pbx/Hox heterodimers (Peltenburg and Murre, 1996; van Dijk *et al.,* 1995). The region in Hox proteins that is required for the interaction is located at the N-terminal of the homeodomain and contains a highly conserved hexapeptide while EH2 and EH3 domains are needed for En-2 and Pbx interaction. Both the Engrailed and Hox homeodomain themselves are important for cooperativity because chimeric proteins containing either the Hox hexapeptide or the EH-2 domain when grafted onto a heterologous DNA binding domain do not show cooperative DNA binding (Neuteboom *et al.,* 1995; Peltenburg and Murre, 1996). However, the *Pbx*

interaction motifs present in Hox and engrailed recognize a common structure present in the *Pbx* family (Peltenburg and Murre, 1996).

Pbx proteins usually work as Hox partners but there are also other several mechanisms for the action of Pbx. For instance, *Pbx1*-mutant mice was reported to have craniofacial malformations that affect maxillary and frontonasal mass derivatives but none of the 39 Hox genes are expressed there (Krumlauf, 1993). This shows that Pbx does not work as Hox partners in that situation. *Pbx* can interact with non-homeodomain proteins, in the both the presence or absence of Hox proteins. For example, *Emx2* and *Pbx1* interact genetically in the patterning of the most proximal part of the mouse forelimb, which is an area that does not depend on Hox gene activity (Kmita *et al.,* 2005). Besides that, Pbx can also act together with Engrailed alone or form trimeric structure with Hox proteins. For instance, the interaction of Engrailed with Pbx proteins was shown to establish the midbrain-hindbrain boundary in zebrafish embryos (Erickson *et al.,* 2007) but *meis3* (Engrailed) was reported to synergize with *pbx4* and *hoxb1b* in promoting hindbrain fates in zebrafish (Vlachakis *et al.,* 2001). Finally, an even more divergent mechanism for Pbx functional activity is that it might regulate the translation of unknown mRNA. Other homeodomain proteins such as Bicoid and Hoxa9 can interact with the translational machinery (Topisirovic & Borden, 2005) and many homeodomain proteins can be secreted (Prochiantz & Joliot, 2003).

1.5 Expression patterns and functions of *Pbx* genes

Expression of *Pbx* genes has been observed in a wide variety of adult and fetal tissues. *Pbx1* has highest level of expression during early mid gestation, initially at the condensing mesoderm and neuronal tissues. It also displays a dynamic expression

pattern in derivatives of all principle layers. In particular, *Pbx1* localizes to sites of mesenchymal-epithelium interaction during the morphogenesis of lung, kidney, tooth buds and vibrissae follicles in mouse (Schnabel *et al.,* 2001). However, the spatio-temporal domain of expression for *Pbx2* is ubiquitous and widespread during the embryonic development (Selleri *et al.,* 2004). During early organogenesis, until E12.5, *Pbx3* expression is found mostly in the embryonic head, forelimbs, and septum transversum. Conversely, later in organogenesis, *Pbx3* expressions become more widely detectable. The major sites of *Pbx3* expression are in the CNS, as well as epithelial and mesenchyme tissues, similar to *Pbx1* (Giacomo *et al.,* 2006). Mouse *Pbx4* mRNAs have been found preponderantly in testes (Wagner *et al.,* 2001). In zebrafish, only *pbx4/lazarus* has been extensively studied. *pbx4* is expressed broadly during blastula stages but becomes excluded ventroanteriorly during gastrulation and later expressed primarily in neural tissues starting from segmentation stages. At the 3-5 somite stage, a high level of *pbx4* expression was detected in the rostral central nervous system, including the optic primordial. At 28 somite stage, *pbx4* was detected at high levels in the telencephalon and midbrain but at low levels in the olfactory primordial.

