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Characterization of the platelet derived growth factor beta promoter

Author
Shinbrot, Eve,

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Characterization of the Platelet Derived Growth Factor Beta Promoter

by

Eve Shinbrot

DISSERTATION

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This dissertation is dedicated to George and Elyse

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Eve Shinbrot

Characterization Of The Platelet Derived Growth Factor Beta Promoter

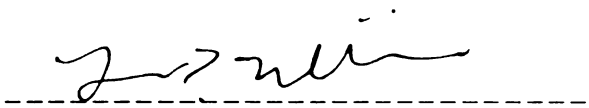
ABSTRACT

This thesis describes my study of the Platelet Derived Growth Factor Beta Receptor (PDGFβr) promoter. The PDGFβr has been implicated in many physiological processes. The correct temporal and spatial expression of this gene is vital for normal cellular functioning. However, aberrant expression of the receptor leads to pathological conditions. Therefore, I chose to study regulation of expression of the PDGFβr gene, the promoter region of the gene.

When I started my thesis work, the expression pattern of the PDGFβr throughout development had not been studied. My initial work consisted of a descriptive study analyzing the expression pattern of the PDGFβr mRNA during mouse embryogenesis. Its expression was high in the mesenchymal component of many developing tissues and organs, particularly where mesenchyme directly supported an epithelium. However as the mesenchyme differentiated into smooth muscle, the receptor was no longer expressed. This implied that the PDGFβr might be involved in mesenchymal-epithelial interactions during organogenesis. It also suggested a role for the PDGFβr in undifferentiated mesenchyme, while the gene appeared to be silenced in differentiated smooth muscle. The gene was also expressed in the endothelium of small blood vessels and vascular structures indicating a role in vasculogenesis.

The next part of my thesis describes the isolation and characterization of the promoter region of the PDGFβr gene. I characterized a 4.5 kb genomic fragment which confers PDGFβr tissue specific promoter activity.

This fragment was able to direct luciferase reporter gene expression in a cell type specific manner. A minimal promoter of 614 bp was identified. I generated transgenic mice which carry the chloramphenicol acetyltransferase (CAT) reporter gene under control of the promoter. The promoter was able to direct reporter gene expression with the same temporal and spatial pattern as the endogenous PDGFβr. The expression pattern of the transgene was high during organogenesis, yet silenced in the adult mouse. Therefore this clone contains the regulatory regions sufficient to direct expression of the PDGFβr. The last chapter of my thesis describes current and future experiments that will further elucidate the regulation of this promoter. A discussion of the uses for this promoter is also included.



Lewis T. Williams

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CHAPTER 1

INTRODUCTION

I. Introduction

The development and maintenance of a multicellular organism involves a highly complex set of events. During development, cellular migration and proliferation are key events leading to the morphogenesis and organization of tissues and organs. In contrast, these are not major events in the adult organism . However, wound healing may cause a reinstatement of embryonic processes involved in tissue organization. While the carefully controlled expression of proteins involved in tissue migration and proliferation is essential for correct cellular function, the loss of this control has detrimental effects on the organism. Reinstatement of embryonic processes involved in tissue organization may become unregulated and pathological resulting in fibrosis or cancer. Polypeptide growth factors and their receptors are major regulators of cell growth, differentiation migration and repair. It has long been known that they are key regulators of processes involved in development and disease such as migration and proliferation.

For my dissertation, I studied one type of polypeptide growth factor receptor, the platelet-derived growth factor beta receptor (PDGFβr). The PDGFβr (Yarden et al., 1986) has been the focus of many studies investigating, its biochemistry, its role in signal transduction, and its role in cellular processes ranging from development to disease. I found the PDGF system intriguing. The expression of PDGF and its receptors is essential for development, however it is also involved in many pathological conditions such as cancer, fibrosis, atherosclerosis. Many studies have focused on the signal transduction pathways of the receptor, however I felt studying the regulation of expression of the receptor was vital to understanding its involvement in cellular events. During development and wound healing, the

transcriptional regulation of the PDGFβr is essential. However, loss of its transcriptional regulation may be involved in its role in disease. The transcriptional regulation of the gene is likely controlled by a complex system of positive and negative regulatory mechanisms, dependent on the cellular environment. The cis-regulatory elements and trans-acting factors that regulate its expression must be highly controlled. It is possible that wound healing causes a reinstatement of positive transcriptional regulatory factors that were present during development. Pathological tissue proliferation, such as fibrosis and cancer, may result from a failure of negative transcriptional regulatory mechanisms to be implemented. Therefore I decided to study the transcriptional regulation of the PDGFβr When I started my thesis, the expression pattern of the PDGFβr throughout development had not been described. Therefore, my initial experiments consisted of a descriptive study examining this pattern of expression (Chapter 2). I then studied the regulation of receptor expression, and focused on isolating the promoter region of the PDGFβr (Chapter 3). These studies will be described in detail after a general introduction into the background of PDGF and its receptors and its role in cellular events.

1. Polypeptide growth factor families

Many essential and diverse cellular processes are modulated by polypeptide growth factors, these include cell growth, migration, differentiation and proliferation. Many of these distinct cellular responses are regulated by tyrosine kinase growth factor receptors. The tyrosine kinase growth factor receptor family includes EGF receptor, FGF receptor, IGF receptor, VEGF receptor, HGF receptor, Neurotrophin receptor and the PDGF receptor (Heldin, 1995)**Fig. 1.** Common features of tyrosine kinase

growth factor receptors include an extracellular ligand binding domain linked to a large cytoplasmic catalytic domain containing a tyrosine kinase region. Binding of the ligand to the receptor induces receptor dimerization which leads to signal transduction. This induces cellular responses which result in cell cycle progression, DNA synthesis, and cellular replication. The PDGF receptor subfamily consists of receptors which have an extracellular region composed of 5 immunoglobulin-like (Ig-like) domains, a single transmembrane region, and a cytoplasmic tyrosine kinase domain. Members of the PDGF receptor family include PDGFα, PDGFβ, SCFR (Kit), CSF-R (Fms), Flk-2(Heldin, 1995).

1A) PDGF

Platelet-derived growth factor (PDGF) and its receptors have been implicated in many cellular processes including mitogenesis, development, wound healing, fibroproliferative disease, neoplasia and atherosclerosis (Ross, 1993; Ross et al., 1986). PDGF stimulates growth, mitogenesis, actin reorganization, membrane ruffling and chemotaxis (Ross et al., 1986). PDGF was initially characterized from platelets as growth-promoting activity for glial cells, fibroblasts, and smooth muscle cells (Claesson-Welsh, 1994). Early studies of PDGF focused on platelets, however many other sources of PDGF have been identified including endothelial cells (Kavanaugh et al., 1988), macrophages (Shimokado et al., 1985) mitogen-stimulated fibroblasts (Paulsson et al., 1987) placental cytotropoblasts, and type-1 astrocytes (Noble et al., 1988; Richardson et al., 1988). PDGF exists as a 30 kD dimer linked by disulfide bonds . It can be found as hetero- or homodimeric complexes of two ligands, PDGF-A and PDGF-B(Heldin and Westermark, 1989). PDGF A and B chains are encoded by separate genes yet share 60 % homology in their

amino acid sequence (Betsholtz et al., 1986). Human platelets contain about 70% PDGF AB and 30% PDGF-BB (Hammacher et al., 1988) while PDGF from porcine platelets contain only PDGF-BB (Stroobant and Waterfield, 1984). These homodimers are closely related to the v-sis oncogene product (Doolittle et al., 1983; Waterfield et al., 1983). PDGF AA is produced and secreted by several cell lines. PDGF exerts its effects by binding to cell surface receptors.

1. B) PDGF receptors

Two receptors for PDGF have been identified, they are structurally related transmembrane glycoproteins **Fig. 2**. The PDGF α (Claesson-Welsh et al., 1989) and β (Yarden et al., 1986) receptors are protein tyrosine kinases with a molecular weight of 170 kD- 180 kD. The human PDGF α receptor is located on chromosome 4q11-4q12 (Matsui et al., 1989) while the β receptor is located on chromosome 5q31-5q32 (Yarden et al., 1986). The receptor extracellular region consists of five Ig-like domains. The three disulfide-bonded N-terminal domains are involved in ligand binding(Heidaran et al., 1990). The receptor contains a single membrane spanning region, a juxtamembrane domain, and a cytoplasmic tyrosine kinase domain. The tyrosine kinase domain exists as two parts separated by a hydrophilic region, with the α and β receptor sharing 80 % identity in this region (Heldin and Westermark, 1989). Binding of PDGF to its receptors induces receptor dimerization. This stimulates receptor autophosphorylation which leads to complexes with cytoplasmic signaling molecules. Many signal transduction molecules that interact with the PDGF receptor have been identified. These include, Scr family kinases, Grb2, Shc Pi3 kinase, Nck, GAP, Shb. PLC-γ(Claesson-Welsh, 1994).**Fig. 3** Activation of the PDGF receptor leads to many cellular responses such as calcium flux, turnover of PI , membrane ruffles and

cytoskeletal rearrangements, cellular migration and proliferation(Claesson-Welsh, 1994).

1. C) PDGF binding to its receptors

The PDGF α and β receptor bind the various forms of PDGF with different affinities. The PDGF alpha receptor (PDGFαr) (Yarden et al., 1986) binds all forms of PDGF (PDGF-AA, PDGF-AB and PDGF-BB), while the PDGF beta (PDGFβr) receptor (Matsui et al., 1989)binds PDGF- BB with high affinity, PDGF-AB with low affinity and does not bind PDGF-AA (Heldin and Westermark, 1989). **Fig..4** The availability of two receptor subtypes and multiple forms of ligand dimers may allow for the pleiotrophic effects of the PDGF system. PDGF and its receptors have been shown to be involved in cellular migration and proliferation, processes which are crucial to distinct conditions such as development, wound healing, neoplasia, and atherosclerosis. The biological effect of PDGF on the cells may depend on the particular isoform of PDGF ligand and receptor that is expressed. In this way the PDGF system may exert different cellular behavior in response to different stimuli. Thus, coordinate tightly controlled temporal and spatial expression of PDGF and its receptors is required for normal cellular functioning.

My thesis work focused on the PDGFβr. However, the biological events caused by PDGFβr also involve PDGF B, A, and the α receptor. Therefore as I discuss the role of PDGFβr in biological processes, I will include PDGF B, A, and the α receptor.

2. PDGF and its receptors in development

It has long been hypothesized that PDGF is involved in embryonic

development. Initial studies described the presence of transcripts of PDGF and its receptors. PDGF A transcripts were described in mouse blastocysts and Xenopus oocytes (Mercola et al., 1988; Rappolee et al., 1988). Transcripts for PDGF A and PDGF α receptor were detected during early development while the β receptor and B chain were not found (Mercola et al., 1990). Later, in situ hybridization was used to describe the expression pattern of PDGF A and the PDGFα receptor during mouse development (Orr-Urtreger et al., 1992; Orr-Urtreger and Lonai, 1992; Schatteman et al., 1992) The PDGFα r mRNA was detected in mesoderm derivatives throughout development, while PDGF A was found in epithelial layers adjacent to those expressing the receptor (Orr-Urtreger and Lonai, 1992). The contrasting expression of the receptor and ligand in adjacent tissue layers suggested that they are involved in developmental interactions. At the time of my thesis work, the expression pattern of the PDGFβr throughout development had not been described. Therefore one of my initial goals was to examine the expression pattern of the PDGFβr throughout development. This study is described in detail in Chapter 2.

Most of the initial evidence linking PDGF and its receptors to embryonic development was largely descriptive, the ligand or receptor were at the right place at the right time. More direct evidence was found in mice carrying mutations. The hypothesized role of PDGF and its receptors in development was supported by examining mice mutant for PDGF and its receptors. Early evidence that PDGF α receptor is essential for development was present in *patch* mouse mutant. The Patch (Ph) mutation was shown to represent a deletion of the PDGF α receptor (Stephenson et al., 1991).The majority of patch homozygous mice die before the 11th day of gestation and display many developmental abnormalities (Orr-Urtreger and Lonai, 1992). Some of

these problems include major abnormalities in mesenchymal structures, for example, the esophagus is missing the submucosa, the skin is missing the dermis. The mutant mice also display defects in neural crest derived structures. In general Ph, mutant mice show disorganized and diminished connective tissue and smooth muscle. However, they do retain partial organization of some tissues that normally express the α receptor. This is probably due to expression overlap of the α and β receptors and to redundancy both in their binding to PDGF ligands and in their functions. One problem with using Ph mutant mice to ascertain the role of PDGFαr in development is the size of the genomic deletion in the *patch* mice. The extent of the PDGFαr deletion has not been mapped, in fact, recent evidence indicates that the deletion breakpoint may be over 10 kb upstream from the PDGFαr initiator ATG(Orr-Urtreger et al., 1992). Therefore, it is possible that the defects seen in Ph mutant mice are due to disruptions of other genes as well as the PDGF α receptor. However, since many of the abnormalities are consistent with the expression pattern of the α receptor, most are probably due to the deletion of the α receptor gene.

Direct evidence for the role of PDGF-B and the β receptor in development has recently been gained by the generation of mice deficient in each of these genes. Homologous recombination was used to create mice carrying a null mutation in the PDGF B (Leveen et al., 1994), and PDGFβr genes (Soriano, 1994). The PDGF B mutant mice had abnormal glomeruli, heart and blood vessels. They also displayed major hematological abnormalities including anemia, thrombocytopenia, and severe hemorrhages leading to death. The majority of the mutants died at birth while some died 1-2 days prior to birth (Leveen et al., 1994). Many of the developmental defects seen in the PDGF B mutant mice were similar to those described for

the PDGFβr mutant mice. This is consistent with the role of PDGFβ receptor primarily binding PDGF B. The PDGFβ receptor homozygous mutant mice also displayed multiple defects and died at or shortly before birth (Soriano, 1994). These mice had multiple hematological, vascular, and kidney glomerulus disorders. In both the PDGF B and PDGFβ receptor mutant mice some tissues that are known to express the gene were normal. This is probably due to the redundant expression pattern of the α receptor and its probable overlap in function. As with the patch mutant mice, some of the abnormalities described might be due to the disruption of other genes. The PDGFβr gene and the CSF-1receptor (*c-fms*) gene are closely linked, the 3' untranslated end of the PDGFβr gene runs into the 5' untranslated region of the CSF-1 receptor gene. It is possible that a disruption of the PDGFβr coding region might also effect transcription of the CSF-1 receptor gene. Since the CSF-1 receptor is involved in hematopoiesis, a mutation in the CSF-1 receptor would probably lead to many hematological abnormalities. Therefore caution must be used in the interpretation of the knock out mice.

Mice mutant in PDGF B, β receptor and α receptor display major developmental abnormalities, culminating in embryonic lethality. Overall the effects are consistent with the expression pattern of the mutant gene. However, caution should be used in attributing all the abnormalities present to the mutant gene. In each case, the α receptor, β receptor and B mutant mice might also show abnormalities due to the disruption of nearby genes. As discussed above, the patch deletion is quite large, and nearby genes may also be deleted. The β receptor knock out might also affect expression of hematopoietic genes such as CSF-1receptor. Another problem with interpretation of the knock out mice lies in the additive nature of

development. One developmental stage directly effects the next. Disruption of an early structure directly affects the formation of later structures. It is difficult to ascertain if the mutation affected the tissue or organ that is abnormal, or acted at an earlier stage affecting precursor structures. Although there are uncertainties with the deletion mice, some of the major abnormalities are probably due to the mutant gene. This is supported by the evidence that the abnormality is in the tissue that has been shown to express the gene. The mutant mice also provide evidence for the redundancy of the PDGF system. Mice lacking the PDGF α or β receptor retain some tissue organization in the regions that express both receptors. This is probably due to the compensation of the α or β receptor for the deleted gene in those tissues. Taken together, the PDGF B, PDGFα receptor and PDGFβ receptor mutant mice give direct evidence that PDGF and its receptors play an essential role in development.

3). PDGF and its receptors in wound healing, fibrosis and neoplasia

The presence of PDGF and its receptors has been described during inflammation and wound healing. Wound healing might cause a reinstatement of transcription of the PDGFβr reflective of the processes active during development. The PDGFβr is not widely expressed in adult tissue, however its expression is increased during many disease processes including atherosclerosis, wound healing and neoplasia (Heldin and Westermark, 1989; Ross et al., 1986). Wound healing, inflammation and tissue repair involve highly regulated cellular responses such as migration, proliferation, and differentiation. PDGF activation of its receptors has been shown to stimulate these processes. The steps involved in wound healing are well characterized. Initially platelets are deposited in the wound, then

macrophages, neutrophils, and fibroblasts migrate into the wound. The fibroblasts proliferate and produce connective tissue, while neovascularization of the region takes place. That PDGF and its receptors play a role in these processes is suggested by the evidence that PDGF is a mitogen for fibroblasts (Senior et al., 1983), is chemotatic for monocytes and neutrophils (Deuel et al., 1982) can regulate collagen synthesis (Narayana and Page 1983) and has been implicated in neovascularization. PDGF has been shown by immunohistochemistry to be present in sites of wound repair and sites of neovascularization in the wound (Antoniades et al., 1991; Reuterdahl et al., 1993; Whitby and Ferguson, 1991). Further evidence for the role of PDGF in wound healing was demonstrated by applying exogenous PDGF directly to incisional wounds in rats, which greatly accelerated wound healing (Pierce et al., 1988). Similarilary, treatment of excisional wounds in rabbits with PDGF caused an increase in granulation tissue consisting of fibroblasts, with an increase in the amount of extracellular matrix at the leading edge of the wound (Mustoe et al., 1991). Thus, PDGF and its receptors may play a role in tissue reconstruction in response to wound healing.

The expression of PDGF and its receptors is crucial for wound healing, however unregulated aberrant expression of PDGF and its receptors leads to pathological conditions such as neoplasia, fibroproliferative disorders and atheroscleorsis. PDGF secreted at the site of tissue injury may induce fibroblast and smooth muscle migration and proliferation which leads to extracellular matrix reorganization. While this is a beneficial process for tissue reorganization and repair, it may become pathological. Under normal circumstances the proliferation and migration of fibroblasts would be halted when the injury is repaired, however these processes may become

unregulated leading to various fibroproliferative conditions. An increase in PDGF and the PDGFβr has been described in proliferative fibroblastic disorders (Smits et al., 1992) and many neoplastic conditions. High levels of the PDGFβr have been described in proliferative disorders including rheumatic synovial tissue (Rubin et al., 1988), vascular cells in rejected kidney transplants (Rubin et al., 1988), mesangial nephritis (Iida et al., 1991) renal inflammation (Fellstrom et al., 1989), proliferative glomerulonephritis (Gesualdo, L., Di Paolo S., Pinzani J. Clin. Invest. 94: 1994 508.), and in fibroblasts and dermal vessels in skin biopsies from patients with scleroderma (Klareskog et al., 1990). However, the non-diseased counterparts of the inflamed tissue do not express the PDGFβr. The condition of inflammation and injury may cause a controlled induction of PDGFβr gene expression, however, uncontrolled gene expression may contribute to pathological unregulated growth. This illustrates the importance of precisely regulated control of PDGFβr gene expression.

Neoplasm may result from similar situations. In neoplasia, proliferating tumor cells modulate tissue stroma, much like an inflammatory reaction. An increase in the expression of the PDGFβr has been found in many neoplasm's including glioblastoma (Hermansson et al., 1988)astrocytomas, (Bronzert *et al.*, 1987; Pantazis *et al.*, 1985; Sariban *et al.*, 1988). Additional support for the role of PDGF in neoplasia comes from an oncogenic form of PDGF-B, v-*sis* (Doolittle, 1983), which is capable of neoplastic transformation. The cellular form of the *sis* oncogene, c-*sis* can transform NIH 3T3 cells when under control of a strong promoter (Clarke et al., 1984; Gazit et al., 1984). This implies that increased expression of PDGF B may lead to oncogenesis. This may be due to loss of transcriptional regulation of the gene, a common mechanism seen in viral oncogenesis.

Further evidence for the induction of PDGF and its receptors in response to cellular stresses comes from examining cells in culture. Expression of the PDGFβr can be induced by simply subjecting cells to the stresses of cell culture . In normal tissues, the expression of PDGFβr in fibroblasts and smooth muscle is low, however, when cells are isolated from these tissues and cultured, they display an increase in PDGFβr levels (Terracio et al., 1988). This phenomena has also been described in smooth muscle cells, which express PDGF B in culture yet do not in vivo (Majesky et al., 1988). The induction of PDGF and its receptors in response to cell culture is probably analogous to its response to injury. Thus, the tightly controlled regulation of expression of PDGF and its receptors has significant consequences.

