Functional Studies on the *PDGFRα* Gene Promoter and Effects of Autocrine PDGF-A Stimulation

*in vivo*

BY

XIAO-QUN ZHANG
Platelet-derived growth factor receptor α (PDGFRα) plays an important role during embryogenesis. After implantation, the patterns of expression of Pdgfrα and its ligand Pdgf-A undergo an “autocrine-paracrine transition”, in that Pdgf-A becomes expressed in the ectoderm and epithelia, while Pdgfrα is expressed in the adjacent mesenchymal tissue. In human tumors, such as malignant glioma, both PDGF and PDGFRα are overexpressed within the same tissue, indicating that an autocrine PDGF loop is generated in the tumors. This thesis is focused on the in vivo functionality of the PDGFRα gene (PDGFRA) promoter, and on the effect of autocrine PDGF-A stimulation in transgenic mice during embryogenesis.

To test the in vivo promoter function of a human PDGFRA 2.2 kb 5´ flanking fragment, we generated transgenic mouse lines and found that the 2.2 kb fragment was able to promote lacZ reporter gene expression in most of the endogenous Pdgfra expressing tissues. Absence of expression and “ectopic” expression of the transgenic lacZ were also observed. To investigate the autocrine PDGF effect, we produced autocrine PDGF-As (A short-chain) transient transgenic embryos. These transgenic embryos carried a 6 kb mouse Pdgfra 5´ flanking sequence linked to a human PDGF-As cDNA. The pattern of expression of the PDGF-As transgene mRNA was similar to that of lacZ. Some of the transgenic embryos exhibited severe abnormal phenotypes, such as midline fusion defects in the cephalic and craniofacial region and small body size, and these embryos die at mid-gestation stage. These findings indicate that a paracrine pattern of expression and the dosage of PDGF are important for sustaining normal embryo development, especially with regard to the midline fusion in craniofacial regions.

The possible signaling pathways that may be involved in regulating Pdgfra activity were also studied by comparison of patterns of mRNA expression of Gli, Ptc, and Pax1 with that of Pdgfra. The results pointed to the possibility that the Shh signaling pathway may be involved in the regulation of Pdgfra expression for example during early bone and foregut development. The specific regulatory mechanisms may vary for different tissues.

Key words: Autocrine PDGF, promoter, PDGFRA, transgenic mice.

Xiao-Qun Zhang, Department of Genetics and Pathology, Rudbeck Laboratory, Uppsala University, University Hospital, SE-75185 Uppsala, Sweden

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ABBREVIATIONS

BCC    basal cell carcinoma
BMP    Bone morphogenetic protein
CNS    central nervous system
FGF    fibroblast growth factor
Hh     hedgehog
Ig     immunoglobulin
Ihh    Indian hedgehog
IL-1   interleukin 1
MCSF-1 macrophage colony stimulating factor-1
ncc    neural crest cells
OPs    oligodendrocyte progenitors
Pax    paired box domain-containing gene
PDGF   platelet-derived growth factor
PDGFR  platelet-derived growth factor receptor
Ph     patch mutant
Ptc    patched
RA     retinoic acid
Shh    sonic hedgehog
Smo    smoothened
TCCE   teratocarcinoma cryptic exon
un     undulated mutant
VEGF   vascular endothelial growth factor
INTRODUCTION

Cell proliferation and differentiation are basic events in embryonic development. Development of a tumor and an embryo may share common ways of sustaining cell proliferation. Many of the genes that play important roles in embryogenesis are therefore also important in tumorigenesis. The gene encoding platelet-derived growth factor receptor α (PDGFRα) is one of the genes that are highly active during both tumor and embryonic development. In the investigations described in this thesis in vivo promoter function has been studied and the temporal-spatial pattern of expression of the PDGFRα gene during mouse embryonic development has been characterized. Using an isolated Pdgfra promoter fragment, we have also created a forced autocrine PDGF-A transgenic mouse model to mimic the autocrine platelet-derived growth factor (PDGF) situation found in tumors. In addition, possible involvement of the Shh-Ptc-Gli signaling pathway in transcriptional regulation of Pdgfra has been studied by comparison of the patterns of mRNA expression of these genes.