Different contribution of *Pbx1*, *Pbx2*, *Pbx3* and *Pbx4* proteins to mammalian development have been established (Selleri *et al.,* 2001, 2004; Rhee *et al.,* 2004; Moens and Selleri, 2006). *Pbx1* has unique and essential functions in embryonic development as *Pbx-1* deficient embryos die at gestational day (E) 15/16 with severe hypoplasia and/or aplasia of multiple organs (Selleri *et al.,* 2001; Kim *et al.,* 2002; Schnabel *et al.,* 2001; Manley *et al.,* 2004; Brendolan *et al.,* 2006), as well as homeotic transformation (Selleri *et al.,* 2001) and hematopoietic abnormalities (Di Martino *et al.,* 2001).

Mice homozygous for a null allele of *Pbx1* (*Pbx1*$^{-/-}$) are used to examine the roles of *Pbx1* in various organs formation and patterning during embryonic development. *Pbx1*$^{-/-}$ mutant embryo was shown to exhibit phenotypes that are similar to or more severe than abnormalities in pharyngeal development caused by single or multiple Hox gene mutation. This suggests that *Pbx1* acts collaboratively with several different Hox proteins in the development of the pharyngeal region (Manley *et al.,* 2004). Moreover, some aspects of the *Pbx1* mutant phenotypes are not as severe as Hox mutant pharyngeal phenotypes, implying either that interaction with *Pbx* proteins are not required for all Hox-dependent aspects of pharyngeal development, or that other *Pbx* proteins are providing redundant function in this region.

Pbx1 was also shown to play a critical role in the differentiation of urogenital organs, where *Pbx1* is widely expressed in mesenchymal tissues. Decreased cellular proliferation in *Pbx1*$^{-/-}$ genital ridge has caused the absence of adrenal glands and formation of gonads displaying rudimentary sexual differentiation. Furthermore, expression of steroidogenic factor-1 (SF-1), a nuclear receptor essential for adrenal organogenesis, was reduced to minimal level in *Pbx1*$^{-/-}$ embryos which indicate that *Pbx1* functions upstream in adrenocortical development. Besides that, the loss of *Pbx1* markedly reduces urogenital ridge outgrowth and results in impaired differentiation of the mesonephros and kidneys, and the absence of Mullerian ducts (Schanabel *et al.,* 2003a). Therefore, *Pbx1* is required early in the urogenital development and regulates the pathway of SF-1 in the adrenal formation and gonadal differentiation.

In addition, *Pbx1* plays an important role in splenic cell specification. The earliest known markers for splenic progenitor cells in mice, *Hox11* and *Nkx2.5* are absent in the splenic anlage of *Pbx1*$^{-/-}$ embryos. *Pbx1* and *Hox11* genetically interact

in spleen formation and loss of either is associated with a similar reduction of progenitor cell proliferation and failed expansion of the splenic anlage. Thus, a *Pbx1-Hox11*-dependent genetic and transcriptional pathway is predicted in spleen ontogeny. *Pbx1* is also involved in the *Nkx3.2* and *Pod1* pathway and therefore *Pbx1* emerges as a central hierarchical co-regulator in spleen genesis (Brendolan *et al.,* 2006).

Pbx1 is essential for normal pancreatic development. The *Pbx1*$^{-/-}$ embryos had pancreatic hypoplasia and marked defects in exocrine and endocrine cell differentiation before E15 or E16. The expression of *Isl1* and *Atoh5* which are the essential regulators for pancreatic morphogenesis and differentiation was severely reduced in these embryos. *In vitro* studies showed that *Pbx1* regulates the activity of *Pdx1*, a Para-Hox homeodomain transcription factor required for the development and function of the pancreas in mice and human (Kim *et al.,* 2002).

Besides that, *Pbx1*$^{-/-}$ embryos show widespread patterning detects of the axial and appendicular skeleton. *Pbx* is important for limb axis patterning apparent from the malformations of proximal skeletal elements. Additionally, homeotic transformation can be seen at the second neural crest cell-derived skeletal structures of the second branchial arch which was morphologically transformed into elements reminiscent of first arch-derived cartilages. The skeletal malformations which were restricted to domains of *Hox* proteins have affected *Hox* genes expressions. In addition, *Pbx1* was shown to have a novel function in coordinating the extent and/or timing of proliferation with terminal differentiation in bone formation.