4). PDGF and its receptors in atherosclerosis

Atherosclerosis is the principal initiator of heart attacks. Initially atherosclerosis arises as a protective response to injury of vessels, analogous to the wound healing process described above. Advanced atherosclerosis results from an excessive inflammatory fibroproliferative response (Ross, 1993). In the atherosclerotic lesion, endothelial cells interact with macrophages, platelets, smooth muscle cells and lymphocytes. The formation of the atherosclerotic lesion arises from intimal proliferation of smooth muscle cells followed by formation of new connective tissue by these cells. One difference between wound healing and atherosclerosis is that the sources of arterial injury are chronic (obesity, hypertension, diabetes) (Ross, 1993). Another difference is that the principal source of connective tissue in the arterial wall is smooth muscle cells. The role of PDGF and its receptor in the pathogenesis of atherosclerosis is supported by descriptions of increased expression of PDGF and its receptors in many stages of atherosclerosis.

Increased expression of PDGF and its receptors has been described in cultured rat smooth muscle cells (Sjolund et al., 1988), human atherosclerotic plaques (Wilcox et al., 1988), macrophages in human and non human primate atherosclerosis (Ross et al., 1990), experimentally induced atherosclerosis, smooth muscle accumulation in vascular grafts (Golden et al., 1991; Ross et al., 1990), human microvascular endothelial cells (Beitz et al., 1992). Direct evidence for the role of PDGF in atherosclerosis was demonstrated using neutralizing antibodies against human platelet PDGF (Ferns et al., 1991). In this study, the authors injured the carotid artery of rats using an intra-arterial balloon catheter, then administered anti-PDGF antibody. Rats treated with anti-PDGF displayed reduced cellular content and thickness in the neointima of their artery (Ferns et al., 1991). Therefore, the role of PDGF and its receptors have been linked to atherosclerosis by both indirect and direct evidence. The induction of PDGF and its receptors in atherosclerosis is probably due to the same mechanisms as in inflammatory conditions, possibly a failure to repress the transcription of genes induced during wound healing. Furthermore, the transcriptional induction might represent a reinstatement of conditions that were present during development. This emphasizes the importance of the accurate transcriptional regulation of the PDGFβr gene.

5. Transcriptional Regulation of PDGF and its Receptors

As described above, PDGF and its receptors are key regulators of many essential processes, ranging from normal cellular processes such as development and wound healing to pathological states such as fibrosis and atheroscleorsis. It is essential that the PDGFβr gene is expressed during development and wound healing, however it is equally important that the

gene is repressed at the completion of tissue organization. This precise regulation of gene expression is vital to normal cellular functioning. The correct spatial and temporal expression of this gene is crucial, however the transcriptional regulation of its expression has not been characterized. While the promoter region of the PDGF A gene (Takimoto et al., 1991), the PDGF α receptor (Wang and Stiles, 1994) and the PDGF B gene (Pech et al., 1989) have been defined the promoter region of the PDGFβ receptor has not been described. The PDGF A chain promoter was isolated from a human leukocyte genomic library and found to be very GC rich, to contain a TATA box but no CAAT box . The promoter region contains overlapping AP-2 consensus binding sites. Gel mobility shift assays have demonstrated binding of protein factors to this region, however they have not been identified (Kaetzel et al., 1993). The promoter region (Takimoto et al., 1991)of the PDGF B gene also contains large GC rich regions, with potential Sp-1 binding sites, however binding of Sp-1 has not been demonstrated (Pech et al., 1989). A region of the promoter upstream of the cap site was shown to be involved in megakaryoblast differentiation (Pech et al., 1989) and both positive and negative cis regulatory elements have been characterized in the first intron (Franklin et al., 1991). Recently the α receptor gene was cloned, it was shown to contain a large intron separating the first two exons (Wang and Stiles, 1994). This is similar to the genomic organization of the β receptor. It has been hypothesized that the PDGF α and β receptor arose by gene duplication, their similar genomic organization supports this hypothesis. It is probable that their promoters contain similar regulatory mechanisms. The expression of the α and β receptor is coordinately regulated and they heterodimerize in response to binding PDGF-AB , it is likely that their transcriptional regulation would also be related.

One of my main goals in my thesis research was to isolate and characterize the promoter region of the PDGFβ receptor. The characterization of the promoter region is important for a number of reasons. As described in this introduction, the PDGFβr is important in development, wound healing and fibroproliferative diseases. The defined promoter may be used to assay the transcriptional regulation of the PDGFβr throughout these processes and might provide clues as to its role in tissue organization and morphogenesis. Initially the promoter linked to a reporter gene can be monitored to determine the transcriptional regulation of the PDGFβr gene during development and in response to various pathological states. Eventually, the defined promoter may be used to assay factors that modulate the transcriptional regulation of the PDGFβr gene in response to cellular conditions. This includes examining cis regulatory elements and trans-acting factors that regulate its expression. Finally the characterized promoter may be used as a tool to direct the expression of genes to have the same profile as the endogenous receptor. The promoter may be used to express genes that modulate the PDGFβr, such as a dominant negative PDGFβ receptor, or downstream signaling molecules. Conversely, the promoter may be used to direct the expression of a heterologous gene. Thus, the promoter may be used to study developmental interactions, wound healing or other cellular processes which occur with the same temporal and spatial profile as the PDGFβr.

When I started my project, the expression pattern of the PDGFβr throughout development had not been described, therefore my initial experiments were a descriptive study of the expression pattern of the receptor throughout development (chapter 2). To identify the promoter region, I examined genomic clones of the PDGFβ receptor. I analyzed these clones to determine candidates that might contain transcriptional regulatory regions. I

focused on a 4.5 kb genomic clone that contained putative consensus sequences for regulatory regions. I used this fragment to characterize the promoter region of the PDGFβr gene. Initially I used in vitro analysis to define the promoter region of the gene. Next I analyzed the ability of the putative PDGFβr promoter to direct reporter gene expression in transgenic mice. My study on the isolation and characterization of the PDGFβr promoter and its expression in transgenic mice is described in detail in chapter 3. The final chapter of my thesis (Chapter 4) describes the expression pattern of the promoter-reporter gene in adult transgenic mice. The discussion section of this chapter also describes future directions for this project.

Figure 1 Polypeptide Growth Factor Families

Figure legend:

Striped boxes - Cysteine-rich domains

Half-circle - Immunoglobulin-like domains

Stippled box - Tyrosine kinase domains

Solid box -Transmembrane domains

Checkered box - Fibronectin domains

Reprinted from:

Fantl, W.J., Johnson, D.J., Williams, L.T. (1993) Signaling by Receptor Tyrosine Kinases. Annu. Rev. Biochem. 62, 453-81.

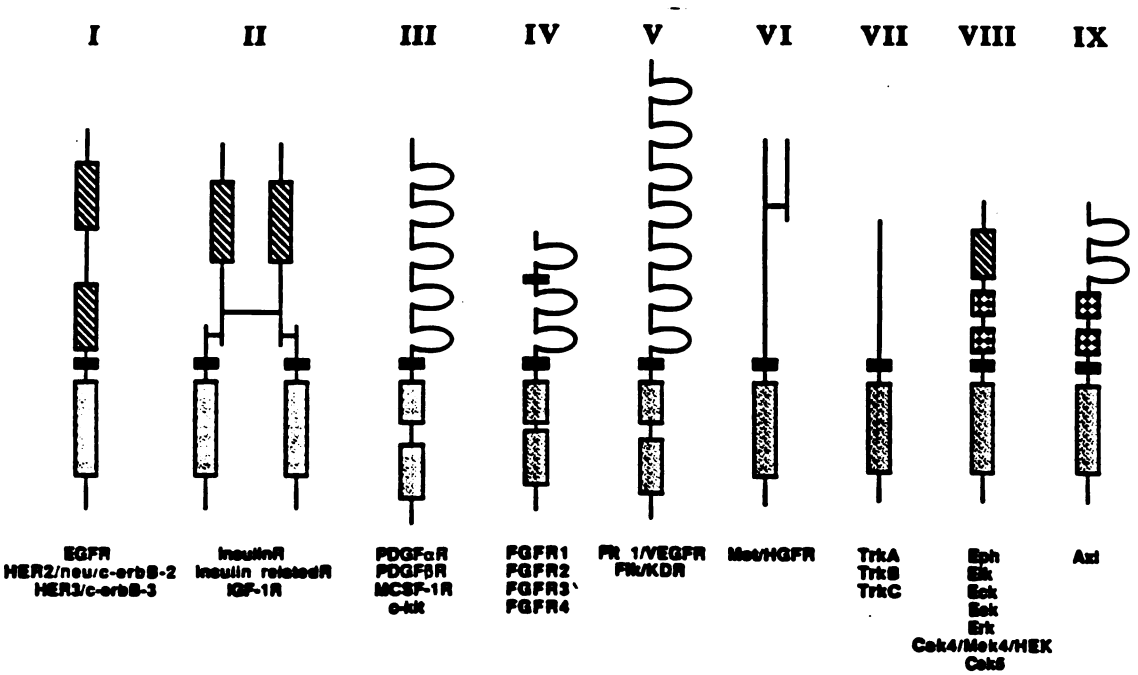

I
II
III
IV
V
VI
VII
VIII
IX
EGFR
HER2/neu/c-erbB-2
HER3/c-erbB-3
InsulinR
Insulin relatedR
IGF-1R
PDGFαR
PDGFβR
MCSF-1R
c-kit
FGFR1
FGFR2
FGFR3
FGFR4
Flt 1/VEGFR
Flk/KDR
Met/HGFR
TrkA
TrkB
TrkC
Eph
Elk
Eek
Eek
Erk
Cek4/Mek4/HEK
Cek5
Axl

Figure 2 Structure of the PDGF Receptor

Figure legend:

Open box - tyrosine kinase domain

Loops - immunoglobulin-like domains

KI - kinase insert region

Reprinted from:

Williams, L.T. (1989) Signal Transduction by the Platelet-Derived Growth Factor Receptor. Science 243, 1564-1570.

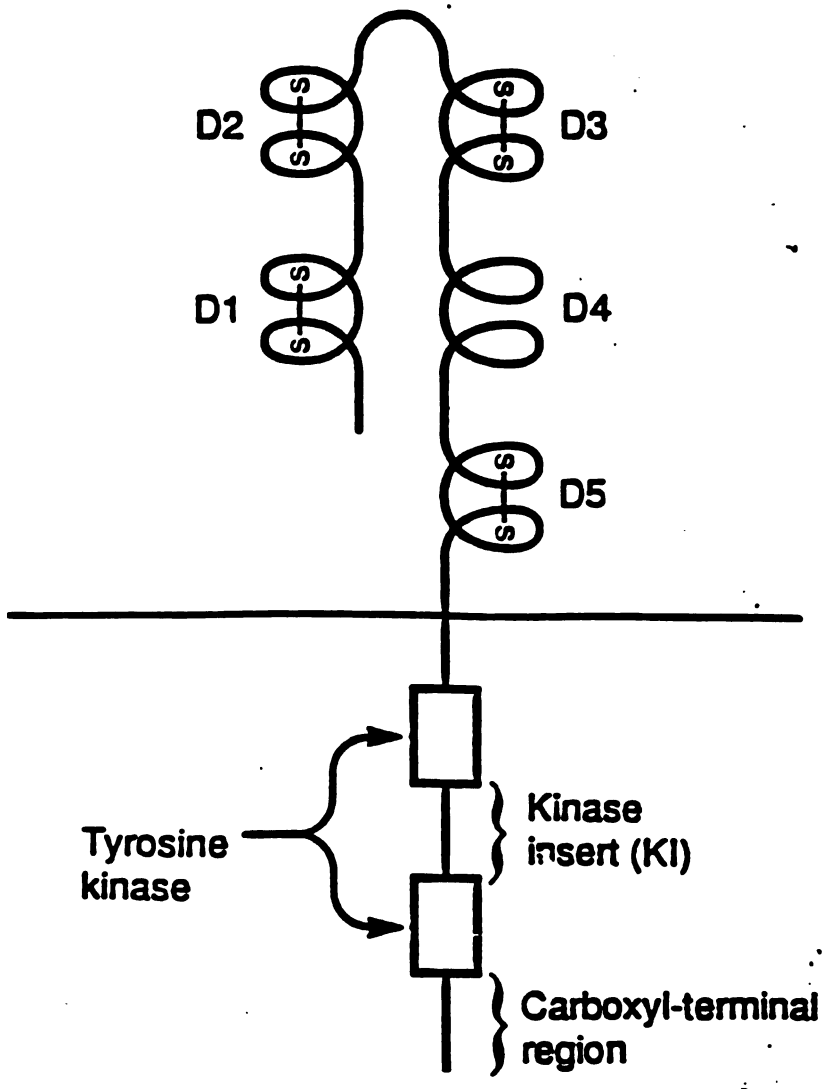

D2
D3
D1
D4
D5
S
Tyrosine
kinase
Kinase
insert (KI)
Carboxyl-terminal
region

Figure 3 Signaling Molecules That Bind the PDGFβ Receptor

Figure Legend:

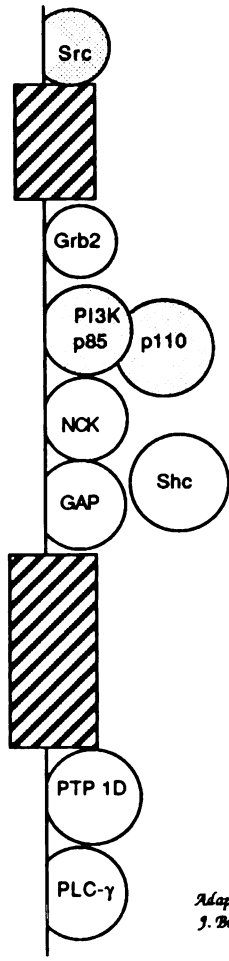
Stripped box - tyrosine kinase domain

the signaling molecules that bind the receptor are named on the figure, they are not drawn to scale.

Adapted from:

Classon-Welsh, L. (1994). Platelet-derived Growth Factor Receptor Signals. J. Biol. Chem. 269, 32023-32026.

Figure 3



Adapted from Classen-Welsh, L. (1994). J. Biol. Chem. 269: 32023-32026.

Figure 4 PDGF Binding to its Receptors

Figure Legend:

PDGF A - light circle

PDGF B- dark circle

PDGFα receptor - light line

PDGFβ receptor - dark line

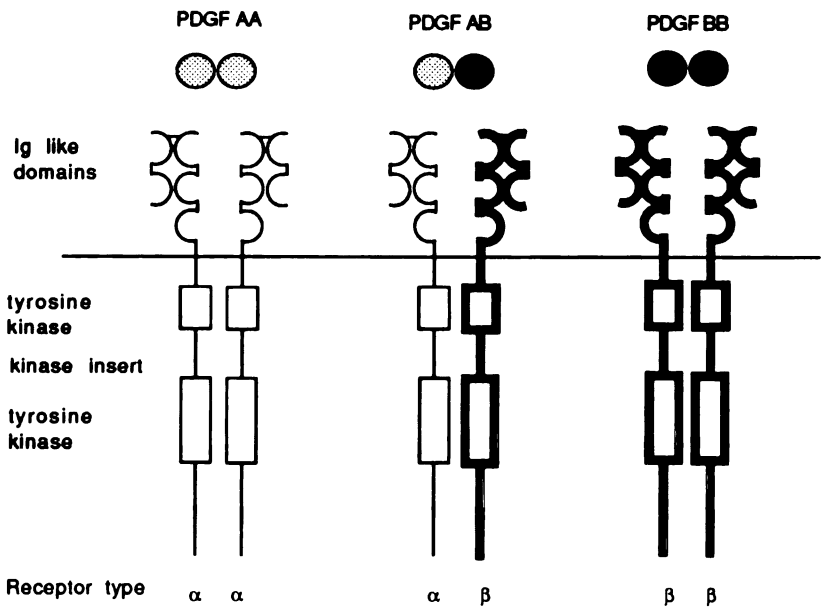
salient features are labeled on the diagram

Adapted from:

Classon-Welsh, L. (1994). Platelet-derived Growth Factor Receptor Signals. J. Biol. Chem. 269, 32023-32026.

Figure 4

PDGF Binding to its Receptors



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CHAPTER 2

EXPRESSION OF THE PDGF BETA RECEPTOR DURING ORGANOGENESIS AND TISSUE DIFFERENTIATION IN THE MOUSE EMBRYO

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EXPRESSION OF THE PDGFβ RECEPTOR DURING ORGANOGENESIS AND TISSUE DIFFERENTIATION IN THE MOUSE EMBRYO

E. SHINBROT, K.G. PETERS, and L.T. WILLIAMS*

Howard Hughes Medical Institute, Program of Excellence in Molecular Biology, Cardiovascular Research Institute at the University of California San Francisco, San Francisco, CA 94143, USA

*Corresponding author

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Key words: PDGF receptor, embryogenesis, growth factor, in situ hybridization.

ABSTRACT

In this study we used in situ hybridization to localize expression of the PDGFβ receptor mRNA during organogenesis in the mouse embryo (E. 9.5 to 16.5). Expression was first seen in periaortic mesenchyme (E. 9.5- 10.5). Later (E.12.5 - E.16.5), the receptor was expressed in the mesenchymal component of many developing tissues and organs, particularly derivatives of the primitive gut. The expression was exceptionally high in mesenchyme directly supporting an epithelium, typical of many developing organs such as the trachea and intestine. However, as the mesenchyme differentiated into smooth muscle, PDGFβ receptor mRNA was no longer detected. The expression of the PDGFβ receptor mRNA in mesenchymal components of developing organs, along with its absence in epithelial tissues indicates it may play a role in mesenchymal-epithelial interactions during organ development. Somewhat unexpectedly, the PDGFβ receptor was highly expressed in the endothelium of small blood vessels and vascular structures such as the hyaloid plexus and choroid plexus. In large blood vessels, PDGFβ receptor mRNA was found in the mesenchyme surrounding the endothelium. This suggests that the PDGFβ receptor is involved in growth and development of blood vessels.

INTRODUCTION

Platelet derived growth factor (PDGF) is a major mitogen for mesenchymal cells. The PDGF ligand consists of a disulfide-linked hetero- or homodimer of two related gene products, PDGF-A and PDGF-B (reviewed in Heldin and Westermark, 1989). Two types of PDGF receptors, α and β, have been identified (Yarden et al., 1986; Matsui et al., 1989). The platelet-derived growth factor α receptor (PDGFαr) binds PDGF-AA, PDGF-AB, and PDGF-BB. While the platelet-derived growth factor β receptor (PDGFβr) binds PDGF-BB with high affinity, PDGF-AB with low affinity, and PDGF-AA not at all (reviewed in Heldin and Westermark, 1989). PDGF and its receptors have been implicated in many cellular process including chemotaxis and mitogenesis of connective tissue cells, wound healing, neoplasia, fibrosis, myeloproliferative disease, atherosclerosis, and embryogenesis (reviewed in Ross et al., 1986).

Since PDGF is known to stimulate cellular proliferation and migration it has been hypothesized that it is an important regulator of embryonic development. Recent reports have described the presence of the PDGF ligand and receptor genes during embryogenesis (reviewed in Bowen-Pope et al., 1991), however the role of PDGF and its receptor in development has not yet been determined. Both PDGF-A and PDGFαr have been detected in pre-implantation mouse blastocyst and early embryos by RNA protection analysis. In contrast, the PDGFβr was not found until later, 7.5 day of gestation (Rappolee et al., 1988; Mercola et al., 1990). These findings suggested that the PDGF-A ligand and α receptor might be important in early embryonic development, while the β receptor is involved in later events. Recent studies using in situ hybridization have described the location of the PDGFαr

mRNA in mesoderm derivatives throughout development (Schatteman et al., 1992; Orr-Urtreger et al., 1992, Orr-Urtreger and Lonai, 1992). The PDGF-A ligand was found in layers adjacent to those expressing the α receptor, primarily in the epithelium of various organs, suggesting developmental interactions between the cell layers (Orr-Urtreger and Lonai, 1992). Recently the PDGFβr was described in growing blood vessels in human placentae, indicating a role in angiogenesis (Holgren, et al., 1991). To gain further insight into the role of PDGF in embryonic development we have analyzed the expression of the PDGFβr in the mouse embryo (E. 9.5-16.5).

RESULTS

Mouse embryonic sections were hybridized with both antisense and sense (control) PDGFβ receptor riboprobes. No specific hybridization signal was seen in any of the sense controls.