EMBRYONIC DEVELOPMENT

Embryonic development starts from one fertilized oocyte. After a series of cell cleavages, the embryo becomes a hollow ball of about 100 cells, called a blastocyst. The blastocyst implants into the uterine wall and the cells constituting the inner cell mass are split into two layers: the primitive ectoderm and the primitive endoderm. Later on, the primitive ectoderm (epiblast) cells near the primitive streak proliferate and flatten and lose their connection with one another. These flattened cells become invaginated and enter between the ectoderm and endoderm to form the mesoderm (Larsen, 1993).

Mesoderm and its derivatives

During early vertebrate embryonic development the mesoderm can be divided into five different parts: the notochord; the dorsal (paraxial), intermediate, and lateral mesoderme; and the head mesenchyme. The notochord, which is located in the center of the mesoderm at the neural plate stage, is a transient midline structure. The signal from the notochord is important for initiation of ventral and dorsal polarity, induction of the ventral neural tube, and sclerotome formation. The paraxial mesoderm, which is located on either side of the notochord, forms somites and gives rise to axial bones, ribs, skeletal muscle, and part of the dermis. The intermediate mesoderm, located in between the lateral and paraxial mesoderm,
produces the kidney and urinary system and part of the genital system. The lateral mesoderm, which is further away from the notochord, differentiates into the heart, connective tissue of internal organs, and body wall (i.e. pleura, pericardium and peritoneal membranes surrounding the cavities), parts of the limbs, and most of the dermis. Finally, the head mesenchyme forms the connective tissues and musculature of the face (Fig. 1) (Larsen 1993).

Figure 1. Schmatic illustration of the development of the mesoderm in the chick embryo. (A) Formation of the notochord and paraxial mesoderm. (B) Differentiation of the somites.
The mesoderm gives rise to many different tissues, including bones, skeletal muscles, smooth muscles, part of the dermis, and connective tissues. During development, mesenchymal tissue that lies beneath an epithelial layer forms the basis of the epithelial-mesenchymal interaction. In that location mesenchyme is responsible for the determination of the fate of the epithelium, and conversely, secreted factors from the epithelium contribute to regulate the proliferation and differentiation of the mesenchymal cells. Such interaction has been shown to play an important role in the induction and development of many tissues and organs, including the teeth, the skin follicles, the limb buds, the lung bronchi, and the digestive tube (Francis-West et al., 1998; Maas and Bei, 1997; Roberts, 2000; Warburton et al., 1999).

**Somite development**

Somites originate from the unsegmented paraxial mesoderm, which is located between the intermediate mesoderm and the neural tube. The somite development includes segmentation, epithelialization, maturation of the segmental plate, and differentiation. The mesenchymal cells of unsegmented paraxial mesoderm form a series of rounded structures called somatomeres, which undergo segmentation and epithelialization. The segmentation of somites (Fig. 1) starts in the occipital region and continues in the caudal direction along the body axis at each side of the neural tube and notochord. After epithelialization, somites differentiate into a ventral and a dorsal portion. Cells in the ventral portion that are oriented toward the notochord lose their epithelial organization and change into mesenchymal tissue through an epithelial-mesenchymal transition, forming the sclerotome. The ventral portion of the sclerotome surrounds the notochord and forms the rudiment of the vertebral body. The dorsal portion of the sclerotome surrounds the neural tube and forms the rudiment of the vertebral arch. During this process, the cells in the dorsal portion underneath the surface ectoderm remain as epithelium and form the dermamyotome. This epithelial portion of the dorsal somite differentiates further into two layers, the dermatome and the myotome. The dermatome, which is located in the outer layer, gives rise to the dermis of the back, while the myotome, located in between the dermato mesenchyme and the sclerotome, gives rise to the skeletal muscles (Brand-Saberi et al., 1996; Christ et al., 1998; Dockter and Ordahl, 2000; Olivera-Martinez et al., 2000; Stockdale et al., 2000). The condensing sclerotome mesenchyme subsequently becomes chondrified and forms the cartilage primordium. After maturation, cartilage undergoes ossification and finally becomes bone.