Furthermore, *Pbx1* is essential for the function of hematopoiesis and its absence results in severe anemia, liver hypoplasia and embryonic lethality. Fetal liver hypoplasia reflects defect in the multi-lineage progenitors and their lineage-restricted progeny. Hematopoietic stem cells from *Pbx1*$^{-/-}$ embryos are unable to establish multi-

lineage hematopoiesis, causing depletion of common myeloid progenitors (CMPs) and reduction of megakaryocyte and erythrocyte-committed progenitors. Therefore, *Pbx1* is important for the function of hematopoietic progenitors with erythropoietic potential and its loss causes a proliferative constriction at CMP level.

Not many studies have been done for other mouse *Pbx* members. *Pbx2* is not functionally important as, when it alone is lost, it does not affect normal organogenesis, fertility, hematopoiesis or immune function (Selleri *et al.,* 2004). This is likely due to the compensation by related *Pbx* family members. Mice deficient of *Pbx3* develop to term, but die within a few hours of birth due to respiratory failure (Rhee *et al.,* 2004). In zebrafish, only *Pbx4/lazarus* has been studied extensively. *Pbx4* is important in promoting hindbrain fates during zebrafish embryogenesis (Vlanchakis *et al.,* 2001; Waskiewicz *et al.,* 2002).

Although *Pbx* proteins are essential for the development of most areas of the organism with individual functions, they have a large degree of redundancy too. In mice, the individual contribution of each *Pbx* member seemed to be different because, whereas the *Pbx1* single mutant had a wide range of organ malformations, there is only minor or no phenotype observed in *Pbx2* and *Pbx3* mutants. However, when *Pbx2* and / or *Pbx3* dosage was added to the $Pbx1^{-/-}$ background, strong exacerbations of the malformations were detected especially in the cardiovascular system, axial skeleton, limbs and craniofacial area (Selleri *et al.,* 2001). Besides that, redundancy was also seen among *Pbx* genes in zebrafish. The *Pbx2* and *Pbx4* gene products seem to overlap in a range of tissues (hindbrain and tectum) and share similar functions during hematopoiesis (Waskiewicz *et al.,* 2002). However, genetic analysis to evaluate the possible extent of redundancy among *Pbx* members are still at an early stage.

1.6 Aims of studies

To date, little has been known about *pbx* genes expression and functions in teleost, except for *pbx4* in zebrafish. In contrast to teleost, the *pbx* family has been relatively well characterized in mammals. However, it is unlikely that the teleost *pbx* genes mimic the mammalian counterpart *in toto* due to the phyletic distance between mammals and teleosts (~420 million years of evolution). Therefore, we sought to characterize and dissect the importance of *pbx* genes in embryogenesis. The zebrafish has been chosen for this purpose because of its numerous advantages as a laboratory model (Wixon, 2000 and Fishman, 2001). The zebrafish embryos are ideal for the study of development because the fertilization and subsequent embryonic development are external and occur synchronously in large clusters. Additionally, the embryos are relatively large and transparent which will facilitate the observation tremendously. In comparison with higher vertebrates, the organ structures are much simpler, using fewer cells to fulfill the equivalent function. Besides that, zebrafish has the advantage of its usefulness for gene identification by mutations due to the short generation time and large progeny number. A few methods and tools were developed over the past few years that allowed new experimental approaches to be undertaken. For example, microinjection and knowdown method using antisense morpholino are better compared to generating homozygous mutants in mice for functional studies.