PDGFβr expression in mesenchyme

The PDGFβ receptor was broadly expressed in mesenchyme at all stages examined (E. 9.5 - 16.5). In addition to other mesenchymal populations, the PDGFβr mRNA was expressed in facial, cephalic, and limb bud mesenchyme (Fig. 1A,B). Expression in facial mesenchyme was highest adjacent to an epithelium. At 16.5 days p.c., when a multilayered epidermis is formed, the PDGFβr was expressed in the dermis of the skin, but was not detected in the epidermis (data not shown). In general, expression was highest in mesenchyme cells adjacent to an epithelial layer (Fig. 2). At 12.5 days post coital (p.c.) the gut consists of a simple tube composed of stratified endodermal cells surrounded by mesenchymal cells which express high levels of PDGFβr mRNA (Fig. 2C,D). From 12.5 days p.c. to 18.5 days p.c. the gut mesenchyme differentiates to form smooth muscle layers. At 14.5 days p.c. cells identified as smooth muscle (see materials and methods) did not contain detectable mRNA for the PDGFβr (Fig. 2E-H). However, high levels of PDGFβr mRNA were present in the mesenchyme between the smooth muscle and the gut epithelium (Fig. 2F,H). Thus, the PDGFβr mRNA was present in the undifferentiated mesenchyme, yet as the mesenchyme differentiated to form antibody positive smooth muscle cells, the

PDGFβr mRNA was no longer detected. This pattern of expression was seen at all levels of the gut and trachea. The receptor was found throughout the mesenchyme in parenchymal organs such as the gonads (Fig. 2C,D) lung and kidney (data not shown).

PDGFβr expression in the heart and developing vasculature

At 10.5 days p.c. the PDGFβr was expressed in the pericardium and endocardium of the developing heart (Fig. 3A,B). The myocardium did not express the receptor. In large blood vessels the expression of the PDGFβr mRNA was highest in the mesenchyme surrounding the endothelium (Fig. 3C,D).Somewhat surprisingly the PDGFβr was expressed in the endothelium and mesenchyme surrounding small blood vessels during all stages of embryonic development examined (Fig. 4). PDGFβr was detected in small vessels in the brain (Fig. 4G-I). The perineural plexus contained high amounts of PDGFβr mRNA (Fig. 4 G-I), as did the hyaloid plexus of the developing eye (Fig. 4 A-F), and the choroid plexus of the developing brain (Fig. 3E). However vessels in the developing liver were not labeled.

PDGFβr expression in developing bone

The PDGFβr mRNA was present in developing bones (Fig. 5A,B) in cells within the presumptive marrow. No PDGFβr mRNA was detected in ossification centers or in hypertrophic cartilage.

DISCUSSION

In this study we found that PDGFβr mRNA was highly expressed in the mesenchyme of many developing tissues. The mesenchymal components of many tubular organs, such as the esophagus, intestine, stomach, trachea, and blood vessels had particularly high expression of the PDGFβr mRNA as did the mesenchymal components of parenchymal organs such as the lung, kidney, and gonads. As the mesenchyme layer of these organs began to differentiate, the PDGFβr mRNA was not expressed in newly formed smooth muscle layers, while adjacent mesenchymal layers retained high levels of PDGFβr mRNA expression. This pattern of expression suggests that the PDGFβr plays a role in the early development and growth of the mesenchymal components of these organs and perhaps in mesenchymal/epithelial interactions.

PDGF has been shown to stimulate proliferation of fibroblasts, smooth muscle and mesenchymal cells in vitro (Ross et al., 1986). High levels of the PDGFβr have been described in many proliferative disorders including skin epithelial cells following skin injury (Antoniades et al., 1991), rheumatic synovial tissue (Reuterdahl et al., 1991), vascular cells in rejected kidney transplants (Rubin et al., 1988), and smooth muscle cells of atherosclerotic lesions (Wilcox et al., 1988). However, the normal non-diseased counterparts of inflamed tissue do not express the PDGFβ receptor. In our study, mesenchyme around newly formed organs had high expression of the PDGFβr mRNA while terminally differentiated cells such as smooth muscle cells no longer express the PDGFβr mRNA. It is possible that inflammation and injury cause an induction of PDGFβr gene expression and

thus contribute to pathologic unregulated growth.

PDGF has been implicated in the process of angiogenesis during placental development (Holmgren, et al., 1991). Our results support the hypothesis that PDGFβr plays a role in embryonic vessel development. In small vessels, the PDGFβr mRNA was detected in both the mesenchyme surrounding the vessels and the endothelium while in large vessels the expression was highest in the mesenchyme surrounding the endothelium. This is consistent with the reported expression of the PDGF ligand and receptor genes in placental development (Holmgren, et al., 1991). PDGFβr mRNA was also expressed in vascular structures such as the hyaloid plexus of the eye and the choroid plexus (both in vascular and mesenchymal components) of the brain.

When the expression pattern of the PDGFβr mRNA is compared to recently described reports of the PDGFαr mRNA (Schatteman et al., 1992; Orr-Urtreger et al., 1992; Orr-Urtreger and Lonai, 1992) it appears that both receptors are expressed in mesenchymal populations of the face, newly formed organs and in the choroid plexus. This presumed co-expression of both receptors is not surprising, since they form heterodimers when bound by the PDGF-A-B heterodimeric ligand (reviewed in Williams, 1989; Heldin and Westermark, 1989). The co-expression of both receptors may allow any form of PDGF (AA, BB, or AB) to be active in that tissue. The expression of PDGFαr in mesenchyme has been correlated with defects seen in mouse Ph/Ph homozygous embryos (Schatteman, et al., 1992). In the homozygous Ph/Ph embryo the connective tissue and smooth muscle components of many organs are disorganized and diminished, yet still present. The presence and partial organization of these tissues many be due to redundant functions of the α and β receptor

types. Since both PDGFα and β type receptors bind PDGF-B, the presence of the PDGFβr in Ph/Ph mutants may allow limited development of mesenchymal structures.

Although the PDGFα and β receptors were co-expressed in many embryonic tissues, there were some differences. The endothelium of small blood vessels expressed the PDGFβr mRNA, yet in recent reports of its expression, the PDGFαr mRNA was not described in blood vessels (Schatteman et al., 1992; Orr-Urtreger et al., 1992; Orr-Urtreger and Loni, 1992). It has previously been shown that the PDGFβr only binds PDGF BB, therefore the sole expression of the PDGFβr mRNA in endothelium of blood vessels suggests that PDGF BB might be important in embryonic blood vessel development.

The expression of the PDGFβr described in our study is consistent with its implicated role in development and disease. Functional studies of both the mRNA and protein products of this gene will be necessary to further ascertain its role in these processes.

EXPERIMENTAL PROCEDURES

Riboprobe generation

A 461 bp sac I fragment of the murine PDGFβr (0-461) cloned in the pGEM (Promega) vector was used. Radiolabeled antisense and sense transcripts were made by in vitro transcription using ^{35}S-labeled UTP (1200 Ci/mmol, Amersham). Plasmids were linearized with the appropriate restriction enzymes and transcribed using either SP6 (for antisense probes) or T7 (for sense probes) RNA polymerase (Promega). Each experiment included a set of sections hybridized with the sense PDGFβr probe.

In situ hybridization

Embryos were collected as described previously (Peters et al., 1992). The embryos were fixed in 4% paraformaldehyde and embedded in paraffin. 5 um serial sections were cut and placed on vectabond coated slides. The slides were dewaxed in xylene, which was removed with ethanol. The ethanol was removed by incubation in saline (5 min), then PBS (5 min). The sections were post-fixed in 4% paraformaldehyde in PBS for 20 min. The sections were washed with PBS (5 min), then acetylated with acetic anhydride in .1 M Triethanolamine-HCL (2 x 5 min). After washing the slides with PBS then saline, they were dehydrated by passing the slides through 30%, 60%, 85% 95% ethanol in saline. The riboprobe (5 x 10^5 cpm/slide) was dissolved in hybridization mix (50% DI formamide; 0.3 M NaCl; 20 mM Tris-HCL pH 8.0; 10 mM Sodium phosphate, pH 8.0; 10% Dextran sulfate; 1X denhardts; 0.5 mg/ml yeast tRNA; 10 mM DTT) and added to each slide. The slides were covered

with a parafilm strip and incubated in a hybridization chamber in a 55°C oven overnight. The next day the slides were incubated in high stringency buffer (5 X SCC, 0.01 M DTT, 50% formamide) at 65°C for 30 min. They were then incubated in wash buffer (0.5 M NaCl, 10 mM Tris-HCL pH 8.0, 5 mM EDTA) at 37°C 3 times for 10 min each time. Next they were incubated with 20 ug/ml RNase A in wash buffer at 37°C for 30 min. The slides were rinsed in wash buffer at 37°C for 15 min, then placed in high stringency buffer for 20 min. The slides were then washed in 2 X SCC, then .1 X SCC for 15 min each at 37°C. The sections were then dehydrated by passing them through 30%, 60% 80% 95% ethanol in .3 M ammonium acetate, then through 100% ethanol twice. The slides were air dried then dipped in Ilford K5 Emulsion diluted 1:1 with water. The slides were incubated at 4°C in a light safe container for 4 weeks. After developing the emulsion, the slides were stained with eosin and hematoxylin.

Immunolocalization of muscle specific actin

5 um paraffin sections (serial sections to those used for in situ hybridization) were stained with antibodies against muscle-specific actin HHF-35 (ENZ diag) using Vectastain ABC-AP - Vector RED reagent kit (Vector Laboratories) according to the vendors instruction. The antibody positive cells are indicated by a red color. The slides were lightly counter stained with Hematoxylin.

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FIGURE LEGENDS

Fig. 1. PDGFβ receptor expression in 10.5 day mouse embryo. (A) Bright field photographs, (B) dark field of para-sagittal section of a 10.5 day mouse embryo (50X magnification), showing positive signal in the perineural plexus, facial mesenchyme, limb bud mesenchyme dorsal aorta (star) and other blood vessels. bv, blood vessel; fm, facial mesenchyme; m, mesenchyme; pn, perineural plexus.

Fig. 2. PDGFβ receptor expression in mesenchymal components of developing organs. (A) Bright field (B) dark field of a coronal section of 10.5 day embryo showing positive signal in blood vessels, mesenchyme surrounding the esophagus and trachea (100X magnification). (C) Light field (D), dark field photograph of a para-sagittal section through a 12.5 day p.c. embryo showing positive signal around the mesenchyme of the gonad and stomach (100X magnification). (E) To evaluate the pattern of expression of the PDGFβr mRNA in differentiating smooth muscle sections were stained with a monoclonal antibody against muscle actin, HHF35 (Enz Diag).(E,F) Frontal section through the gut region of a 14.5 day mouse embryo (100X magnification) showing smooth muscle actin antibody staining in the intestine (E, red color) and mRNA staining in the mesenchyme adjacent to the smooth muscle layer (F, brown silver grains). Staining of muscle specific actin (G,H) appears red. (G,H) Higher magnification (200X) of (E,F) showing antibody stained smooth muscle layer (G), and silver grains in mesenchymal layer (H). bv, blood vessel; ep, epithelium; es,

esophagus; m, mesenchyme; i, intestine; li, liver; sm, smooth muscle layer; st, stomach; tr, trachea.

Fig. 3. PDGFβ receptor in the developing heart and vasculature. (A) Light field, (B) dark field of a 10.5 day embryo, coronal section through the heart, both the pericardium and endocardium show silver grains while the myocardium is unlabeled. (C) Blood vessel in a 10.5 day embryo (400X magnification using epi-illumination with transmitted light the use of a filter makes the silver grains appear green, the mesenchyme surrounding the vessel is indicated with a closed white arrow, the endothelium is indicated by a small closed black arrow. (D) 1,000X magnification of the corner region of the same vessel, the black arrows designate the endothelium and the white arrows, the mesenchyme. (E) High magnification (400X) of the choroid plexus in a 14.5 day embryo, the silver grains appear dark brown. en, endocardium; my, myocardium; pc, pericardium; cp, choroid plexus; ne, neuroepithelium;.

Fig. 4. PDGFβ receptor expression in vascular plexus. (A) Light field, (B) dark field section of a 10.5 day embryo, black arrow in (A) and white open arrow in (B) point to the hyaloid plexus (200X magnification). (C) 1,000X magnification of the hyloid plexus in (A,B), white astrick points out blood cell present in the vasculature. (D) Light field, (E) dark field section of a 16.5 day embryo, black arrow in (D) and white open arrow in (E) point to the hyaloid plexus (100X magnification). (F) 1,000X magnification of the hyloid plexus in (D,E). (G) Light field, (H) dark field coronal section of a 10.5 day embryo showing labeling in the perineural plexus, while arrow in (H) shows labeled blood vessels in the brain. (I) 1,000X magnification of white arrow in (H), white astrick shows a blood cell. bv, blood vessel; le,

lens; pn, perineural plexus.

Fig. 5. PDGFβ receptor expression in developing bone. (A) Light field, (B) dark field illumination of the PDGFβ receptor in a long bone in a 16.5 day embryo (200X magnification) pm, presumptive marrow.