Many genes are involved in the regulation of the somite development. For
sclerotome formation, ventral signals such as Shh are important for initiating and sustaining the sclerotome (Fan et al., 1995; Fan and Tessier-Lavigne, 1994). Transcription factors such as Twist, Pax1 and Pax9 also play a critical role in the development of the vertebral column. For dorsal somite development, signals from the surface ectoderm, such as the Wnts and BMPs, are required to maintain the dermamyotome, while Myf-5 and MyoD are important genes specific for the myotome and muscle formation (Dietrich et al., 1997; Stockdale et al., 2000; Tajbakhsh and Sporle, 1998).

**Neural crest cells**

Neural crest cells (ncc) arise from cells at the border between the neural plate and non-neural surface ectoderm, and are characterized by expression of the zinc-finger transcription factor Slug. Just before or soon after the neural tube closure, ncc detach from the dorsal neural tube, pass through a stage of epithelial-mesenchymal transition, and migrate ventrally to specific locations in the body. The migrated neural crest cells give rise to numerous types of tissue, including peripheral nerves, glia, facial bones and dermis, cardiac septum, and pigment cells (Bronner-Fraser, 1995; Le Douarin et al., 1993).

The question of what molecular mechanisms are involved in ncc induction, migration, proliferation, and apoptosis is still not fully clarified. However, some genes have been found to play a specific role in these processes. For example, the Wnt, FGF and BMP genes are required for induction of early ncc, while BMP genes and Rho are important for migration of ncc. The interaction between Eph and ephrin is involved in directing migration of the trunk ncc in a segmental fashion. Other genes, such as Hox, Msx, Pax, Twist, and AP2 genes, as well as genes for several growth factors, and for retinoic acid (RA), for example, are also involved in the distribution, proliferation, and differentiation of neural crest cells (Christiansen et al., 2000; Garcia-Castro and Bronner-Fraser, 1999; Maschhoff and Baldwin, 2000; Selleck et al., 1998). Since the craniofacial and cardiac neural crest cells express the Pdgfra, they are of interest in our current studies.

The craniofacial neural crest cells migrate from the fore-, mid- and hindbrain. The ncc migrate from the dorsal neural tube, through the cranial mesenchyme, into the branchial arches. The ncc from the fore- and midbrain become the nasal structures, the palate, and the mesenchyme of the first pharyngeal pouch, and are later differentiated into maxillary, and mandibular bones, while the ncc from the hindbrain migrate and differentiate into the second pharyngeal pouch and the facial cartilage. The cervical neural crest cells give rise
to muscles and bones of the neck. It has been shown that the fate of the ncc is specified by Hox genes (Chai et al., 2000; LeDouarin, 1999).

The cardiac neural crest cell is a subtype of neural crest cell that originally comes from the vagal nerve. The cardiac neural crest is required for formation of the aorticopulmonary septum and of conotruncal cushions separating the outflow tract, and it also surrounds the thymus and thyroid during the development of these organs (Epstein et al., 2000; Jiang et al., 2000).

Targeted mutations of different genes have led to an accumulation of mouse mutants with affected neural crest cells and derivatives. In the formation of the craniofacial structures, the homozygous mutant mice for Pax3, Pax7, Msx, Shh and AP2 genes exhibit defects in neural crest-derived mesenchymal cells, including defects in the head and neck region (Ahlgren and Bronner-Fraser, 1999; Francis-West et al., 1998; Schorle et al., 1996; Serbedzija and McMahon, 1997; LeDouarin, 1999).
Platelet-derived growth factor was originally described as a platelet-derived mitogen for fibroblasts and smooth muscle cells (Kohler and Lipton, 1974). Subsequently, PDGF was found to be synthesized by a number of different cell types.