In the course of this work, we look into the zebrafish *pbx* family members generally and *pbx1* in particular. The study aims to provide a comprehensive analysis of *pbx1*, with characterization at various levels including gene structure, spatio-temporal expression profile and gene functions. The cDNA of *pbx1* will be characterized and compared with other *pbx* members. In addition, the basic properties

of the encoded protein will be analyzed. The evolutionary relationship between teleost *pbx* members and with other vertebrates will also be determined by phylogenetic analysis. A detailed analysis of the spatio-temporal expression of *pbx1* during embryogenesis and larval development will be carried out using whole mount *in situ* hybridization. The information of the expression pattern will help to understand and decipher the gene functions. Finally, the ultimate goal of this study is to dissect the functions of *pbx1*. The roles of *pbx1* during development will be analyzed by gene knockdown using antisense morpholino oligonucleotide targeting. Based on previous information of mammalian *pbx1*, particular attention will be focused on investigating *pbx1* role in the development of swim bladder. The availability of hedgehog pathway signaling mutants will facilitate the study of *pbx1* involvement in Hedgehog pathway during swim bladder development. Besides that, we will examine the roles of *pbx1* in pharyngeal, tooth and skeletal development, and homeotic effects in comparison with mammals. It is hope that with this study, we would be able to address the question of whether a conserved role of *pbx* in development exist between teleost and mammals.

CHAPTER 2

MATERIALS AND METHODS

2.1 Fish culture and embryos collection

The adult zebrafish are kept on a 14-hour light and 10–hour dark cycle in 'overflow containers system' tanks (Westerfield, 1995). Fishes were set up for mating in the afternoon using mating containers. The mating container consists of a 1-litre acrylic box, a removable inner container with sieve to hold the fish and also a barrier to separate the pair of fish. The barrier will be removed the next morning to allow the fishes to mate, and then embryos were collected and staged according to Kimmel *et al.,* (1995). Embryos meant for *in situ* hybridization and histological analyses were cultured in 0.03% phenythiourea (Sigma, Mo, USA) egg water from 18 hpf onwards to inhibit melanin formation. It is important to inhibit the melanin formation as these pigments will restrict the observation clarity.

Zebrafish mutants embryos, *syu* and *smu*, were provided by Dr Korzh, IMCB, Singapore.

2.2 Isolation of total RNA

Embryos or tissues weighing 50-100 mg were homogenized in 1 ml of TRI Reagent® according to the protocol described by the manufacturer (Molecular Research Centre, Inc.). The integrity and concentration of total RNA was determined by 1% (w/v) formaldehyde agarose gel electrophoresis and A_{260}/A_{280} measurement. Total RNA was then treated with RQ1 RNase-Free DNase (*Promega*) to remove all the DNA contaminants. The treated total RNA was used as templates in RT-PCR to amplify the target gene fragments.

2.3 Analysis of gene expression by RT-PCR

cDNA was synthesized from 1 μg of total RNA in 20 μl reactions using the Superscript™ II First-Strand Synthesis System. For RT-PCR (Invitrogen) denaturing was performed on 1 μg of cDNA template in 10 μl reactions containing 1μM random hexamers, 10mM of dNTP mix at 65°C for 5 minutes and then on ice for at least 1 minute. This was followed by a 2 minute annealing at 42°C in 10 X RT buffer, $MgCl_2$, DTT and RNase OUT™ Recombinant RNase Inhibitor. SuperScript II RT was then added and cDNA was synthesized by incubating at 42°C for 50 minutes and the reaction was terminated at 70°C for 15 minutes. RNase H was added to remove access RNA. The sequences of the primers used for RT-PCR experiments are provided in Table 2.1. Thermocycling program consisted of initial denaturing step of 94°C for 2 minutes, followed by 35 cycles of 94°C for 30 seconds, 55°C for 1 minute, and 72°C for 1 min, and final step of 72°C for 10 minutes.