Figure 1

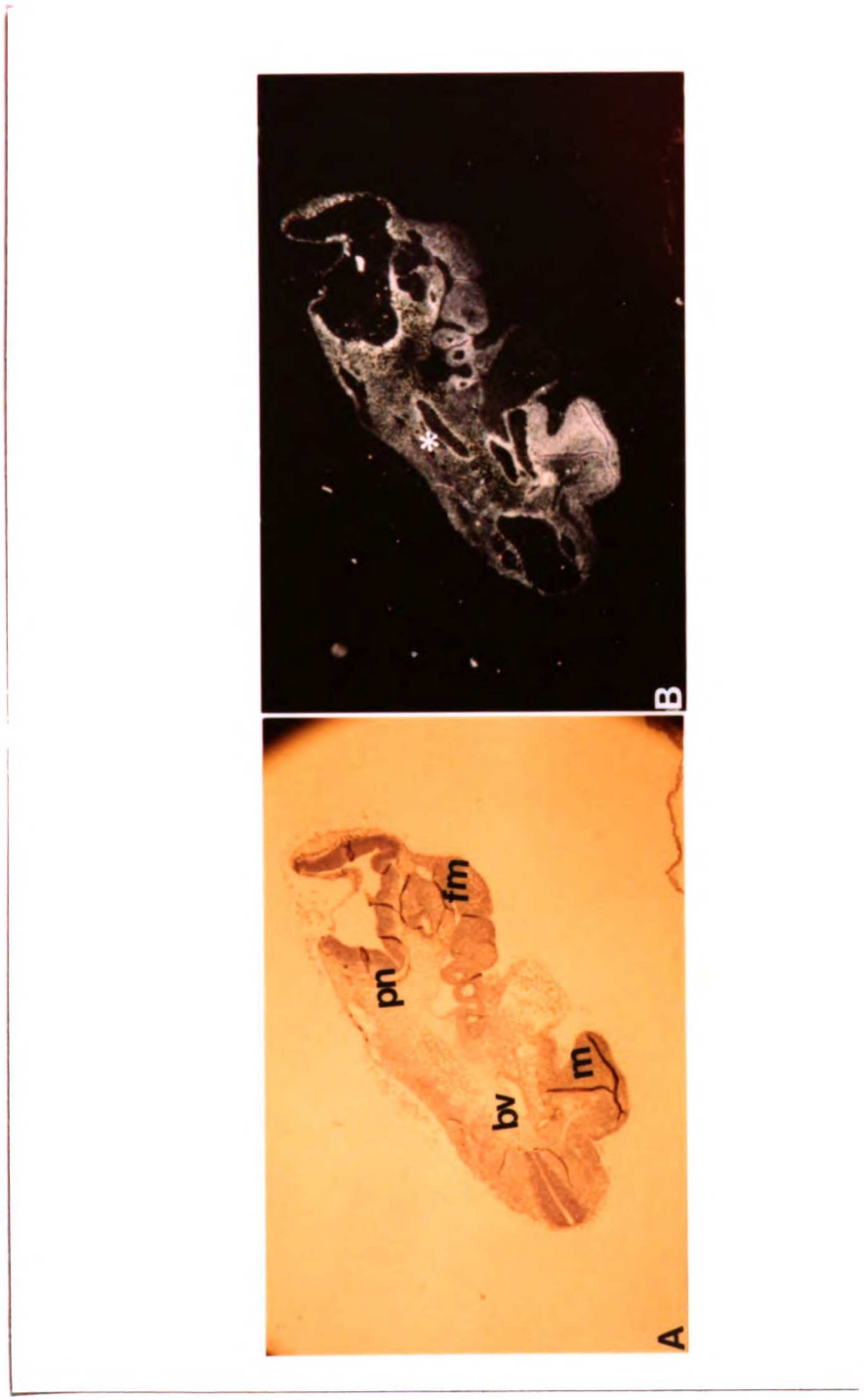


Figure 2

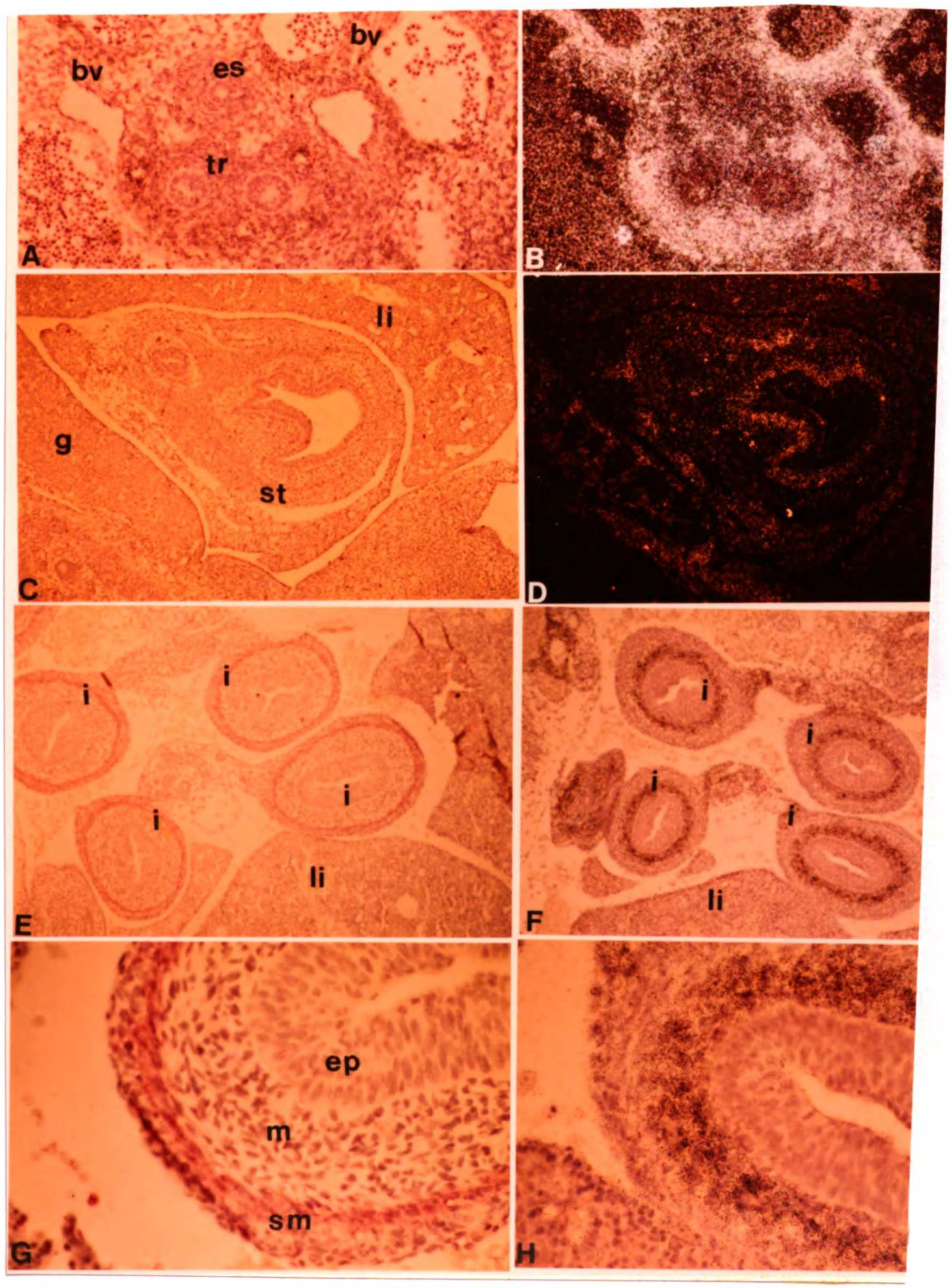


Figure 3

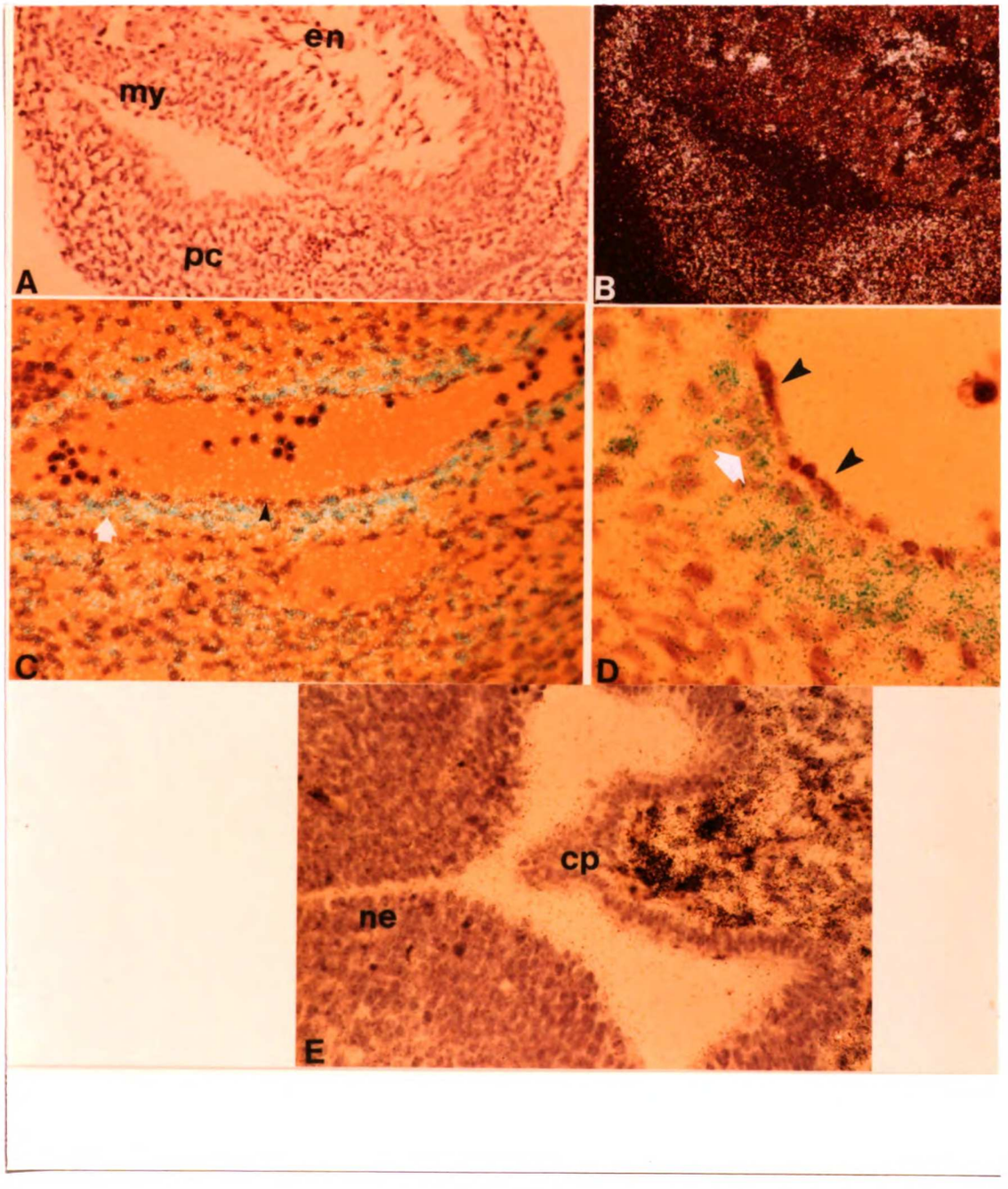


Figure 4

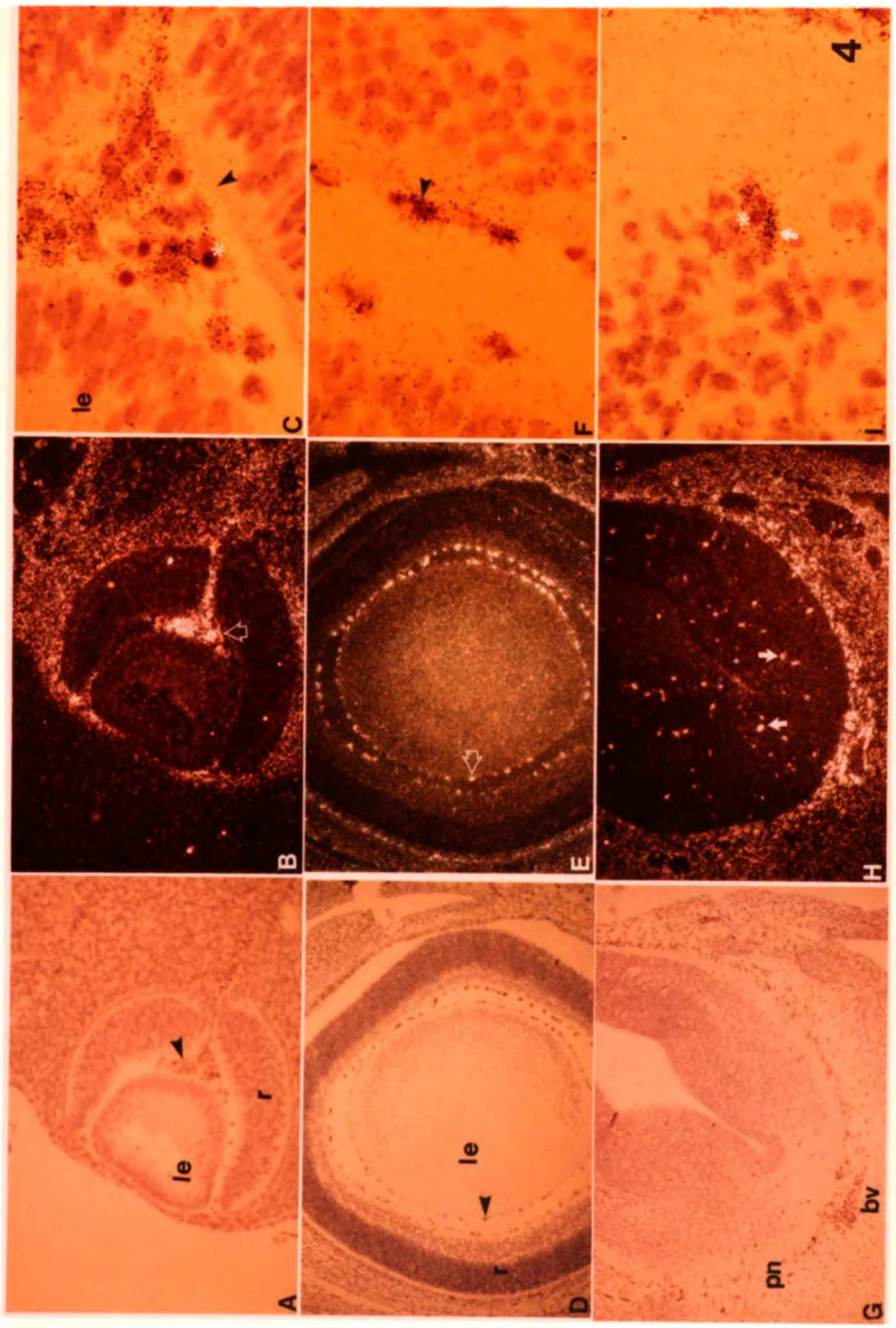
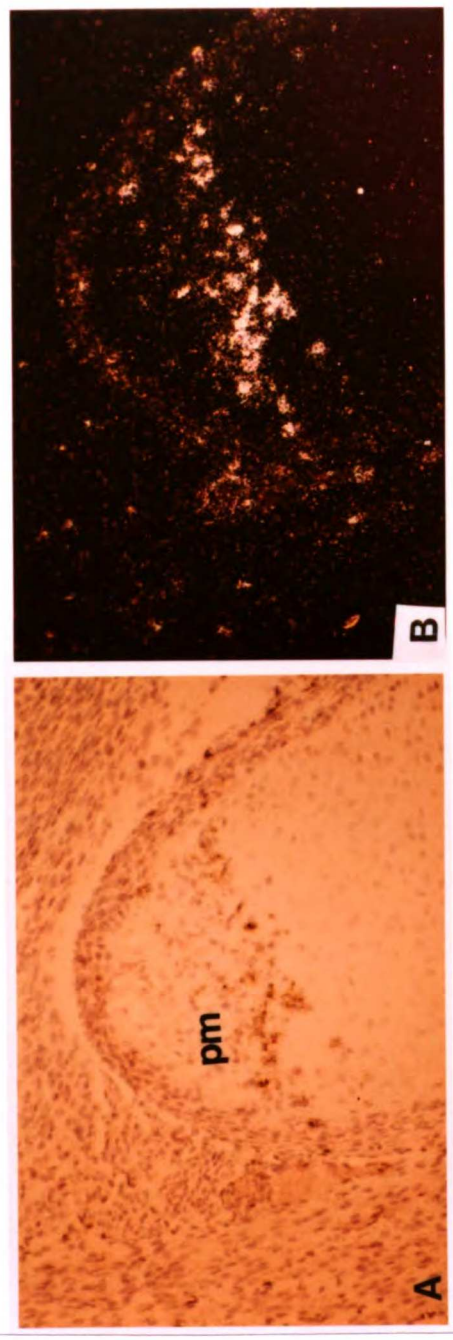


Figure 5



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CHAPTER 3

ISOLATION AND CHARACTERIZATION OF THE PLATELET-DERIVED GROWTH FACTOR BETA RECEPTOR PROMOTER

MANUSCRIPT IN PREPARATION

Isolation and Characterization of the Platelet-Derived Growth Factor Beta Receptor Promoter

Eve Shinbrot, Xiang Liao, and L.T. Williams*

Daiichi Research Center,

Cardiovacular Research Institute,

University of California at San Francisco,

San Francisco, California 94143

*Corresponding author:

Lewis T. Williams, M.D., Ph.D.

Professor of Medicine

University of California

505 Parnassus Ave., San Francisco, CA 94143

Phone: (415) 476-4402

Fax: (415) 476-0429

Abstract

We have cloned and characterized the promoter region of the platelet-derived growth factor beta receptor (PDGFβr). We isolated a 4.5 Kb genomic fragment which confers PDGFβr tissue-specific promoter activity. This fragment can direct transcription of a luciferase reporter gene in a cell-specific manner which correlates well with the known pattern of expression of the PDGFβr. The specificity of this clone was demonstrated by its high activity in NIH 3T3 fibroblasts and lack of activity in N-MUNG epithelial cells. NIH 3T3 cells are known to express high levels of endogenous PDGFβr, while N-MUNG epithelial cells do not express the receptor. We have defined a 614 bp region encompassing the 5 ' UT region of the gene which includes the basal promoter region. We have also determined that the 4.5 Kb promoter fragment is sufficient to direct reporter gene expression with the same profile as the endogenous PDGFβr. We generated transgenic mice that carry the chloramphenicol acetyltransferase (CAT) reporter gene under the control of the promoter. The expression pattern of the reporter gene was compared to the endogenous PDGFβr gene. The promoter was able to direct reporter gene expression with the same temporal and spatial pattern as the endogenous PDGFβr. The most prominant expression was in condensing mesenchyme of developing blood vessels, bone and tissues adjacent to epithelium. We conclude that this clone contains the regulatory regions sufficient to direct expression of the PDGFβr. Therefore, this PDGFβr promoter may be used to monitor the transcriptional regulation of the PDGFβr in response to normal and pathological conditions. This promoter should be a useful tool to deliver genes with the same expression pattern as the PDGFβr. These may be genes

that modulate the PDGFβr or heterologous genes. The further analysis of this promoter will help elucidate the transcriptional regulation of expression of the PDGFβr, and provide a useful tool for the tissue specific delivery of genes.

Introduction

Platelet-derived growth factor (PDGF) and its receptors have been implicated in many cellular processes including mitogenesis, development, wound healing, fibroproliferative disease, neoplasia and atherosclerosis (Ross, 1993; Ross et al., 1986). PDGF exists as hetero- or homodimeric complexes of two ligands, PDGF-A and PDGF-B (Heldin and Westermark, 1989). Two receptors for PDGF have been identified, they bind the various forms of PDGF with different affinities. The PDGF alpha receptor (PDGFαr) (Yarden, et al., 1986) binds all forms of PDGF (PDGF-AA, PDGF-AB and PDGF-BB), while the PDGF beta (PDGFβr) receptor (Matsui et al., 1989) binds PDGF- BB with high affinity, PDGF-AB with low affinity and does not bind PDGF-AA (Heldin and Westermark, 1989).

The expression of the PDGFβr is regulated in both development and many disease processes. During development the PDGFβ receptor is widely expressed in mesenchyme, endothelium, and vascular structures such as the hyaloid plexus and choroid plexus (Shinbrot et al., 1994). The expression of the PDGFβr is exceptionally high in mesenchymal tissues which directly support an epithelium, typical of many developing organs. The PDGFβr is not expressed in epithelium at any stage of development analyzed. Recently, mice deficient in the PDGFβr have been generated by homologous recombination in ES cells (Soriano, 1994). The homozygous mutant embryos displayed multiple defects and died at or shortly before birth. These mice had multiple hematological, vascular, and kidney glomerulus disorders, indicating the PDGFβr plays an important role in the development of these tissues.

The expression of the PDGFβr is restricted to specific cell types in

response to cellular states. The PDGFβr is highly expressed and is essential for the development of some tissues and organs, yet it is not widely expressed in adult tissue . However its expression is greatly increased during wound healing and disease processes including atherosclerosis , fibroproliferative disease, and neoplasia (Ross et al., 1986);(Heldin and Westermark, 1989). It is possible this increased expression is due to transcriptional regulatory mechanisms. The examintion of the promoter region of the PDGFβr would help ascertain the mechanisms of its regulation and its role in both normal and pathological cellular processes. Therefore we isolated and characterized the promoter region of the PDGFβr gene.

There are many uses for the characterized PDGFβr promoter. Initially it may be used to monitor the transcriptional regulation of the PDGFβr during normal cellular processes such as development and wound healing. Ultimately, the characterized PDGFβr promoter may be used as a tool to direct expression of a gene to have the same temporal and spatial pattern as the endogenous PDGFβr. This may be a gene which would modulate the PDGFβr or a heterologous gene which may be delivered to specific cell types at a specific time.

To study the mechanisms of PDGFβr regulation, we identified the regulatory regions of the gene. In this paper we show the analysis of a genomic clone including 4.5 kb of upstream genomic sequence which is able to direct reporter gene expression in a tissue specific manner in both *in vitro* and *in vivo* analysis. The predominant activity appeared to be in a 614 bp segment of the gene. This clone is able to direct the expression of a reporter gene with the same spatial and temporal pattern as the endogenous PDGFβr.

RESULTS

Genomic Organization of the 5 prime end of the PDGFβr

Mouse genomic clones of the PDGFβr were isolated by probing a mouse genomic EMBL3 library (Clonetech) with the 5' end of the PDGFβr cDNA (462 bp SacI fragment encompassing the 5' end of the cDNA). Southern blot analysis and restriction digestion indicated that the genomic organization of the PDGFβr diverges from the cDNA by a large intron (over 10 Kb) which separates the 5' untranslated end of the cDNA from the first ATG. We focused on a 4.5 Kb EcoR1-Bam H1 clone that hybridized strongly to the 5' end of the cDNA . Sequence analysis indicated that this clone contains about 2,500 bp of upstream genomic sequence (-2,500), the entire 5 ' UT region of the cDNA (132 bp, exon 1) and about 2,000 bp downstream that is not encoded by the cDNA sequence (intron 1). The genomic structure of this region of the PDGFβr is shown in Fig. 1. This clone (designated -2500/+2,000) was used for all of the experiments reported in this paper.

Luciferase activity of the -2,500/+2,000 clone

The -2,500/+2,000 clone was able to direct reporter gene expression in cells wich display high expression of the endogenous PDGFβr. We cloned the -2,500/+2,000 genomic clone into a promoterless luciferase vector pGL-basic Luc (Promega). This construct was transiently transfected into NIH3T3 cells using calcium phosphate precipitation. A pSV-β-galactosidase plasmid (Promega) was co-transfected to monitor transfection efficiency. Twenty-four hours after transfection the cell lysates were harvested and assayed for β–galactosidase activity (β-gal). The volume of extract was normalized for β-gal activity and then assayed for luciferase activity. The -2,500/+2,000 genomic

clone showed high reporter gene activity in these cells (Fig. 2 lane A).

Deletional analysis

We localized key regions involved in promoter activity using deletion analysis of restriction sites present in the 4.5 Kb EcoR1-Bam H1 genomic fragment. The deletion constructs were transfected into NIH 3T3 cells and analyzed for luciferase activity (Fig. 2). When 1.9 Kb was removed from the 5' end (-600/+2,000-Luc) there was no effect on luciferase activity (Fig. 2, lane B). However when a 880 bp fragment was removed from the center region of the gene, luciferase activity was greatly reduced to 10-20% of the full length EcoR1-BamH1 fragment (Fig 2 lane C). PCR was used to obtain a 614 bp region (-354/+260) surrounding the 5 prime untranslated region. The -354/+260 clone was ligated into the luciferase vector and luciferase activity was analyzed. This clone was able to restore luciferase activity, however the activity was 50-60% of the full length -2,500/+2,000 or the -600/+2,000 clone (Fig. 2 lane D). This indicates that a 600 bp region surrounding the 5 ' end of the gene is necessary for reporter gene expression, however it is not sufficient to restore total reporter gene expression. This region of DNA likely encompasses a basal promoter element while positive regulatory elements probably reside within 600 bp upstream of the 5' untranslated region and possibly within the first intron.

The specificity of the 4.5 Kb clone andthe deletion constructs was analyzed in N-MUNG epithelial cells which do not possess PDGFβ receptors. In these cells, the genomic clones were not able to direct luciferase expression (Fig. 2).

Transgenic analysis

The ability of the 4.5 Kb clone to direct reporter gene expression in vivo was examined using transgenic mice. The -2,500/+2,000 clone was ligated to pbasicCAT (Promega). This construct (Fig. 3) was used to generate transgenic mice as described in materials and methods. Transgenic mouse line 41 was used for this study. Both in situ hybridization of the bacterial CAT mRNA and immunocytochemistry of the bacterial CAT protein were used to examine the expression pattern of the transgene. The expression pattern of the transgene was compared with the expression pattern of the endogenous PDGFβr during late embryogenesis (day 12.5-16.5). There was no difference in the expression pattern in either the transgene or endogenous PDGFβr during those time points, therefore embryonic day 14.5 was used for most of these studies. In all tissue examined, the transgene had the identical expression pattern as the endogenous PDGFβr mRNA and protein.

Expression in mesenchyme

The CAT reporter gene was seen in mesenchymal tissues throughout the embryo. The endogenous PDGFβr is highly expressed in the mesenchyme of developing mouse embryos, as previously reported (Shinbrot et al., 1994). The transgene was highly expressed in mesenchyme, both in developing organs such as the lung (Fig. 4A,B) and facial mesenchyme (Fig. 4C-F). The expression in mesenchyme was particularly striking in regions where mesenchyme was supporting an epithelium such as the dermal papillae (Fig. 4 G,H). This pattern of expression of both the 4.5 kb-CAT transgene and the PDGFβr was seen in mesenchyme throughout the embryo.

Expression in developing bone

Expression of both the CAT transgene and the endogenous PDGFβr was

seen in the mesenchyme condensing to form developing bone. Expression was high in the mesenchyme surrounding the bone, however, neither transcript was detected in ossification centers or hypertrophied cartilage. Both the CAT transgene and the endogenous PDGFβr were expressed in vascular regions surrounding developing bone. This pattern of expression was seen throughout the embryo (Fig. 5).

Expression in vasculature and vascular plexus

The CAT transgene and endogenous PDGFβr were detected in vasculature and vascular structures throughout the embryo. Vascular plexus such as the choroid plexus (Fig. 6), had high expression of the transgene. The choroid plexus consists of both mesenchyme and capillaries, both mesenchyme and capillary endothelium (these small blood vessels do not possess smooth muscle) show localization of both the CAT transgene and the endogenous PDGFβr protein (Fig. 6). The same expression pattern was seen in the hyloid plexus and perineural plexus (not shown) In large blood vessels the transgene expression was high in mesenchyme condensing around the blood vessel (Fig. 7). The transgene was also expressed in capillaries throughout the embryo.

Discussion

We have cloned and characterized the promoter region of the PDGFβr gene. Cell culture analysis indicated the 4.5 Kb genomic clone was able to direct reporter gene expression in a tissue-specific manner. Examination of the clone in transgenic mice indicated it is able to direct reporter gene expression with the same temporal and spatial profile as the endogenous PDGFβr.