Hitherto, four different PDGF chains have been characterized, namely: PDGF-A, -B, -C, and -D (Bergsten et al., 2001; Betsholtz et al., 1986; Ding et al., 2000; Doolittle et al., 1983; LaRochelle et al., 2001; Li et al., 2000; Waterfield et al., 1983). In vitro studies have shown that PDGFs induce multiple cellular effects, e.g., cell proliferation, transformation, migration and survival (Heldin and Westermark, 1999). They also play important roles during embryonic development and in many pathological processes, such as in wound healing, angiogenesis, atherosclerosis, and oncogenesis (Ataliotis and Mercola, 1997; Heldin and Westermark, 1999; Ross et al., 1990; Ross et al., 1986).

PDGFs are active as dimers. The PDGF-A and -B chains form homo- and heterodimers, e.g. AA, AB and BB, through disulfide bonds. The genes encoding the PDGF-A and -B chains are located on human chromosomes 7 and 22 respectively (Betsholtz et al., 1986; Dalla-Favera et al., 1982; Stenman et al., 1992; Swan et al., 1982). Both the PDGF-A and -B genes contain seven exons and show about 60% amino acid sequence identity. Exons 4 and 5 encode most of the mature protein sequence. Exon 6 encodes a COOH-terminal basic sequence that may cause retention of the PDGF protein inside the producer cells (Robbins et al., 1985). The A chain has two splice forms, resulting in a long and a short A chain, depending on whether the exon 6 sequence is included or not. The short A chain, which lacks the COOH-terminal retention sequence, is secreted and may pass more easily through cell sheets and influence the tissue at a distance from the producing cells, while the location of the long form of the A chain, with the retention sequence, is more restricted to the producing cells themselves (LaRochelle et al., 1991; Pollock and Richardson, 1992; Raines et al., 1992). The short A chain is the most common form of PDGF-A in vivo (Betsholtz et al., 1986).

PDGF-C and PDGF-D form a subgroup of PDGFs. They consist of a two-domain structure with an amino-terminal CUB domain and a C-terminal PDGF/vascular endothelial growth factor (VEGF)-homology domain (also called the core domain) (Bergsten et al., 2001; LaRochelle et al., 2001; Li et al., 2000). PDGF-C and –D share 27-35% and 20-23% identity with PDGF-A and -B, respectively, while the amino-acid sequence identity in between PDGF-C and –D
is about 50% in the core domain and 43% if the full-length protein is compared. Mouse Pdgf-C protein shares 95% amino acid identity with the human PDGF-C, while mouse Pdgf-A and –B proteins share 91% and 85% amino acid identity with human PDGF-A and –B, respectively (Ding et al., 2000; and Locuslink at NCBI).

The full-length proteins PDGF-C and –D do not bind and activate receptors, while the core domain alone, i.e., when it is separated from the CUB domain by proteolysis, is able to bind and activate the respective receptors. PDGF-C and PDGF-D are secreted proteins and they also exist as dimers. Activated PDGF-CC binds to PDGFR-α, while PDGF-DD binds to PDGFR-β. PDGF-C does not seem to heterodimerize to PDGF-A and –B (Li et al., 2000). Whether the –C and –D ligands heterodimerize together is not clear, though they are structurally closely related.

**PDGF receptor α**

PDGF receptors are members of the tyrosine kinase receptor family type III. Two PDGF receptors, named α and β, have been identified (Claesson Welsh et al., 1989; Matsui et al., 1989; Yarden et al., 1986). Both the α and β receptors are encoded by structurally related genes and consist of extracellular, transmembrane, and intracellular portions. The extracellular portion contains a five immunoglobulin (Ig)-like domain, and the intracellular portion contains a tyrosine kinase domain, which is interrupted by a kinase insert region (Claesson Welsh et al., 1989; Matsui et al., 1989; Yarden et al., 1986).

The PDGFRα gene is located on the human chromosome 4 and mouse chromosome 5 (Hsieh et al., 1991; Matsui et al., 1989; Spritz et al., 1994; Stephenson et al., 1991). The human gene consists of a total of 23 exons, which include a short and untranslated first exon and a long, approximately 23 kb, first intron (Kawagishi et al., 1995). The translation initiation codon ATG is located within exon 2. The five Ig-like domains are encoded by exons 3-10, while exon 10 also encodes the transmembrane domain. The two parts of the tyrosine kinase domain are encoded by exons 13-15 and 17-21, respectively, while exon 16 encodes the kinase insert. The translation termination codon is located within exon 23 and is followed by a large 3’ untranslated sequence of approximately 3kb (Kawagishi et al., 1995) (Fig. 3). The molecular weight of the precursor of the encoded PDGFR-α is 140,000 Da and that of the mature protein is 170,000 Da. The cDNA sequences of PDGFR α and β share 44% similarity in overall, with 87% and 74% similarity, respectively, in the first and second tyrosine kinase domains.
The overall amino acid sequence identity between mouse and human receptor proteins is about 91\% (Do et al., 1992).