Several genes were identified to mark specific tissue by *in situ* hybridization and the primers were designed using Primer3 software, website address available at page 28, (Rosen and Skaletsky, 2000). Primers and information for *cyp11a1*, *pdx-1*, *prox1*, *rx3*, *six3* and *wt-1* were obtained from a previous lab member, (Chai and Chan, 2000). *Anxa5* was used as a marker for swim bladder mesenchyme tissue (Thisse *et al.,* 2001), *dlx2a* for pharyngeal arches (Akimenko *et al.,* 1994), *pax2.1* for pronephric tubules (Majumdar *et al.,* 2000), *pitx2a* for tooth germ layer (Jackman *et al.,* 2004), *cyp11a1* for steroidogenic cells, *pdx-1* for pancreas, *prox1* for liver and brain, *rx3* for diencephalons, *six3* for telencephalon and *wt-1* for kidney.

Table 2.1 PCR primers used for RT-PCR

RT-PCR expression analysis primers		
β-actin	beta-actin/G720F	5' CCGTGACATCAAGGAGAAGCT 3'
	beta-actin/G920R	5' TCGTGGATACCGCAAGATTCC 3'
pbx1a	pbx1b/G514F	5'-TAGAGAAGTATGAGCAGGCGTGTA-3'
	pbx1a/G1029R	5'-TCCCCGGAGTTCATGTTAAA-3'
pbx1b	pbx1b/G514F	5'-TAGAGAAGTATGAGCAGGCGTGTA-3'
	pbx1b/G1006R	5'-AGAGTATCCACCGGCCGAAT-3'
pbx2	pbx2/G471F	5' - GATGTCCCCTGACAGCTCAT - 3'
	pbx2/G872R	5' - TGCTTGGCCAGTTCTTCTTT - 3'
pbx3a	pbx3ab/G435F	5'-CATCGAGCACTCCGACTACA-3'
	pbx3a/G1054R	5'-TGTTCTCTGCAGAATCCAGC-3'
pbx3b	pbx3ab/G435F	5'-CATCGAGCACTCCGACTACA-3'
	pbx3b/G1000R	5'-CGCAGGGCTATCCCCGGAGTT-3'
pbx4	pbx4insitu/G892F	5' GAGGAAGCAAACCTGTACGC 3'
	pbx4insitu/G1694R	5' CATCTGACAGACCCCGTTTT 3'

pbx gene sequences were aligned using ClustalW software, website address available at page 29 (Thompson *et al.,* 1994), so that the identity, similarity and differences can be seen. As *pbx* genes are highly identical at the 5' end, probes were designed at the distinctive 3' end of each genes to avoid non-specific hybridization. Primer3 software was used to design the primers for the *in situ* hybridization probes. The parameters for the primers designed were fixed at 60-62°C annealing temperature, 50% GC content, 19-23 base-pair primer length and product size at 700-1000bp. The products of the designed primers were blast using NCBI blast system, the website address available at page 29, (Altschul *et al.,* 1990) to confirm that there are specific.

Table 2.2 PCR primers used for *in situ* hybridization probe synthesis

Isolation of cDNA of marker genes for in situ hybridization		
Anxa5 (Thisse *et al.,* 2001)	anxa5G37F	5' TTCAATGCTAACAGCGATGC 3'
	anxa5G839R	5' ATGTCCAGCAAGTCCACCTC 3'
Cyp11a1 (Chai and Chan, 2000)	zfP450SCCF	5' ATGGCCCGCTGGAATGTGAC 3'
	zfP450SCCR	5' CTATCTGCTGGCATTCAGTGG 3'
Dlx2a (Akimenko *et al.,* 1994)	dlx2a/G6F	5' TGGAGTTTTTGACAGCCTCA 3'
	dlx2a/G800R	5' CCGGCGCTAACAGTTGTATT 3'
Ff1b	ff1b/g106F	5' ACGGTGATGGACTTCAGAGC 3'
	ff1b/g2365R	5' AAGTTGCAGACGCCTTTACC 3'
FoxA3 (Odenthal and Nusslein-Volhard 1998)	foxA3/G280F	5'- CTAGGCTCATCTGCCTCCAC -3'
	foxA3/G1191R	5'- CACCTGGTCCTGGTACGACT -3'
Pax2.1 (Majumdar *et al.,* 2000)	pax2.1/G71F	5' ACCAGCTAGGAGGGGTGTTT 3'
	pax2.1/G1069R	5' TTGTGTACTGCGGGTGAGAG 3'
Pdx-1 (Chai and Chan, 2000)	zfPdx1F	5' TGTGCTCGTGTACGGCACGGT 3'
	zfPdx1R	5' TTAAGAGTCTTGTTGGACTGA 3'
Pitx2a (Jackman *et al.,* 2004)	Pitx2a/G21F	5' ACTTGCATCAACGTGTGCTC 3'
	Pitx2a/G707R	5' TTGCTTGGCTTTCAGTCTCA 3'
Prox-1 (Chai and Chan, 2000)	zfProx1F	5' GTGATGCCTGACCATGACAGC 3'
	zfProx1R	5' CTATTCATGCAGAAGCTCCTGC 3'