The genomic organization of the PDGFβr gene consists of upstream genomic DNA, the 5' untranslated region of the gene, and a large intron which spans over 10 kb which separates the first exon from the translation initiation site. This genomic organization is similar to two closely related genes, the PDGFαr (Wang and Stiles, 1994), and c-fms (Roberts et al., 1992; Yue et al., 1993)). The similar genomic organization of the PDGFβr, α receptor and c-fms genes supports the hypothesis that these genes arose due to gene duplication (Giebel et al., 1992). The 5' flanking sequence of the PDGFβr does not contain a TATA box, however it has a GC-rich region and potential binding sites for transcription factors, this is similar to the c-fms gene (Yue et al., 1993). We are currently analyzing this region of the gene.

We analyzed the ability of regulatory regions surrounding the PDGFβr gene to direct reporter gene expression. Using a fibroblast cell line (NIH3T3) which highly expresses the PDGFβr, the -2,500/+2,000 genomic clone was able to direct luciferase activity. Deletional analysis indicated that a 614 bp fragment which encompasses the 5' UT region can direct luciferase activity, however the expression level was lower than the entire -2,500/+2,000 clone.

Removal of this region from the -2,500/+2,000 clone reduces its ability to direct reporter gene activity. This indicates that the 614 bp fragment contains a basal promoter . Positive regulatory elements are located within the -2,500/+2,000 clone. Further studies are underway to identify these elements. Cell type specificity was demonstrated using N-MUNG epithelial cells which lack the PDGFβr. None of the clones analyzed (the 4.5 Kb clone or its deletional constructs) were able to direct luciferase activity in epithelial cells. These experiments indicated that we had isolated regulatory regions of the PDGFβr gene which possess cell-type specificity.

To determine if the 4.5 Kb clone contained regulatory regions capable of directing reporter gene expression with the same profile as the endogenous PDGFβr gene, we analyzed the -2,500/+2,000 clone in transgenic mice. The -2,500/+2,000 clone was ligated to a promoterless CAT vector (pCat-Basic, Promega), and used to produce transgenic mice. Both in situ hybridization and immunocytochemistry were used to detect the CAT transgene. The expression profile of the CAT transgene was compared to the endogenous PDGFβr gene. Both mRNA and protein localization showed that the -2,500/+2,000 fragment was able to direct CAT expression with the same temporal and spatial pattern as the endogenous gene. The most prevalent site of reporter gene expression was mesenchyme. The CAT transgene was expressed in mesenchymal populations throughout development, however its expression was highest in regions where mesenchyme is condensing such as surrounding developing blood vessels, bone, and mesenchyme supporting an epithelium. This pattern is consistent with the expression pattern of the PDGFβr and its proposed role in the development of these tissues.

These results indicate that the -2,500/+2,000 clone contains the cis

regulatory elements necessary to control the temporal and spatial expression of the PDGFβr gene. The expression of the PDGFβr is highly regulated. Many disease processes including atherosclerosis, neoplasia, and fibrosis show aberrant expression of this gene, while the non-diseased tissue counterparts do not express the gene. The further study of the promoter region of the PDGFβr may provide clues as to its role in these disease processes. Our future experiments include the examination of the PDGFβr promoter-CAT transgene in response to vascular injury, cell transformation and other pathologial processes. We are also interested in identifying the cis regulatory elements present in the 4.5 Kb clone and the protein factors that contribute to the control of expression of this gene under both normal and pathological cellular conditions.

Oncogenic transformation has long been thought to have an effect on growth factor and growth factor receptor genes. Transformed fibroblasts possess lower amounts of PDGF-BB binding sites (Bowen-Pope et al., 1984). Recently, v-ras and v-src transformed 3T3 cells were shown to express significantly lower amounts of PDGFβr than their parental cell lines (Vaziri, 1995). Many proto-oncogenes are derived from signalling molecules which act downstream from activated receptors, such as *ras*, *src*, and *abl.*, this might circumvent the need for the ligand activated receptor. It is possible that oncogenic transformation activates transcriptional silencers that repress the expression of the PDGFβr gene. The PDGFβr promoter may be used to determine the mechanisms of transcriptional repression due to oncogenic transformation. These studies might uncover significant mechanisms in ocogenesis and also might reveal transcriptional feedback mechanisms that are active in normal cells.

The mechanisms involved in the control of the PDGFβr may be relevant to other growth factor receptor genes particularly similar genes such as the PDGFαr and c-fms. It is possible similar regulatory molecules control these genes. During development, the PDGFαr has a similar expression profile as the PDGFβr (Orr-Urtreger and Lonai, 1992; Schatteman et al., 1992). The co-expression of both the α and β receptor may allow any form of PDGF to be active in that tissue. The PDGFβr knock out mice displayed major abnormalities in the organization of many organs, however, some organs that are known to express the PDGFβr were normal (Soriano, 1994). This may be due to the expression of the α receptor in those tissues and the redundancy it may provide. The further understanding of the promoter region of both these genes will help to ascertain the mechanisms involved in their co-expression.

We have shown this promoter can direct gene expression to specific cell types. This promoter may be used to study the PDGFβ receptor, however, it also may be used to study other proteins involved in mesenchymal-epithelial interactions. The major site of transgene expression is in mesenchyme, particularly where it is supporting an epithelium or condensing around newly developing structures. During development there are many mesenchymal-epithilial inductive interactions taking place at these sites. This promoter should be a useful tool for the expression of heterologous genes at this interface.

In conclusion, we have isolated the regulatory regions of the PDGFβr gene. The -2,500/+2,000 clone has been shown to direct reporter gene expression in a tissue specific manner with the same temporal and spatial profile as the endogenous PDGFβr. This clone may be used as a tool to direct the expression of genes to have the same temporal and spatial profile as the

endogenous PDGFβr.

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MATERIALS AND METHODS

Characterization of genomic clones

A mouse genomic EMBL3 library (Clonetech) was screened by plaque hybridization (Sambrook, 1989) using a probe consisting of the 5 prime end of the PDGFβr cDNA (1-461 bp, sac1 fragment). Overlapping genomic clones were obtained. Clones that strongly hybridized to the 5 prime end of the cDNA in Southern blot analysis were subcloned into Bluescript KS (Stratagene) . We focused on a 4.5 Kb Eco R1-Bam H1 fragment and analyzed it using restriction mapping, Southern blot analysis and DNA sequencing analysis. This 4.5 KbEco R1-Bam H1 clone contains the entire 5 prime region of the cDNA, about 2,500 bp of upstream genomic sequence and 2,000 bp that extends into the first intron sequence.

Construction of Luciferase Constructs

The 4.5 Kb Eco R1- Bam H1 fragment was cut out of pBluescript (Promega) using the Xho1 site in the pBluescript polylinker (5') and the Bam H1 site on the 3' end of the 4.5 Kb clone. It was subcloned into the promoterless pGL-basic (Promega) vector using the Xho1 (5') and Bgl2 (3' Bgl2 has compatible ends with BamH1) sites in pGL-basic Luc. This construct was designated -2,500/+2,000 -Luc. Deletional constructs were made by restriction site deletion. 1.9 Kb was removed from the 5' end by digesting the 4.5 Kb Luc construct with Kpn (there is a Kpn site in the p basic luc polylinker and 2 sites in the mouse genomic clone, about 2 Kbfrom the 5' Eco R1 site). The Kpn fragments were removed by gel purification and the resulting construct was re - ligated. This construct was designated

-600/+2,000-Luc, it consists of the entire 5' untranslated region of the cDNA, about 2 KB of genomic sequence extending downstream (including intron sequence) and about 600 bp of upstream genomic sequence. A construct missing 880 bp encompassing the 5' untranslated region was constructed by digesting the 4.5 Kb-Luc construct with Nco1, (there are only 2 Nco1 sites in the 4.5 Kb construct, these span 883 bp and bracket the 5' untranslated region). The 883 bp region was removed using gel purification, the remainder of the construct was re-ligated, the resulting construct consisted of the 4.5 Kb gene minus 883 bp surrounding the 5' untranslated region of the cDNA. The resulting construct was designated Δ 880-Luc. PCR primers were designed in the regions surrounding the 5' UT region (spanning 600 bp), these were used to obtain a 600 bp fragment which included 354 bp of upstream genomic sequence, the 132 bp 5' UT region, and 128 bp of the first intron. This fragment was blunt ended and cloned into the Sma1 site of pGL-basic Luc. This construct was designated -354/+260.

Cell culture and transfections

NIH 3T3 fibroblasts and N-MUNG epithelial cells were grown in Dulbecco's modified essential medium (DMEM) supplemented with 10% bovine calf serum . The cells were transiently transfected with reporter plasmids using the calcium phosphate method(Ausubel, 1987). (Plasmid DNA was isolated using QIAGEN maxi preparation kits as described in their protocol). N-MUNG or NIH3T3 cells were seeded on 10 mm tissue culture dishes at a density of about 50% confluence. 10 mg of reporter plasmid and 2 mg of β-Galactosidase DNA (Promega) were co-transfected. β-galactosidase was used to monitor transfection efficiency.

Reporter gene assays

The transfected cells were harvested 24 hours after transfection. The transfected cells were rinsed three times with ice cold PBS, and lysed with 1 ml of 100 mM potassium phosphate (ph7.8)/ 0.6% Triton-X 100. The insoluble cell debris was removed by centrifugation. The cell lysate was used to measure both B-galactosidase activity and luciferase activity. β–galactosidase activity was assayed using standard methods as described in Molecular Cloning (Sambrook, 1989)

The volume of cell extract used for the luciferase assay was normalized according to β-galactosidase activity. Luciferase activity was analyzed using a Moonolight 2010 luminometer using Luciferase Assay Reagent (Promega). The luciferase assay was performed according to the Promega protocol. The results represent at least 3 separate transfections for each data point analyzed.

Construction of transgene

The 4.5 Kb genomic fragment was ligated to pCAT-Basic (Promega), a promoterless reporter plasmid which contains the CAT coding region and a SV 40 poly A region.. The 4.5 Kb plasmid in pBluescript KS+(the 4.5 Kb Eco R1 - Bam H1 fragment was ligated into Bluescript KS cut with EcoR1 - Bam H1) The 4.5 Kb insert was removed from pBluescript KS using the Sal1 (5 prime) and Spe 1 (3 prime) multiple cloning sites in pBluescript KS (Promega) The pCAT basic plasmid was cut with Sal 1 (5 prime) and Xba1 (3 prime, a compatible site with Spe 1) The 4.5 Kb fragment was ligated in using standard techniques. The resulting plasmid designated as PDGFβr promoter-CAT (PDGFβr-P-CAT). The 4.5 Kb insert, CAT reporter gene, and SV 40poly (A) region was excised using Eco RV (5' carried over with KS bluescript multiple cloning site) and PVU1 (3' site of SV40 poly A region). The 6.2 Kb construct was gel purified and prepared for microinjection into fertilized

eggs.

Transgenic mice

Standard procedures were used to generate transgenic mice (Hogan, 1986) Female B6SJLF1/J (Jackson Laboratories) were super ovulated the fertilized eggs were obtained after mating with stud males. The purified 6.2 Kb insert DNA was injected into the pronuclei of one -cell stage embryos at a concentration of 1.5 μg/ml. The micro injected eggs were transferred at the 2 cell stage into the oviducts of pseudo pregnant females on day one of gestation. Positive transgenic mice were identified by Southern blot analysis of tail DNA using the 1.6 KB CAT gene as a probe.

immunocytochemistry/ in situ analysis

In situ hybridization of paraffin sections was carried out according to (Wilkinson et al., 1987). To detect the endogenous PDGF receptor mRNA, a 461 bp sac I fragment of the murine PDGFβr (1-461) cloned into pGEM (Promega) vector was used. Radiolabeled antisense and sense transcripts were made by in vitro transcription using 35S-labeled UTP (1,200 Ci/mmol, Amersham). To detect the transgene, a riboprobe was generated from the CAT sequence using a 1.6 Kb fragment using in vitro transcription as described above. Both sense and antisense hybridizations were performed using both probes.

Immunocytochemistry was performed using the Vectastain ABC-AP reagent (Vector Laboratories) as per their protocol. The CAT antibody used was a purified rabbit polyclonal antibody(5 Prime- 3 Prime). The PDGFβr antibody was made in our lab by Anke Kipple and is a rabbit polyclonal against the extracellular domain of the PDGFβr.

Figure Legends

Figure 1

Genomic Organization of the 5' region of the PDGFβr gene

The 4.5 Kb R1-Bam H1 genomic clone contains 2,500 bp of upstream genomic sequence, the entire 5' untranslated region of the cDNA (1-132 bp) and 2,000 of the first intron. Southern blot analysis of overlapping genomic clones indicated intron 1 is at least 10 Kb. The 5' untranslated region is denoted as a stippled box, while the first coding exon which contains the initiator ATG is denoted with a closed box. R1, Eco R1; S, Sac 1; K, kpn; N, Nco; B, Bam H1.

Figure 2

Deletional and Cell-Type Specificity Analysis of Genomic Clones

The 4.5 Kb Eco R1-Bam H1-Luciferase construct and its deletion constructs were transfected into NIH 3T3 cells (stippled box) or N-MUNG epithelial cells (striped box). Luciferase activity (normalized to β-galactocidise activity as described in Materials and Methods) was measured. A) - 2,500/+2,000, the full length 4.5 Kb construct. B) -600/+2,000, 1.9 Kb was removed from the 5' end of the full length construct using the kpn site. C) Δ880, 880 bp was removed from the full length construct using the Nco 1 sites bracketing that region. D) - 354/+260, 600 bp surrounding the 5' untranslated region linked to the luciferase gene. E) p Basic-Luc. R1= Eco R1; K= kpn; N=Nco1; Luc=Luciferase gene.

Figure 3

4.5 Kb-CAT Transgenic Construct

The -2,500/+2,000 fragment was ligated into pCAT-Basic (Promega) as described in Materials and Methods. The -2,500/+2,000 clone, the CAT region , and the SV40 poly A fusion gene was removed from the pCAT-Basic vector. Unique Eco RV and Pvu1 sites were used to remove the transgene from the plasmid vector. RV= Eco R V; R1= Eco R1; Bam = Bam H1; PDGFβr-P = 4.5 Kb EcoR1-Bam H1 genomic fragment, the promoter . CAT= the bacterial CAT gene; AAA= the SV40 poly A region.

Figure 4

Localization of the -2,500/+2,000 transgene in mesenchyme in transgenic mice. In situ hybridization of para-sagittal sections of a 14.5 day transgenic mouse embryo using the 1.6 Kb CAT antisense RNA probe (A-D). Light field photography (A,C) dark field (B.D). Sections through the developing lung (A,B) , nasal region and tongue (C,D) show hybridization in the mesenchyme . Immunocytochemistry of frontal sections through the facial region of a 14.5 day transgenic mouse embryo (E-H). Immunocytochemistry using the CAT antibody (E,G) or PDGFβr antibody (F,H) show an identical pattern of localization with strong signal in the facial mesenchyme and mesenchyme condensing around the facial bones (E,F) arrowheads point to hair follicles. Higher power (G,H) of the hair follicles in (E,F) show strong hybridization in the dermal papillae while the hair epidermis is completely negative for expression of either gene. dp, dermal papillae; e, epithelium; m, mesenchyme.

Figure 5

Localization of PDGFβr and CAT protein in developing bone in transgenic mice. Immunocytochemistry using the PDGFβr antibody (A, C) and CAT antibody (B, D) in E. 14.5 day transgenic mouse embryos. The mesenchyme surrounding the bone is condensing to form the periosteum at this stage of development, the antibody is detected in these regions of mesenchyme condensation (A, PDGFβr antibody; B, CAT antibody) and in regions of vasculature surrounding the bone (C, PDGFβr antibody; D, CAT antibody). White arrows point to vasculature surrounding the bone, black arrowheads point to mesenchyme condensing around the bone.

Figure 6

Localization of the PDGFβr protein and CAT protein in the choroid plexus of a 14.5 day transgenic mouse. Immunocytochemistry using the PDGFβr antibody (A, C) and CAT antibody (B, D) The choroid plexus consists of a mass of capillaries projecting into the ventricle. Higher power (C, D) of (A, B) showing the localization of both antibodies in the capillaries and their supporting connective tissue. The choroid epithelial cells are negative for expression of either gene. Arrowheads point to capillaries.

Figure 7

Localization of the CAT protein in blood vessels in transgenic mouse embryo. A) The CAT antibody is localized to the vasculature and is highest in the mesenchyme surrounding the blood vessels. (B) In situ hybridization of a small blood vessel (x 1,000 magnification) showing the CAT mRNA

expressed in both the endothelium and supporting mesenchyme. Expression of the CAT antibody to the endothelium surrounding a small capillary is designated by an arrow in (B).

Figure 1

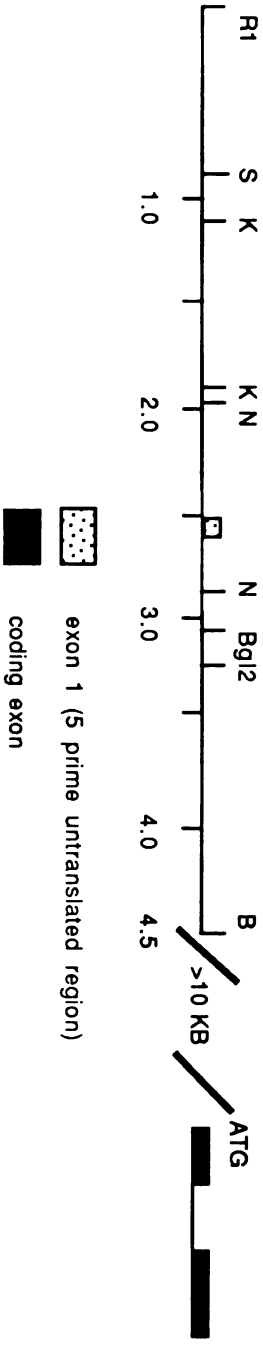


Figure 1 Genomic Organization of the 5' region of the PDGFβr gene

Figure 2

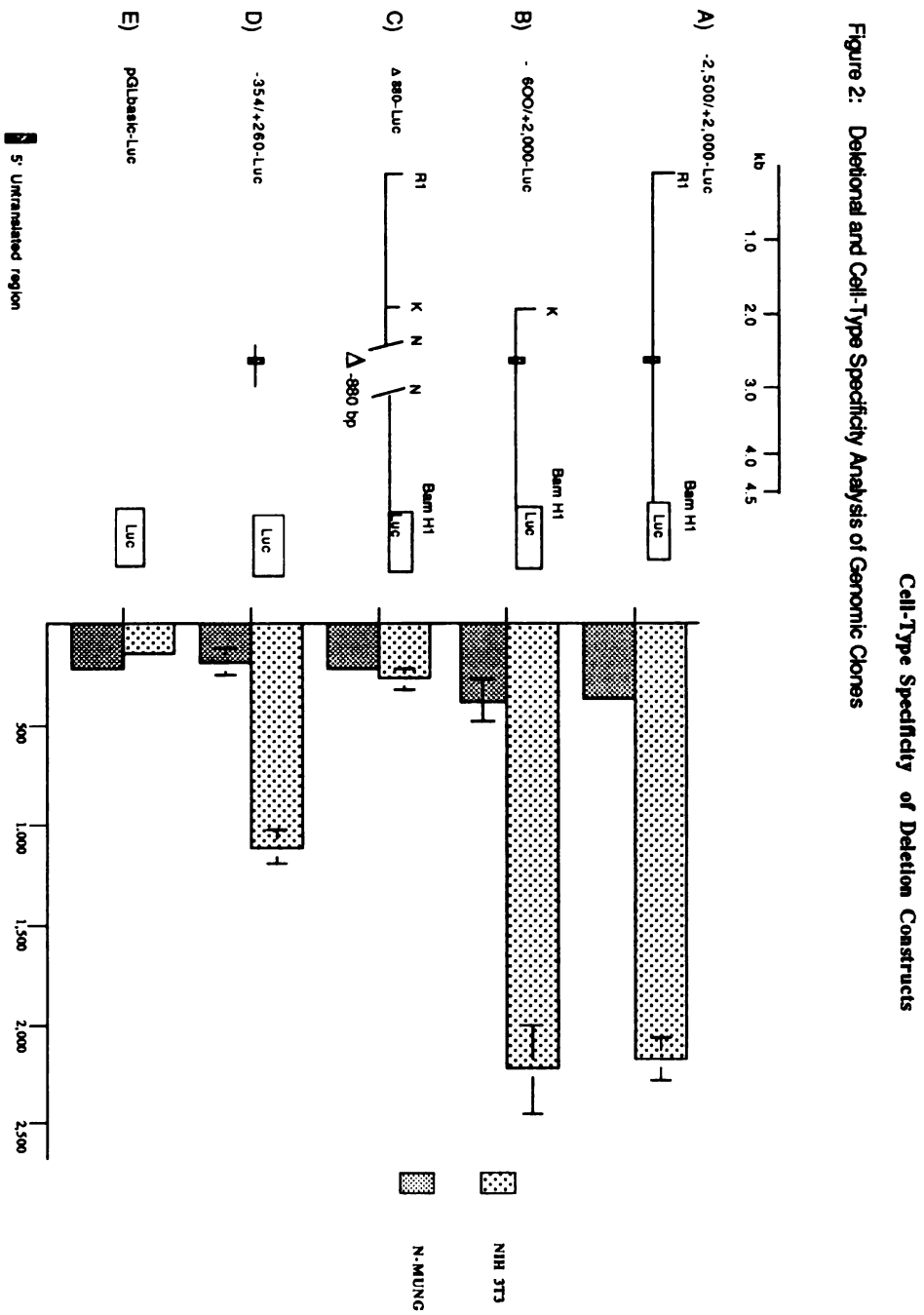

Cell-Type Specificity of Deletion Constructs
Figure 2: Deletional and Cell-Type Specificity Analysis of Genomic Clones
A) -2,500/+2,000-Luc
B) -600/+2,000-Luc
C) Δ880-Luc
D) -354/+260-Luc
E) pGLbasic-Luc
kb
1.0
2.0
3.0
4.0
4.5
R1
K
N
Bam H1
Luc
Δ -880 bp
5' Untranslated region
500
1,000
1,500
2,000
2,500
NIH 3T3
N-MUNG