Two other growth factor receptor genes, c-kit and macrophage colony stimulating factor-1 (MCSF-1) receptor, have been found to be very similar to PDGFRα both in structure and in their chromosomal localization. The PDGFRα and c-kit genes are closely located on human chromosome 4q11-13 (Giebel et al., 1992), whereas the PDGFRβ and MCSF-1 receptor genes are both located on chromosome 5q31-q33 (Roberts et al., 1988).

Four forms of PDGF dimers, i.e., PDGF-AA, -AB, -BB, and -CC, are able to bind to the PDGF receptor- α with high affinity, whereas PDGF-BB and -DD only bind with high affinity to PDGF receptor-β (Claesson Welsh and Heldin, 1989; LaRochelle et al., 2001; Li et al., 2000). Ligand binding induces the receptor proteins to dimerize as αα, ββ, and αβ units and subsequently triggers phosphorylation of conserved tyrosine residues within the receptor. This autophosphorylation induces a downstream intracellular signaling cascade through a number of signaling pathways and leads to different cellular responses (Heldin and Westermark, 1999).

**Distribution of Pdgf-A and Pdgfra expression during mouse embryogenesis**

Studies on the distribution of PDGFs and PDGF receptors suggest that both paracrine and autocrine PDGF signaling can occur during embryogenesis. Pdgfra and Pdgf-A are coexpressed in the mouse embryo from the two-cell stage to the blastocyst stage both at the mRNA and protein level (Palmieri et al., 1992; Rappolee et al., 1988). After implantation, the coexpression of receptor and ligand changes to an adjacent expression pattern such that Pdgfra expression becomes mainly confined to the mesoderm and non-neuronal neural crest, whereas Pdgf-A is expressed in the adjacent ectoderm and endoderm (Orr-Urtreger and Lonai, 1992; Palmieri et al., 1992; Schatteman et al., 1992). The implication of the shifting pattern of expression from autocrine to paracrine Pdgf-A is unclear. However, blocking the autocrine Pdg loop in early embryonic development is lethal in the Xenopus (Ataliotis et al., 1995). It may be speculated that PDGF-A and its receptor might act in an autocrine manner to stimulate cell proliferation at a high rate. A paracrine PDGF-A might induce mesenchymal cell proliferation through an interaction between epithelium and mesenchyme; such an interaction is known to play an important role during induction and development of many organs. The paracrine PDGF can also function as a chemotactic factor for cell migration, which
is thought to be the case in PDGF-A-induced proliferation and migration of the oligodendrocyte progenitors (OPs) (Fruttiger et al., 1996).

During somite development, Pdgfα-receptor mRNA is present in the somites and later in the differentiated sclerotome and dermatome from E8 onward, but not in the myotome (Morrison-Graham et al., 1992; Orr-Urtreger and Lonai, 1992; Schatteman et al., 1992). The expression of Pdgfra is reduced and undetectable when the sclerotome differentiates into precartilage condensations and cartilage stage, respectively (Orr-Urtreger and Lonai, 1992), but the gene is still strongly expressed in the perichondrium and intervertebral disc. Pdgf-A, on the other hand, is strongly expressed in the myotome and in the whole skeletal muscle primordia (Orr-Urtreger and Lonai, 1992).

In the development of limb buds, Pdgf-A is expressed in the surface ectoderm and muscles, while Pdgfα is diffusely expressed in the mesenchyme, especially that surrounding the developing bones (Orr-Urtreger and Lonai, 1992). Interestingly, both Pdgf-A and Pdgfα have been found to be expressed in the apical ectodermal ridge (AER), an ectoderm-derived tissue important for the out-growing of limb buds (Orr-Urtreger and Lonai, 1992).