Rx3 (Chai and Chan, 2000)	zfRx3F	5’ GAATTCATGAGGCTTGTTGGATCTC 3’
	zfRx3R	5’ GTCGACTACCACGTCTTCCCTATAG 5’
Six3 (Chai and Chan, 2000)	zfSix3F	5’ GCTGGTGTCATTAGGCGATAG 3’
	zfSix3R	5’ AAGGATTGCATCCTGGCTGC 3’
Wt1 (Chai and Chan, 2000)	zfWt1F2	5’ ATGGGTTCTGATGTTCGTGAC 3’
	zfWt1R2	5’ GGTTCAGATGGTTAGCTGGAG 3’
pbx1	pbx1UTR/G929F	5’ CTAACGCAACCAGCGTCTC 3’
	pbx1UTR/G1623R	5’ GCTGAGAGGTAGAATGAAGCA 3’
pbx2	pbx2insitu/G1042F	5' GGGTCGTTCTCTCTGTCAGG 3'
	pbx2insitu/G2009R	5' TGGCTCAGACAAAGGCTACA 3'
pbx3	pbx3insitu/G960F	5' CGCCGTCCAGAATAGTCAGA 3'
	pbx3insitu/G1809R	5' AAGCGTCCAACAAAATGTCC 3'
pbx4	pbx4insitu/G892F	5' GAGGAAGCAAACCTGTACGC 3'
	pbx4insitu/G1694R	5' CATCTGACAGACCCCGTTTT 3'

2.4 Cloning and analysis of PCR amplified products

PCR amplified products were analyzed on agarose gel and the bands of interest were gel purified using the Qiaquick Gel extraction kit (*Qiagen, Germany*). The purified fragments were then ligated into plasmids, pGEM-T Easy Vector (*Promega*), transformed into *E. coli* DH5α and propagated on LB-broth containing ampicillin, IPTG and X-Gal plates. Upon successful recombinant bacteria cloning, positive white colonies were selected and purified by using Qiaprep Spin Miniprep Kit (*Qiagen, Germany*).

2.5 DNA sequencing

DNA sequencing was used to identify and confirm the correct sequences. DNA cycle sequencing was performed with the ABI PRISM™ BigDye Terminator kit (Applied Biosystems). Sequencing reaction consisted of 50ng of DNA, 0.4pmol of primer, 1X Sequencing buffer and 1µl BigDye in a total volume of 10 µl. Thermocycling program consisted of 35 cycles of 94°C for 10 sec, 50°C for 10 sec, and 60°C for 4 min. Final products were EtOH precipitated, washed once with ice-cold 70% EtOH, and vacuum dried. Pellets were resuspended in 2 µl loading dye and samples were run on ABI PRISM 377 or ABI PRISM 3700 (Applied Biosystems Inc., CA, USA). Service for the sequencing runs were provided by Department of Biological Sciences, National University of Singapore.