Figure 3

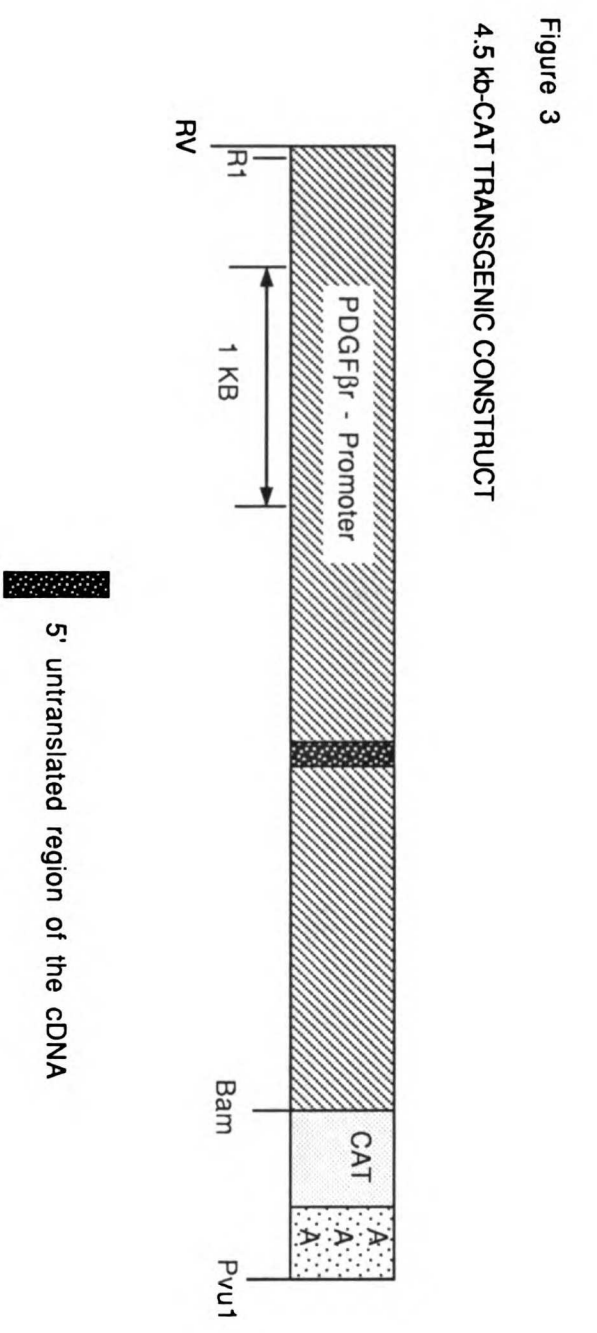


Figure 3
4.5 kb-CAT TRANSGENIC CONSTRUCT
RV
R1
1 KB
PDGFβr - Promoter
CAT
A
A
A
Bam
Pvu1
5' untranslated region of the cDNA

Figure 4

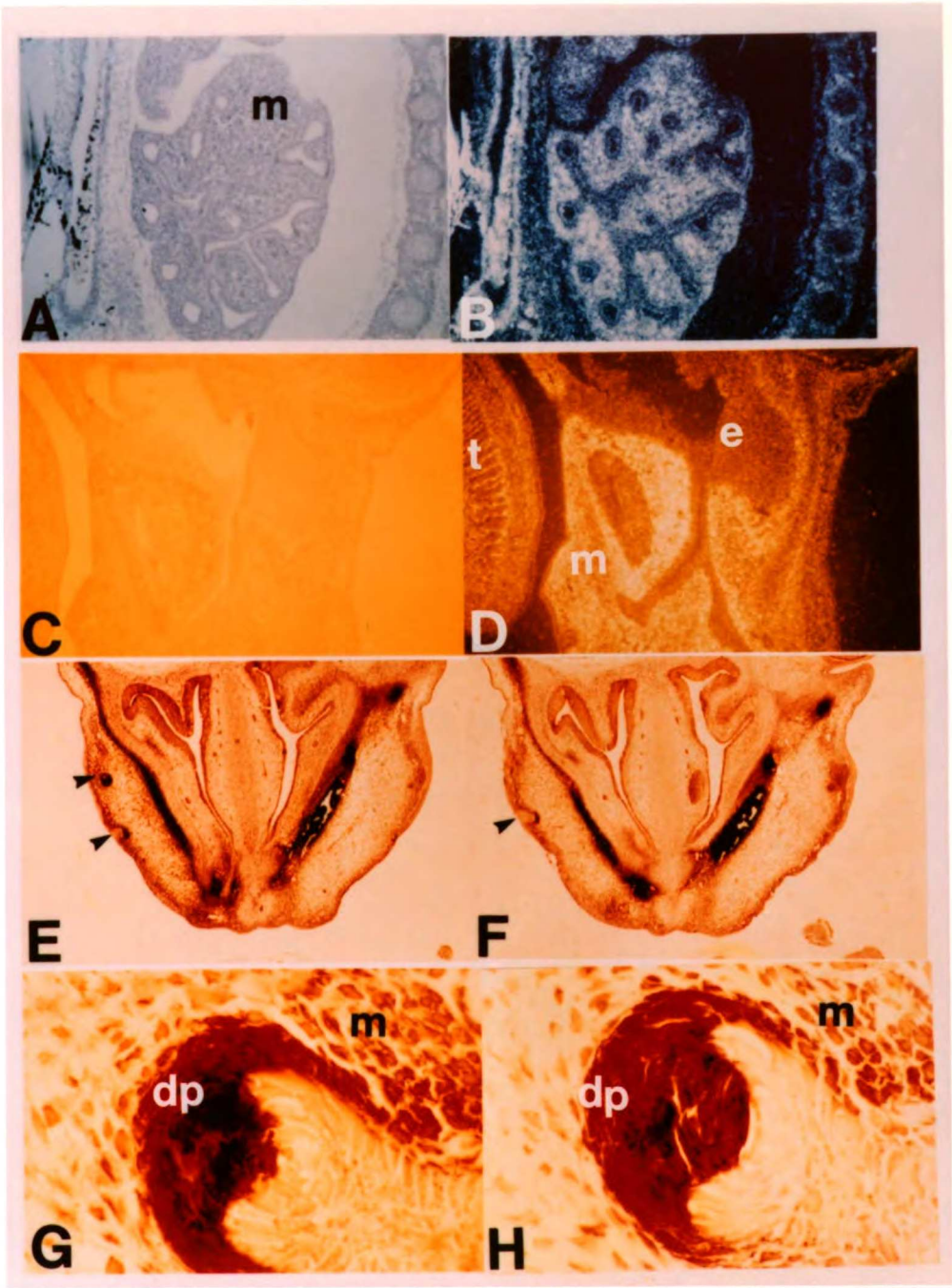


Figure 5

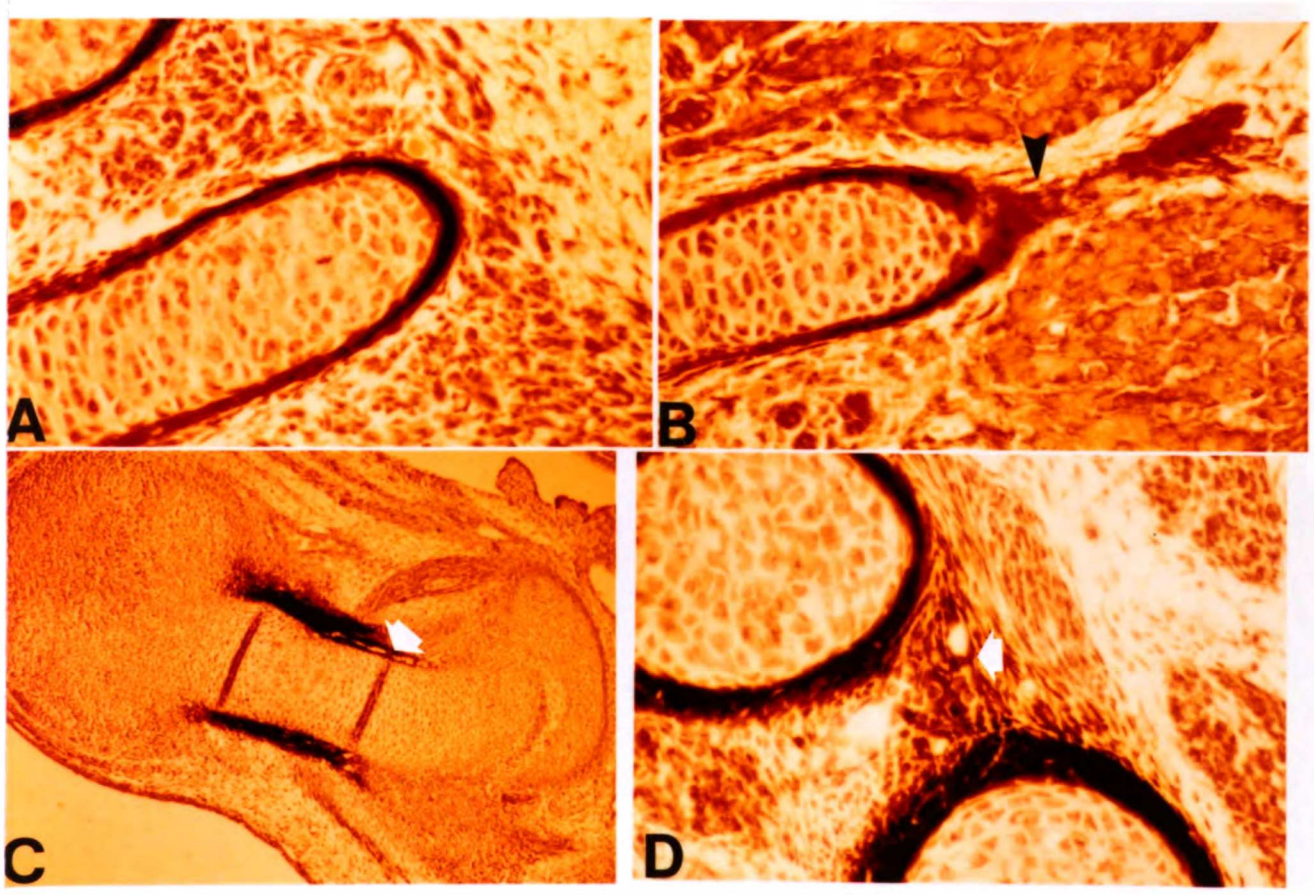


Figure 6

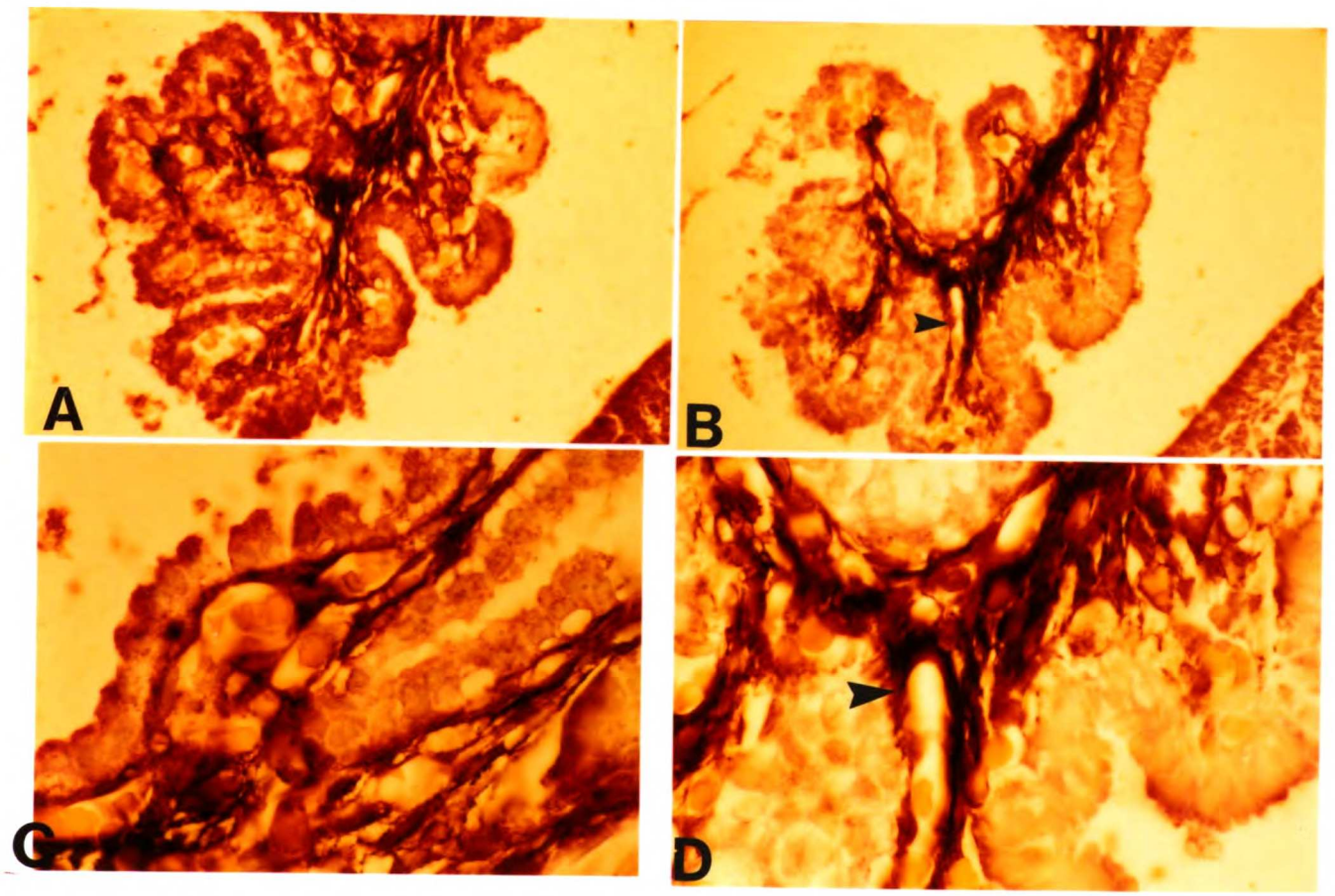
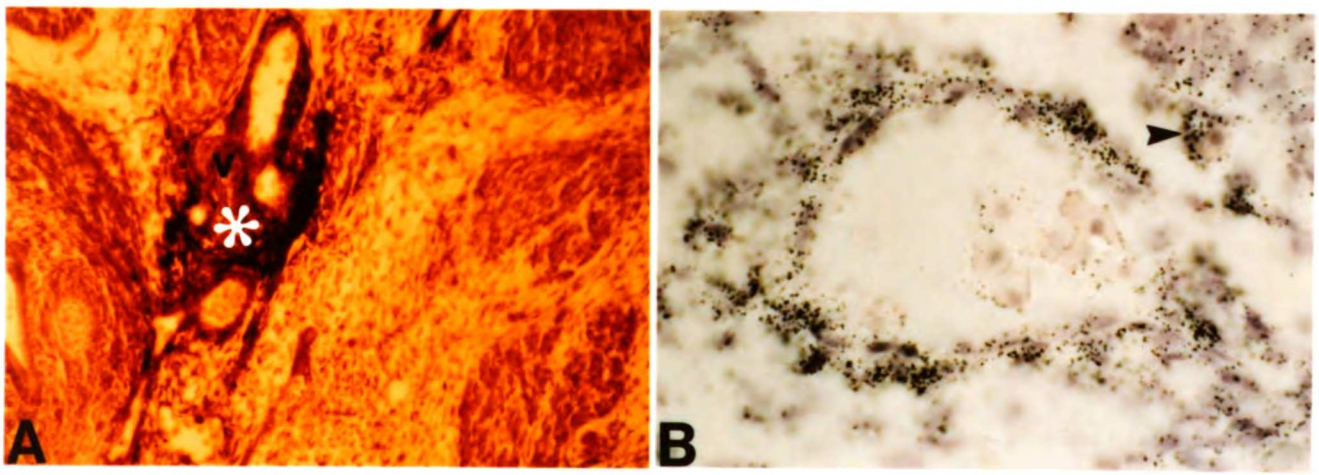


Figure 7



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CHAPTER 4

EXPRESSION OF THE PLATELET DERIVED GROWTH FACTOR BETA RECEPTOR PROMOTER IN AN ADULT TRANSGENIC MOUSE

General Conclusions and Future Directions

Introduction

PDGF and its receptors have been implicated in many cellular processes ranging from development to disease. It is probable that transcriptional regulation of the PDGFβr plays a key role in these processes. The PDGFβr is highly expressed during tissue organization and morphogenesis during development. However, in the adult organism, the receptor is expressed at very low levels. Various cellular conditions such as wound healing cause a re-expression of the PDGFβr. An interesting hypothesis is that this is due to a reinstatement of transcriptional mechanisms that were active during development. This may be due to the activation of positive transcriptional regulatory elements or removal of negative regulatory elements. Furthermore, the progression from wound healing to pathological fibroproliferative disease may be due to loss of this transcriptional regulation. Analysis of the promoter region of the PDGFβr gene would help define its role in these processes.

In the previous chapter I described the isolation and characterization of the promoter region of the PDGFβr. My previous study included the analysis of the putative promoter region in vivo using transgenic mice. I found the promoter contained the elements necessary to direct reporter gene expression with the same temporal and spatial profile as the endogenous receptor in the developing mouse embryo. However, I had not characterized the expression pattern of the reporter transgene in the adult mouse. In this chapter I examined the expression pattern of the PDGFβr promoter-CAT transgene. The only areas in skin that stained positive for PDGFβr were inflammatory loci which contained large amounts of T-lymphocytes and macrophages (Poulter et al., 1986). In areas

of mononuclear infiltrates, the receptor was detected in areas surrounding vessels. In the dermal papillae, the PDGFβr was observed in some capillaries, while dermal fibroblasts did not display any detectable PDGFβr expression (Terracio et al., 1988). The expression of the PDGFβr was also examined in non inflamed synovium, it was undetectable (Rubin et al., 1988; Rubin et al., 1988). This was also seen in normal kidney, where no significant PDGFβ receptor staining was observed. However, in some specimens, low amounts of PDGFβ receptor staining was observed on glomerular cells (Fellstrom et al., 1989). The expression of the PDGFβr was also examined in blood vessels. Smooth muscle cells in the tunica media of large vessels did not express PDGF receptors(Wilcox et al., 1988). In general, the PDGFβr has not been detected in normal adult tissue.

These reports of low expression of the PDGFβr in normal adult tissue can be contrasted to the high expression of the receptor during development, wound healing and fibroproliferative disease. This supports the hypothesis that expression of the PDGFβr is involved in development, wound healing and pathogenesis, yet it is silenced in normal adult tissue. To determine if the 4.5 kb EcoR1-Bam H1 PDGFβr promoter has the regulatory elements necessary to suppress its expression in adult tissue, I monitored its ability to direct reporter gene expression in adult transgenic mice.

RESULTS

In all of the tissues and organs I examined, the expression pattern of the PDGFβr and CAT transgene was identical. Therefore, to avoid redundancy, immunocytochemistry of either the PDGFβr or CAT protein are shown, however the expression pattern of both proteins was thoroughly examined.