Pdgfα is clearly expressed in the mesenchyme of branchial arches of E9-11.5 embryos, a type of tissue which is of ectoderm-derived neural crest cell origin (Orr-Urtreger and Lonai, 1992). The branchial arch mesenchyme later contributes to the craniofacial neural crest cells and gives rise to facial bone primordia, dermis, papillae of skin follicles, and the connective tissue surrounding the eye. Whereas these tissues express Pdgfα, the neuronal neural crest derivatives, e.g., the peripheral neurons or ganglia, parasympathetic ganglia, and pigment cells, do not. Pdgfα is also expressed in the cardiac neural crest cells, including the aorticopulmonary septum, conotruncal cushions and valve of the outflow tract, and the neural crest-derived mesenchyme surrounding the thymus (Morrison-Graham et al., 1992; Orr-Urtreger and Lonai, 1992). Transient Pdgfα expression has been observed in the most dorsal portion of the neural tube at E9. This coincided with neural tube closure, and was thought to represent the neural crest cells before their migration from the neural crest (Schatteman et al., 1992).

During foregut development, the mesenchyme adjacent to the epithelia of the lung and digestive tract strongly expresses Pdgfα, while the epithelial layer expresses Pdgf-A mRNA. Other mesenchymal tissues, such as the developing diaphragm, the pleural and pericardial membrane, and the urogenital primordium also express Pdgfα (Orr-Urtreger et al., 1992; Orr-Urtreger and Lonai, 1992).

The distribution of Pdgfα in the central nervous system (CNS) is not

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completely clear, since there is still a doubt as to whether the neuronal cells express Pdgfra. Most results have shown that Pdgfra mRNA is not present until after E12.5 in the mouse CNS. A spotted Pdgfra signal starts from the ventral area of the central canal of the spinal cord and spreads throughout the whole spinal cord and restricted areas in the brain. These Pdgfra-expressing cells have been identified as OPs (Pringle et al., 1992; Pringle and Richardson, 1993; Yeh et al., 1993). However, others have shown that Pdgfra is also expressed in some neurons, e.g., in the neurons of the retina, Purkinje cells of the cerebellum, and in some ventricular zone cells of the brain (Mudhar et al, 1993; Andrae et al., 2001; Oumesmar et al., 1997). Pdgf-A is first detected in the floor plate at E11.5. After E13, Pdgf-A mRNA can be detected in motor neurons and other neurons in all parts of the spinal cord and in many neurons in the brain (Calver et al., 1998; Orr-Urtreger and Lonai, 1992; Yeh et al., 1991). In addition, Pdgfra mRNA can be clearly detected in the ectoderm-derived choroid plexus and in the eye lens (Orr-Urtreger and Lonai, 1992; Pringle et al., 1992), while Pdgf-A expression is seen adjacent to the Pdgfra in the surface ectoderm of the developing eye at E10.5-14.5 and in the epithelia of the choroid plexus (Orr-Urtreger and Lonai, 1992).

Thus, Pdgf-A and Pdgfra are seen in separate, adjacent cell layers. Pdgfra is mainly restricted to the mesenchymal tissue while Pdgf-A is seen within the epithelia and muscle tissues.

The patterns of expression of Pdgf-A and Pdgf-C are distinct and partly overlap during embryogenesis. Both Pdgf-A and Pdgf-C are expressed in the epithelia of the branchial arches and pouches, in the oral cavity and nasal placode, and in the myotome of the somites at about E9.5-12.5. However, Pdgf-A expression is much broader than that of Pdgf-C, in that most of the surface ectoderm strongly expresses Pdgf-A, while Pdgf-C is mainly restricted to the surface ectoderm of the craniofacial region. In early foregut development, Pdgf-C and Pdgf-A are co-expressed in the endoderm-derived epithelium of the intestines and in the bronchi of the lung. After E13.5, Pdgf-C expression changes from epithelium to mesenchyme, i.e., in developing smooth muscle of the lung and gut. The expression in the smooth muscle of the bronchial branches from E13.5 onward is unique to Pdgf-C. A different tissue expression of Pdgf-C and A has also been found in the developing kidney. In addition, Pdgf-C is not detectable in the CNS from E12.5 to E15.5 (Ding et al., 2000).