2.6 Preparation of plasmid

Plasmids were obtained and purified by the alkaline lysis method according to Sambrook *et al.,* 1989. For DNA preparations on a larger scale, QIAGEN Tip-100 or Tip-500 plasmid purification columns (QIAGEN, Germany) were used. The

endotoxin removal procedure included in the QIAGEN Plasmid Purification Handbook was followed for plasmids intended for microinjection into zebrafish embryos.

2.7 Riboprobe synthesis

Plasmids were first linearized completely with the appropriate restriction enzyme and then purified by phenol/chloroform using phase lock gel. 0.6μg of DNA was used as template for riboprobe synthesis in 10μl reaction containing 1X Roche transcription buffer, 1X NTP dig labelling mix or 1X flourecein labelling mix, (Roche, Switzerland), 20 U of rRNAsin ® RNAse inhibitor (Promega Corp., WI, USA) and 10 U of the appropriate RNA polymerase. T7, SP6 and T3 polymerase are from Roche. The restriction enzyme site chosen for linearizing each plasmid and the respective RNA polymerase used for transcription for marker genes and *pbx* genes are listed in Table 2.3.

Table 2.3 List of restriction enzyme sites chosen for linearizing each plasmid and the respective RNA polymerase used for transcription for each marker genes and *pbx* genes.

Marker	Expression site	Plasmid	Linearizing enzyme	RNA polymerase
Anxa5	Swim bladder	pGTzfAnxa5	*Nco1*	SP6
cyp11a1	Steroidogenic cells	pGTzfCyp11a	*Nco1*	SP6
Dlx2a	Pharyngeal arches	pGTzfDlx2a	*Nco1*	SP6
Ff1b	Interregnal	pGTzfFf1b	*Nco1*	SP6
foxA3	Gut derived organs	pGTzfFoxA3	*Nde1*	T7
Pax2.1	Pronephric tubules	pGTzfPax2.1	*Sac11*	SP6
Pdx-1	Pancreas	pGTzfPdx-1	*Nco1*	SP6
Pitx2a	Tooth germ	pGTzfPitx2a	*Nde1*	T7
Prox1	Liver	pGTzfProx1	*Nco1*	SP6
Rx3	Diencephalons	pGTzfRx3	*Spe1*	T7
Six3	Telencephalon	pGTzfSix3	*Not1*	SP6
Wt1	Kidney	pGTzfWt1	*Spe1*	T7
pbx1	-	pGTzfPbx1	*Nde1*	T7
pbx2	-	pGTzfPbx2	*Nde1*	T7
pbx3	-	pGTzfPbx3	*Nco1*	SP6
pbx4	-	pGTzfPbx4	*Nco1*	SP6

Transcription reactions were incubated at 37°C for 2 hours followed by digestion of DNA template by adding 1 μl of RNase free DNAse I (Promega) and incubated for another 15 minutes. Then 0.4μl of 0.5M EDTA (pH8.0) was added to stop the reaction. Riboprobes were precipitated using lithium chloride and ethanol method. Aliquots of purified riboprobes were analyzed on denaturing formaldehyde gel.

2.8 *In situ* hybridization

Embryos after 24 hpf were dechorionated manually using a pair of 26 gauge hypodermic needles and fixed overnight at 4°C in 4% paraformaldehyde (PFA) dissolved in phosphate buffered saline (PBS). The embryos were then washed with PBST (PBS added with 1% Tween 20) twice for 1 minute and 4 times for 15 minutes on a nutator. At this stage, embryos can be treated with methanol and kept at -20°C for later use. To recover stored embryos for *in situ* hybridization, specimens were passed through 50% and 30% methanol in PBST for 5 minutes each followed by two 5 minute washes in PBST.

Embryos were permeabilized by digestion of proteinase K (10μg/ml in PBST) according to the schemes in Table 2.4. Proteinase K treatment is necessary for embryos older than 20 hpf. Proteinase K digestion increases the sensitivity of *in situ* hybridization significantly because by digesting away proteins from mRNA, it allows better access of the probe, thereby allowing better penetration of the antibody.