Expression in the intestine

I was unable to detect the PDGFβr and the CAT transgene in any layers of the adult intestine (Fig. 1a). This is in direct contrast to the expression pattern seen in embryonic intestine, where there is high expression of the PDGFβ receptor (Fig. 1b). In the developing embryo, the intestine initially develops from an epithelium surrounded by loose mesenchyme. Eventually this mesenchyme differentiates to form the lamina propria and muscular layers, while the epithelium differentiates into the crypts and villi. In the developing embryo, the PDGFβr is highly expressed in the mesenchyme directly supporting the epithelium, however as the mesenchyme differentiates into antibody positive smooth muscle, the receptor is no longer expressed (Fig, 1b,c). In the adult intestine, the lamina propria and muscular layers which differentiate from the embryonic mesenchyme are negative for PDGFβr and CAT transgene antibody staining. Therefore the 4.5 Kb Ecor1-BamH1 PDGFβr promoter contains the regulatory elements necessary to restrict its expression in adult intestine.

Comparison of PDGFbr promoter-CAT transgene with muscle-specific actin

The differential staining pattern of muscle-specific actin with the CAT transgene in developing bone is compared in Fig. 2 and Fig. 3. The CAT transgene (brown stain, Fig. 2a, 3a) is detected in the mesenchyme condensing to form the developing bone. The muscle specific actin (red stain, Fig. 2b, 3b) clearly stains the muscle outside of the bone. This distinct pattern of staining clearly shows the CAT transgene (and PDGFbr protein, not shown) is not present in mature muscle.

Expression in the Lung, Liver and Spleen

Both the PDGFβ receptor and CAT transgene were undetectable in the adult lung, liver and spleen(Fig 4) .

Expression in the heart and vasculature

The PDGFβr and CAT proteins were undetectable cardiac myocytes in the adult heart (Fig. 5). This is consistent with their developmental expression. However both the PDGFβr and CAT protein were expressed in small vessels of the microcirculation (Fig. 5). Both the CAT protein and PDGFβr were seen in small arterioles and capillaries. The expression of both proteins was high in the microvessels in all the organs examined. This is consistant with other reports that microvascular endothelial cells

posses PDGF receptors (Bar et al., 1989; Beitz et al., 1992; Hermansson et al., 1988; Smits et al., 1989), while macro -vessels do not. The expression of the CAT transgene in the endothelium and intimal region of the microvessel in Fig. 6 (brown stain) can be compared with the staining pattern of muscle specific actin (red stain) in Fig. 7. The actin antibody (red color, Fig. 6) clearly stains smooth muscle surrounding the vessel, while the CAT antibody (brown color, Fig. 7) stains the endothelium. Thus, in normal adults, the PDGFβr is expressed in microvascular endothelium.

Discussion

The 4.5 Kb EcoR1-BamH1 PDGFβr promoter is able to direct CAT transgene expression with the same pattern as the endogenous PDGFβ receptor. This indicates that the 4.5 kb EcoR1-BamH1 promoter contains the regulatory elements necessary to restrict the expression of the PDGFβr. It is unknown at this time if this is due to the activation of negative regulatory elements or repression of positive elements. However, a temporally restricted pattern of PDGFβr and CAT transgene expression was seen. In most of the adult tissues and organs surveyed, the CAT transgene and PDGFβr proteins were undetectable. This is consistant with the reported expression pattern of the PDGFβr in normal adult tissue. This is significant, indicating that the PDGFβr does not play a major role in normal adult tissue. However, during wound healing, its expression is greatly increased (Heldin and Westermark, 1989). This supports the idea that PDGFβr plays a major role in the tissue remolding and reconstruction involved in wound repair. The only adult tissue that expressed the PDGFβr and CAT transgene was small arterioles and capillaries of the microcirculation. The continued presence of PDGFβr on microvascular endothelial cells suggests the receptor is involved in neovascular events. The restricted pattern of expression of the CAT transgene indicates that the 4.5 kb EcoR1-BamH1 fragment encompasses the PDGFβr promoter and contains regulatory elements involved with its temporal and tissue specific expression.

The low expression of the PDGFβr and CAT transgene in the adult can be contrasted to its high expression in developing embryos. The

development of the intestine may be used to illustrate the temporal changes in PDGFβr expression. The intestine initially develops from epithelium surrounded by mesenchyme. As development proceeds, the epithelium differentiates into the crypts and villus of the intestinal epithelium. At the same time, the mesenchyme surrounding the epithelium differentiates into the mature intestinal layers, the muscular mucous, submucosa, lamina propria, and circular muscular layers. In the embryo, the PDGFβr is highly expressed in undifferentiated mesenchyme of the developing intestine. However, it is no longer expressed in the differentiated smooth muscle layers of the adult. This general pattern may be applied to most of the organs of the airways and gut. The lack of expression in the adult differentiated smooth muscle and high expression in undifferentiated mesenchyme supports the role of PDGFβr in organogenesis and tissue morphogenesis. The PDGFβr is involved in proliferation and migration of connective tissue cells, vital processes in tissue morphogenesis. However, this must be highly regulated. In the adult, cellular proliferation is detrimental, and usually seen in pathological conditions such as fibrosis and cancer. In most pathological conditions involving tissue proliferation, the PDGFβr is highly expressed. Thus, the PDGFβr plays a role in and is highly expressed during organogenesis, tissue morphogenesis, and repair, however at the completion of those processes the receptor is no longer expressed, furthermore, its aberrant expression is involved in tissue pathologies.

The re-expression of the PDGFβr during wound healing, fibroproliferative disease and cancer may be due to restoration of processes that were active during development. Proliferating cells at the

site of tissue injury appear immature when compared to their non-injured counterparts. The phenotypic changes of arterial smooth muscle cells in atherosclerotic lesions illustrates this phenomena. Normal arterial smooth muscle cells have two phenotypic stages, contractile and synthetic. In the fetus they are in the synthetic state, they appear fibroblast-like, secrete extracellular matrix, and divide. In the adult, they are in the contractile state, they contract in response to stimuli, and take part in the control of blood pressure. However, during atherosclerosis, the contractile smooth muscle cells take on a more synthetic phenotype. In the early stages, the contractile smooth muscle cells become modified, they appear more fibroblast-like and proliferate, a key event in formation of the lesion (Sjolund et al., 1988)In this more synthetic state, the cells express genes for many growth regulatory molecules and cytokines (Libby et al., 1988). Their synthetic activity determines the matrix content of the lesion, a major component of the disease. In this state they express the PDGFβr. Both phenotypically and biochemically, the cells resemble their developmental precursors. This supports the idea that the re-expression of the PDGFβr on adult cells reflects developmental conditions. However, it is unknown if this is due to proliferation of undifferentiated progenitor cells that are present in the smooth muscle or a change in the phenotype of mature cells. It will be interesting to follow the activity of the PDGFβr promoter in response to various pathological conditions.

Future Usage of the PDGFβr Promoter

The PDGFβr promoter has many potential usage's. The PDGFβr promoter-CAT transgene may be used to monitor the transcriptional regulation of the PDGFβr in response to various stimuli. This could include cellular states such as pathological conditions, or normal cellular cycles.

The regulation of expression of the PDGFβr in various stages of the cell cycle may be monitored. The transcriptional regulation of the PDGFβr in response to perturbing the cell cycle may also be examined. It is probable that a change in transcriptional regulation of the PDGRFβr will be seen in various stages of the cell cycle. The transcriptional regulatory mechanisms may be studied.

The expression pattern of the PDGFβr-promoter-CAT transgene in response to tissue injury will be examined in future experiments in our laboratory. Other future experiments include monitoring the activity of the PDGFβr promoter in response to various fibroproliferative diseases including atherosclerosis. It is probable that wound healing and fibroproliferative disease cause an induction of the PDGFβr promoter-CAT gene. The results of these experiments might help ascertain the mechanisms involved in the re-expression of the PDGFβr in response to cellular stresses. Once it is established that transcriptional regulatory mechanisms are involved, cis regulatory elements and trans acting factors that regulate the expression of the PDGFβr may be identified.

The transcriptional regulation of the PDGFβr in response to oncogenic transformation will be examined. Recent reports indicate that cell lines transformed with *v-src* and *v-ras* exhibit reduced levels of PDGFβr mRNA when compared to the parental cell lines (Vaziri, 1995). The mechanisms of this regulation may be studied using the PDGFβr promoter. Both *v-src* and *v-ras* are oncogenic forms of downstream signaling molecules that are involved in signal transduction through the PDGFβ receptor. It is possible the negative regulation of the PDGRβr in transformed cells represents a negative feedback mechanism. Since the second messengers are already active, the ligand activated receptor is unnecessary, therefore its expression is silenced. This may represent transcriptional regulatory mechanisms that are also used in non-transformed cells, such as normal adult tissue. The study of the transcriptional regulation of the PDGFβr in response to transformation should reveal clues to its expression and regulation both in normal and pathological conditions, and may reveal pathways involved in signal transduction.

The PDGFβr-promoter-CAT transgenic mice may be mated to other mice lines to study the effect on the promoter regulation. One example is to mate the mice to oncogene containing transgenic mice. The effect on promoter regulation can be monitored by assaying CAT expression.

The response of the PDGFβr in response to exogenously added agents may also be followed. This can be diagnostic when applied to potential

therapeutic agents. One obvious application is therapeutic agents designed to modulate the progression of atherosclerosis.

The PDGFβr promoter may be used as a tool to direct the expression of genes to have the same temporal and spatial pattern as the endogenous receptor. The promoter may be used to deliver proteins that modulate the PDGFβr and its involvement in development, wound healing or disease. These include dominant negative forms of the receptor, various signaling proteins, or agents that interfere with them.

An alternate use for the promoter is to use it to deliver heterologous genes. The expression pattern of the promoter-CAT transgene is both temporally and spatially restricted. During development the gene expression is high in mesenchyme, particularly where it supports an epithelium. This promoter can be used to deliver genes to ascertain their role in mesenchymal-epithelial interactions during development. It also may be used to perturb tissue at the site of mesenchymal-epithelial interactions, the effect of disrupting these events at specific times in development might provide information as to the mechanisms involved in those interactions.

Figure 1 Expression of the CAT Transgene in the Intestine

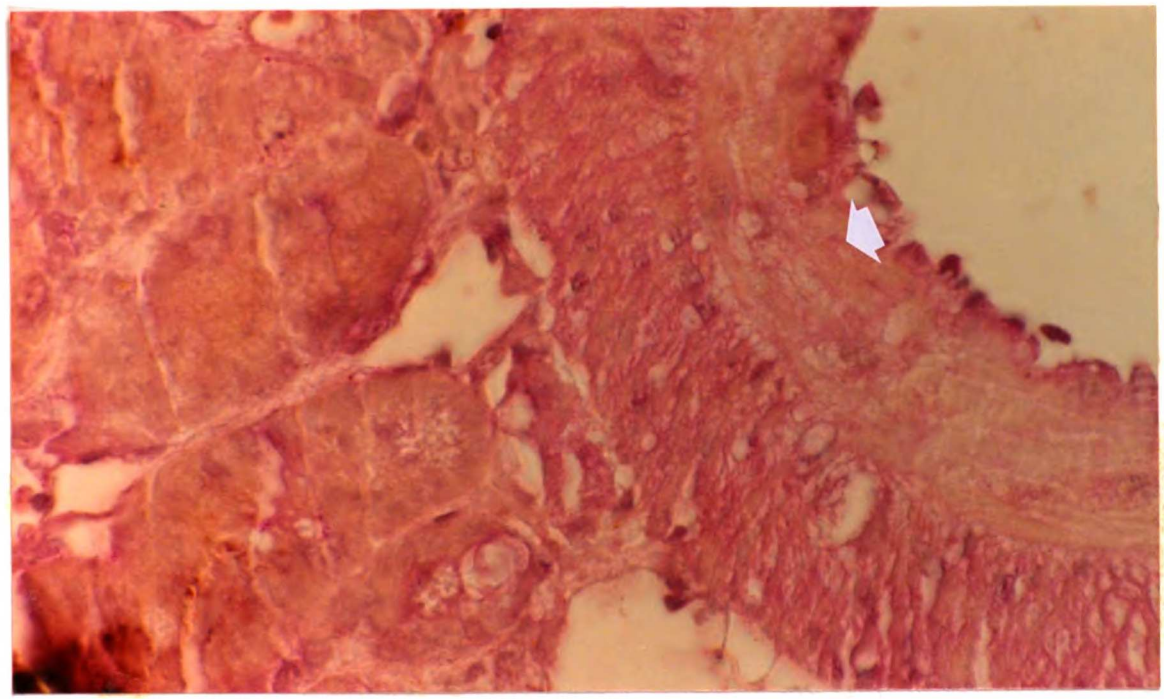
Figure legend:

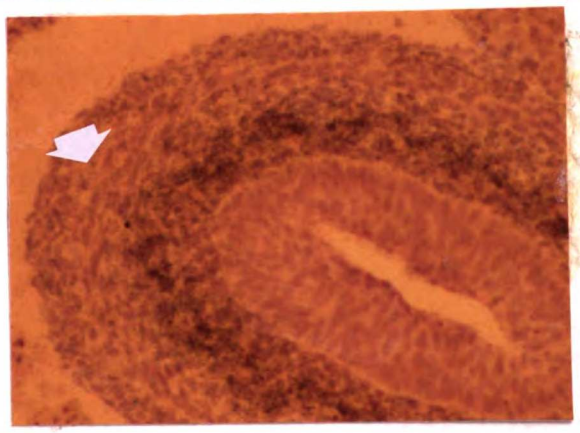
1A) Cross section through a transgenic adult intestine. Immunocytochemistry using the CAT antibody is shown. The antibody is visualized using a black color, no staining is seen. The section is stained with Eosin and Hematoxylin.

1B) Cross section through a 14.5 day mouse embryo intestine. In situ hybridization of the PDGFβr mRNA is shown. Brown silvergrains stain the mRNA. Section is stained with Eosin and Hematoxylin.

1C) Cross section through a 14.5 day mouse embryo intestine. Immunocytochemistry using the Smooth Muscle antibody (HHF-35) is shown. Red color indicates antibody positive smooth muscle. Section is stained only with Hematoxylin.

The white arrow points to the smooth muscle layer in A,B,C.





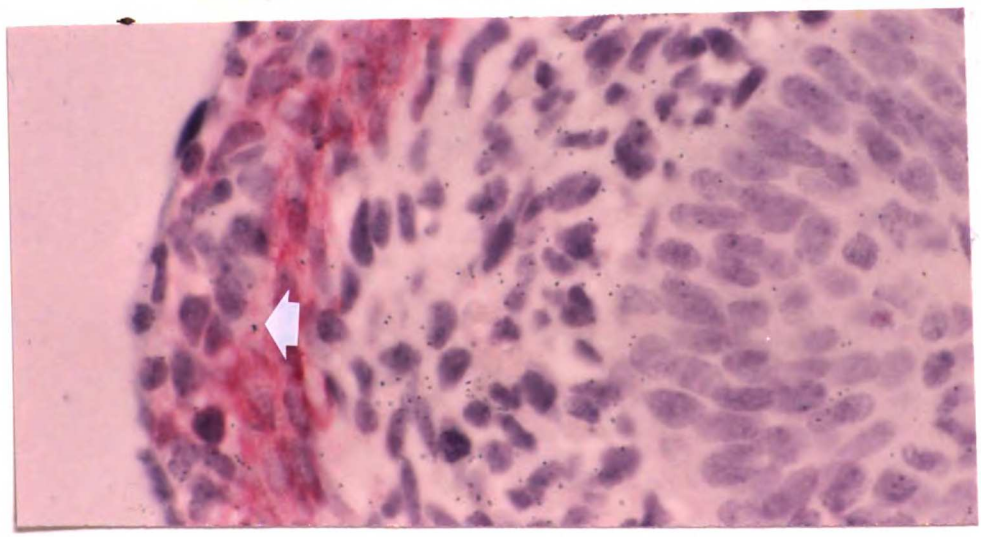
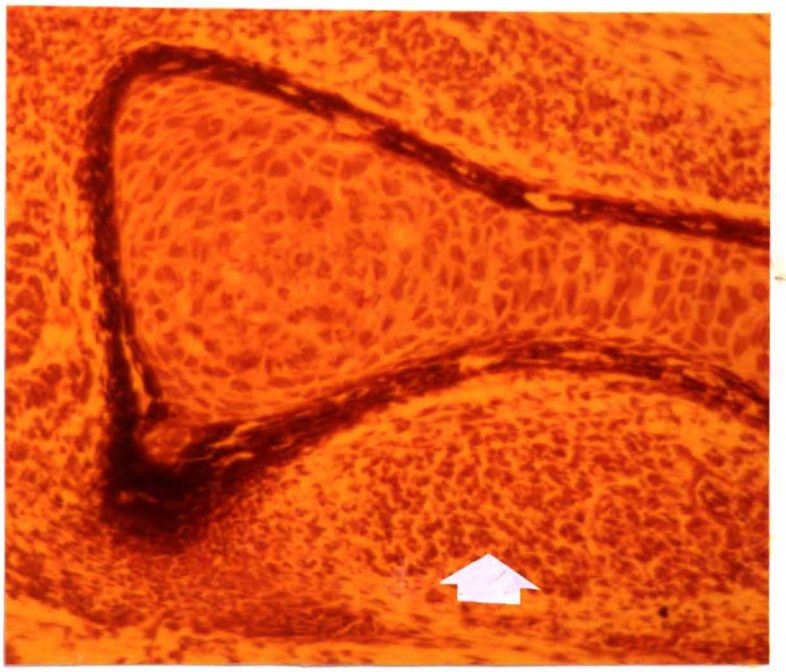


Figure 2
Comparison of CAT Antibody Staining with Muscle Specific Actin in Embryonic Long Bone

2A) 14.5 day transgenic embryo, para-sagittal section.
Immunocytochemistry using the CAT antibody, visualized using a dark brown stain.

2B) Serial section of (2A). Immunocytochemistry using muscle specific actin, visualized using a red stain.

White arrows designate antibody positive muscle



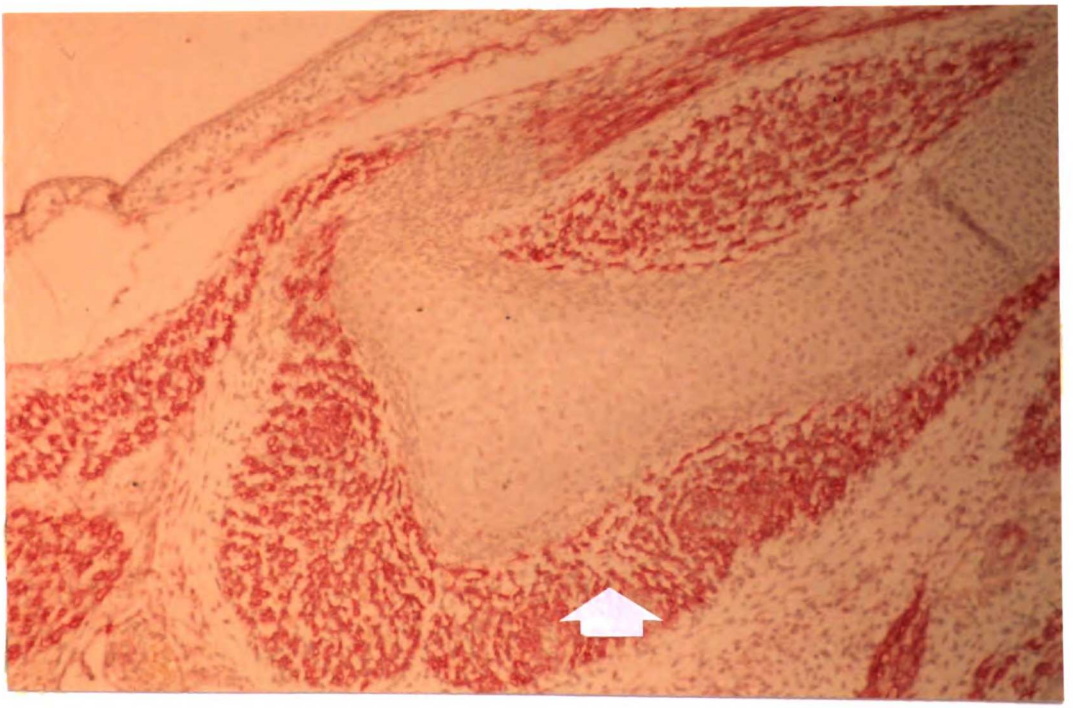


Figure 3
Comparison of CAT and Muscle Actin Antibody Detection in Embryonic Vertebrae

3A) 14.5 day transgenic mouse embryo. Immunocytochemistry using the CAT antibody visualized using a dark brown stain.

3B) 14.5 day transgenic embryo. Immunocytochemistry using the muscle specific actin (HHF-35) antibody, visualized with a red stain.

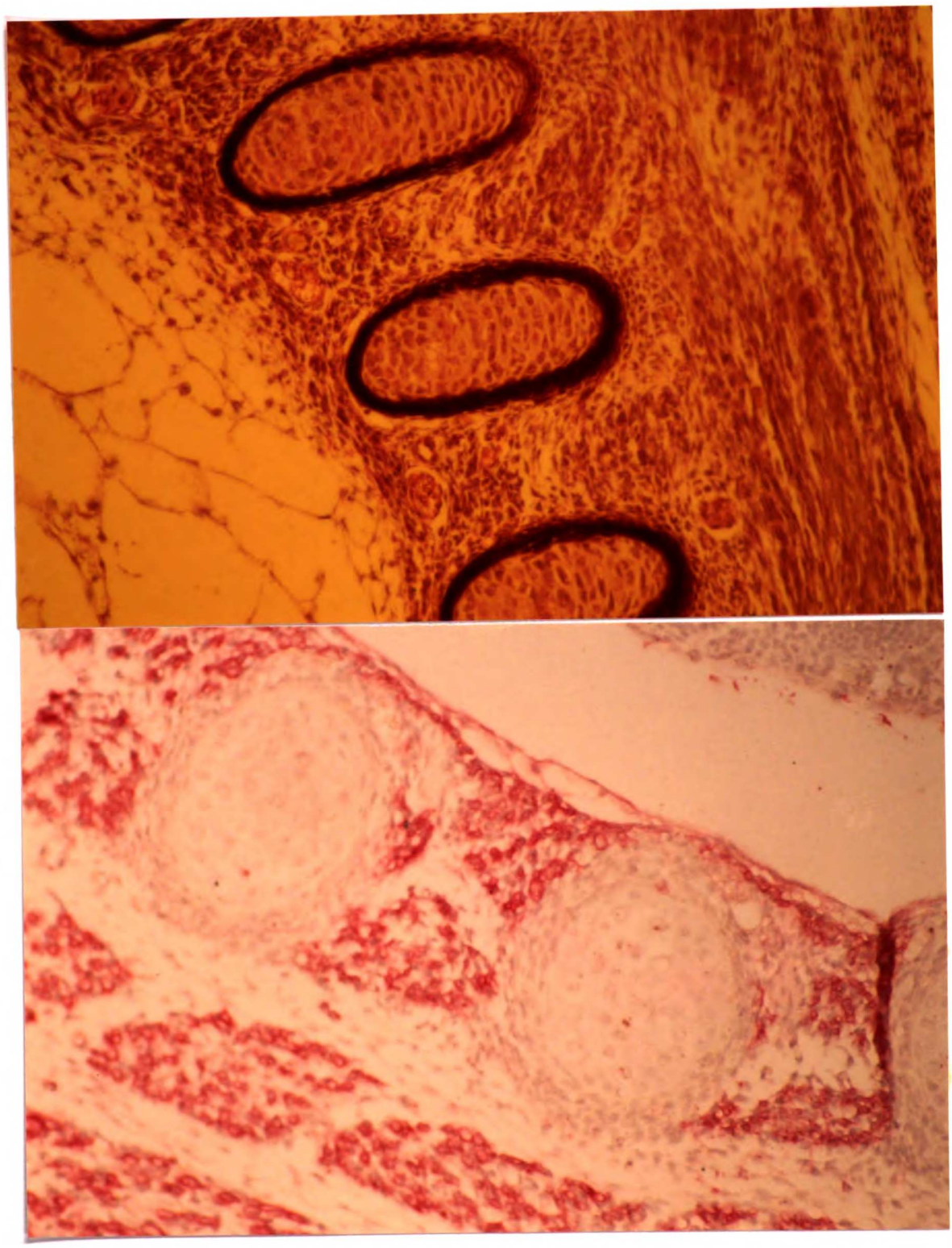
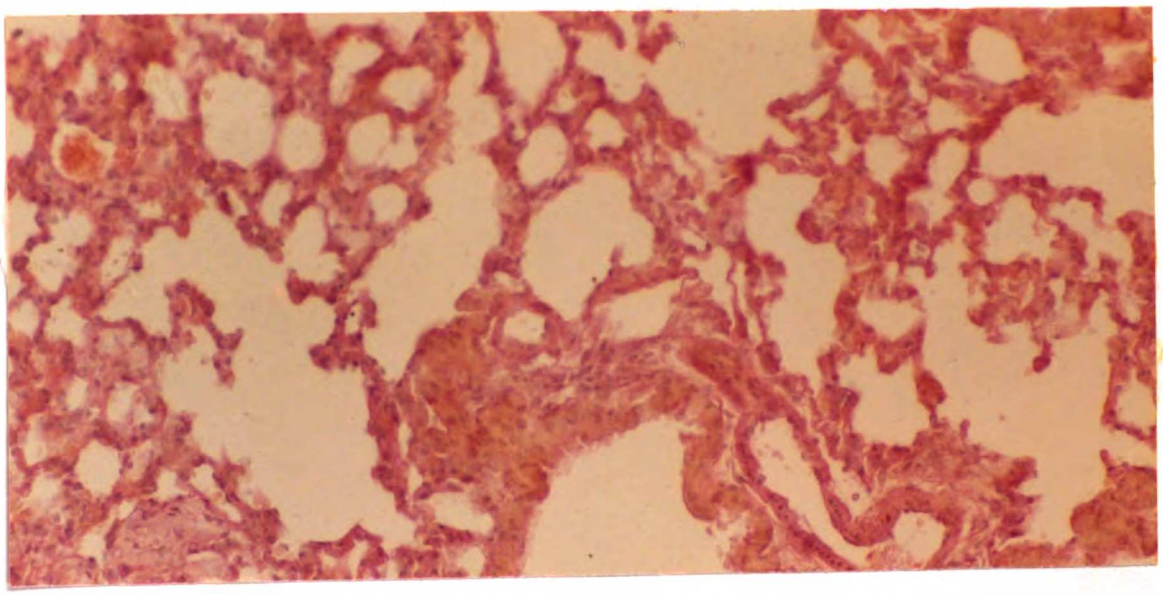


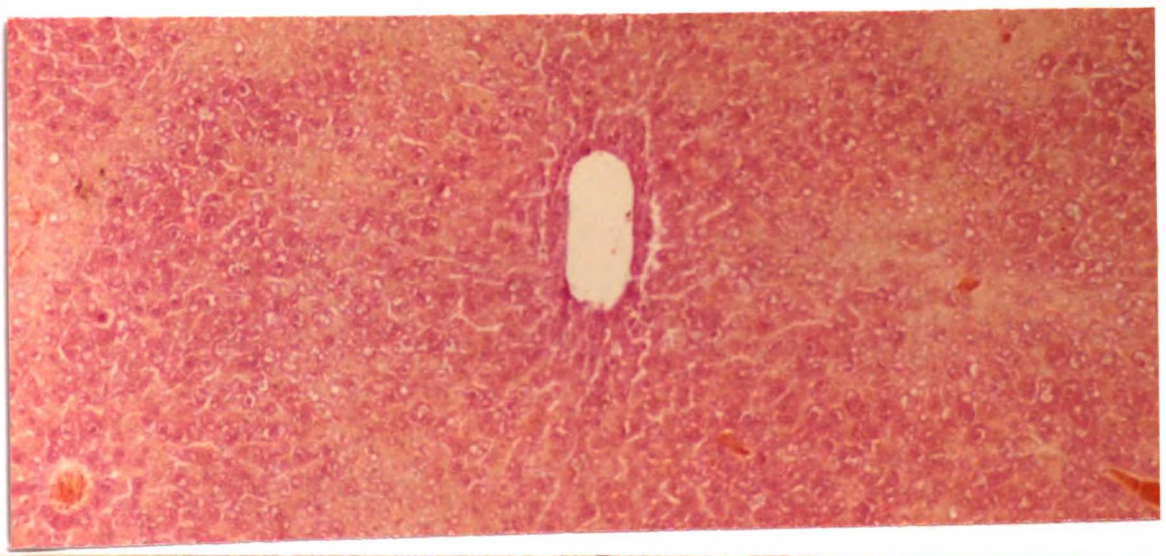
Figure 4
Examination of the CAT Transgene in Adult Lung, Liver and Spleen

4A) Cross section through the lung from an adult transgenic mouse. The CAT antibody is visualized with a dark brown color, however no staining is seen.

4B) Section through the liver from an adult transgenic mouse. The CAT antibody is visualized using a dark brown stain, it is undetectable.

4C) Section through the spleen from an adult transgenic mouse. As with the liver and lung, no CAT staining is detected.





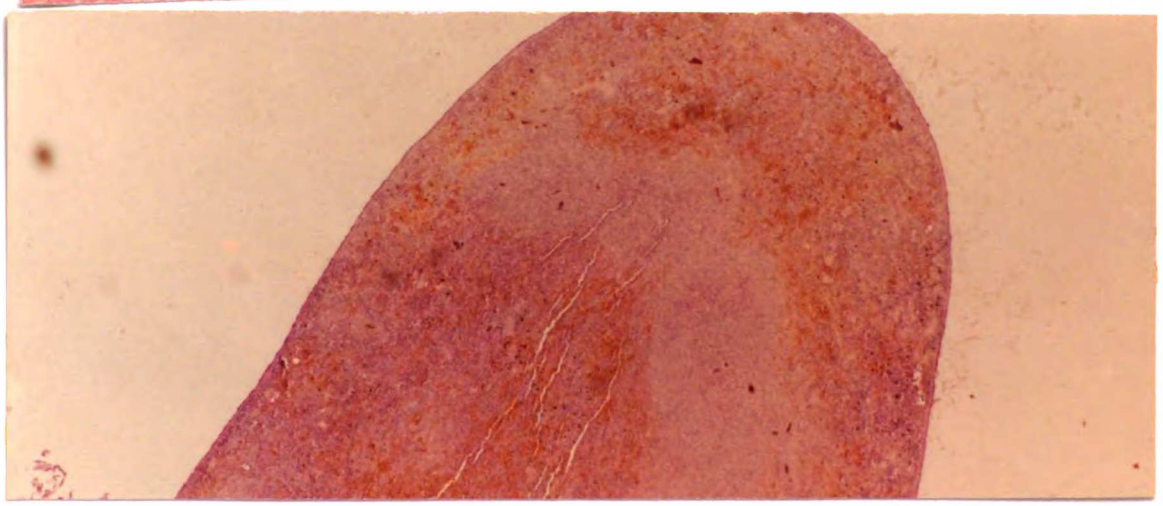


Figure 5

Expression of the CAT Transgene in the Adult Microcirculation

Immunocytochemistry on an adult transgenic mouse heart using the CAT antibody. The antibody is visualized using a dark brown stain. The cardiac myocytes (white arrow) are negative for transgene expression. The endothelium and intimal region of a small vessel stains positive (white star). Capillaries (black arrow), also show positive staining. 1,000 x magnification.

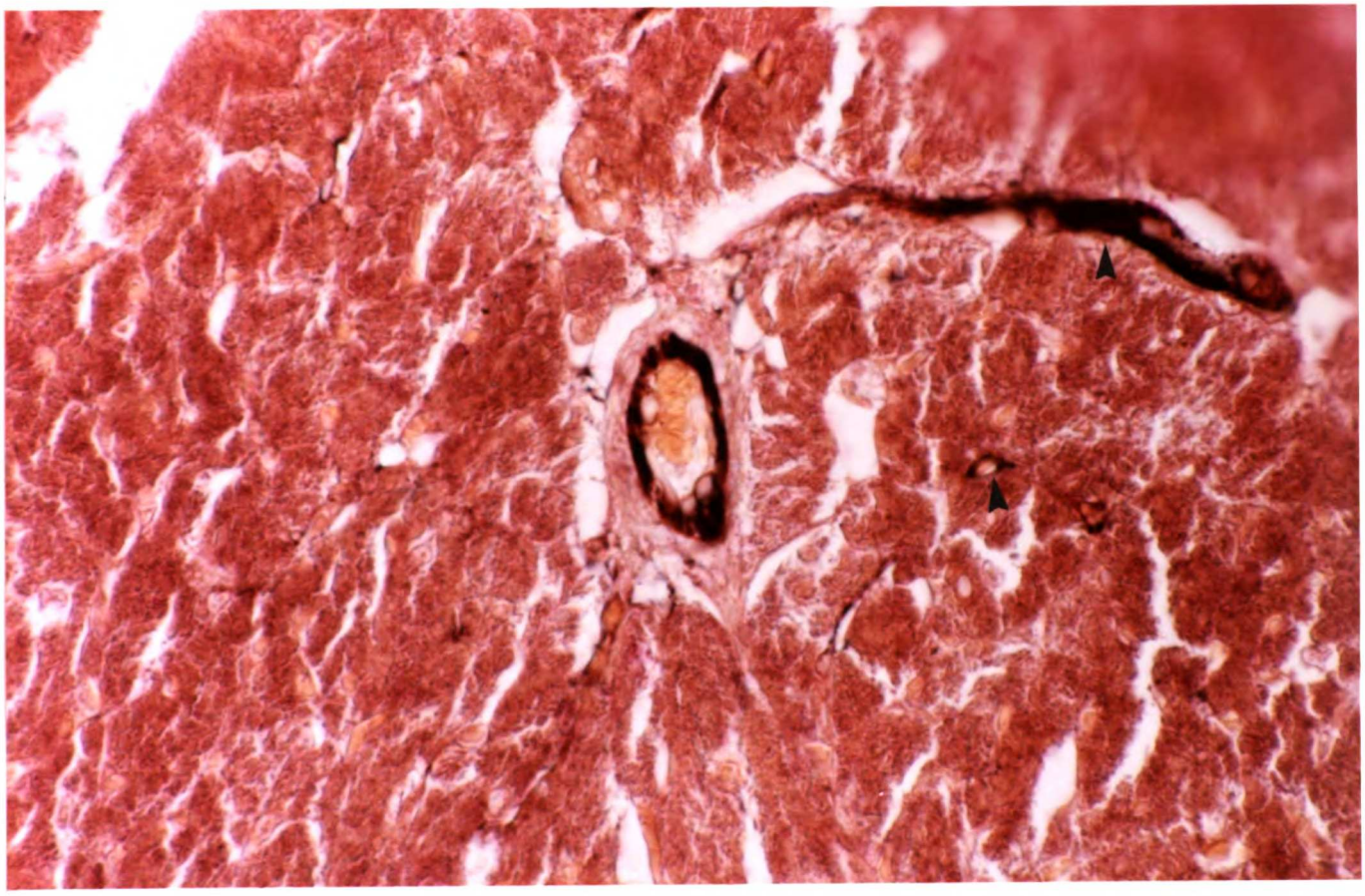
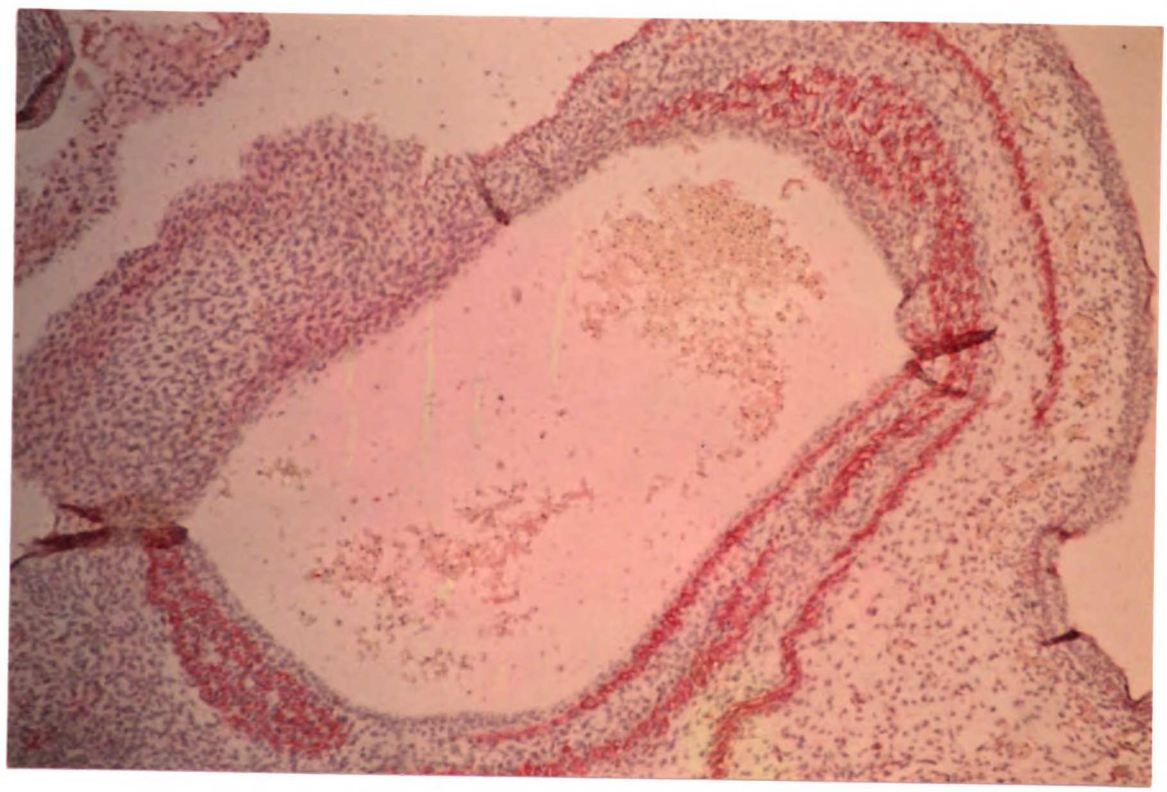


Figure 6

Expression of Muscle Specific Actin in a Large Blood Vessel

Immunocytochemistry using muscle specific actin antibody (HHF-35) on a coronal section through the dorsal aorta in a 14.5 d transgenic embryo. The smooth muscle surrounding the vessel is stained with a red color.



Materials and Methods

Construction of transgene

The 4.5 Kb genomic Eco R1-Bam H1 PDGFβr promoter was ligated to pCAT-Basic (Promega), a promoterless reporter plasmid which contains the CAT coding region and a SV 40 poly A region. . The 4.5 Kb plasmid in pBluescript KS+(the 4.5 Kb Eco R1 - Bam H1 fragment was ligated into Bluescript KS cut with EcoR1 - Bam H1) The 4.5 Kb insert was removed from pBluescript KS using the Sal1 (5 prime) and Spe 1 (3 prime) multiple cloning sites in pBluescript KS (Promega) The pCAT basic plasmid was cut with Sal 1 (5 prime) and Xba1 (3 prime, a compatible site with Spe 1) The 4.5 Kb fragment was ligated in using standard techniques. The resulting plasmid designated as PDGFβr promoter-CAT (PDGFβr-P-CAT). The 4.5 Kb insert, CAT reporter gene, and SV 40poly (A) region was excised using Eco RV (5' carried over with KS bluescript multiple cloning site) and PVU1 (3' site of SV40 poly A region). The 6.2 Kb construct was gel purified and prepared for microinjection into fertilized eggs.

Transgenic mice

Standard procedures were used to generate transgenic mice (Hogan, 1986) Female B6SJLF1/J (Jackson Laboratories) were super ovulated the fertilized eggs were obtained after mating with stud males. The purified 6.2 Kb insert DNA was injected into the pronuclei of one -cell stage embryos

at a concentration of 1.5 μg/ml. The micro injected eggs were transferred at the 2 cell stage into the oviducts of pseudo pregnant females on day one of gestation. Positive transgenic mice were identified by Southern blot analysis of tail DNA using the 1.6 KB CAT gene as a probe.

Immunocytochemistry/ in situ analysis

In situ hybridization of paraffin sections was carried out according to (Wilkinson et al., 1987). To detect the endogenous PDGF receptor mRNA, a 461 bp sac I fragment of the murine PDGFβr (1-461) cloned into pGEM (Promega) vector was used. Radiolabeled antisense and sense transcripts were made by in vitro transcription using 35S-labeled UTP (1,200 Ci/mmol, Amersham). To detect the transgene, a riboprobe was generated from the CAT sequence using a 1.6 Kb fragment using in vitro transcription as described above. Both sense and antisense hybridizations were performed using both probes.

Immunocytochemistry was performed using the Vectastain ABC-AP reagent (Vector Laboratories) as per their protocol. The CAT antibody used was a purified rabbit polyclonal antibody(5 Prime- 3 Prime). The PDGFβr antibody was made in our lab by Anke Kipple and is a rabbit polyclonal against the extracellular domain of the PDGFβr.

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