The partly overlapping and partly distinct patterns of expression of Pdgf-A and -C, and their use of a common receptor, Pdgfra, indicate that PDGF-A and PDGF-C are functionally both distinct and redundant genes.
Functional studies on the role of Pdgfra during development

Studies in null mutant mice have shown that both Pdgf-A and Pdgfra play an important role in the development of many mesoderm- and non-neuronal neural crest-derived tissues (Bostrom et al., 1996; Soriano, 1997).

*Pdgf-A* knockout mice die either before E10 or after birth. The reason for the early death before E10 is not clear. Those that die after birth exhibit several specific cellular defects, including loss of alveolar smooth muscle cells, leading to failure of alveolar septation and an emphysema-like situation (Bostrom et al., 1996; Lindahl et al., 1997). *Pdgf-A* mice also display mesenchymal depletion in the formation of the skin hair follicle and the gastrointestinal mucosal lining, with consequently fewer and misshaped skin hair follicles and intestinal villi (Karlsson et al., 1999; Karlsson et al., 2000). In addition, *Pdgf-A* knockout mice show a dramatically reduced number of OPs in the CNS, and a reduced number of oligodendrocytes together with a dysmyelinating phenotype (Fruttiger et al., 1996). The reduction of OPs is not evenly distributed, the most severely affected tissue being found in the peripheral region of the CNS, i.e., in the optic nerve, spinal cord, and cerebellum. This indicates that Pdgf-A is a major mitogen for OPs and is important for the migration of OPs *in vivo* (Fruttiger et al., 1996). Interestingly, neuronally secreted Pdgf-A has no known function in the proliferation of the neuronal cells themselves, but serves to stimulate the adjacent oligodendrocytes and their precursors to proliferate. This phenomenon mimics the stimulatory action of epithelially secreted Pdgf-A on the adjacent Pdgfra-positive mesenchymal cells within the regions of epithelial-mesenchymal interaction. Thus, all the tissues that are defective in *Pdgf-A* mice are Pdgfra-expressing mesenchymal tissues, not epithelial tissues, confirming the previous theory that Pdgf-A functions only through activation of the α-receptor.

*Pdgfra* knockout mice are also embryonic lethal. A wavy neural tube, subepidermal blebs, and a dilated pericardium are the major defects at E9-10. A cleft face, bleeding, and oedema are observed at E12.5-14. The Pdgfra mutants also exhibit increased apoptosis in the somite and cephalic regions, multiple skeletal abnormalities, including spina bifida, and incomplete fusion of the nasal and skull bones. These defects together lead to the death of the mutants between E8 and E16 (Soriano, 1997). Like the *Pdgf-A* null mice, *Pdgfra* null mice show depletion of Pdgfra-positive mesenchymal cells in the skin hair follicles and gastrointestinal villi, but with more severe and earlier developed skin phenotypes. Besides the hair follicle defect, Pdgfra null mice also exhibit severe dermal mesenchymal hypoplasia and regional detachment of the epidermal from the
dermal layer (subepidermal bleb) before birth, which is not observed in the *Pdgf-A* null mice (Boström et al., 1996; Karlsson et al., 2000; Soriano, 1997). The lung smooth muscle cell phenotype cannot be detected in *Patch (Ph)* and *Pdgfra* null mutants, since they die before the lung smooth muscle cells normally develop. It was not until a human YAC transgenic mouse was established, in which the null *Pdgfra* mutant was partly rescued to live up to birth, that the similar lung phenotype was found in *Pdgf-A* and *Pdgfra* knockouts. The lung phenotype of *Pdgfra* null-PDGFRα-YAC mice is very similar to that of *Pdgf-A* mice (Sun et al., 2000).

*Ph* mice, which carry a naturally occurring deletion of the *Pdgfra* gene, display a phenotype similar overall to that of *Pdgfra* knockout homozygotes (Morrison-Graham et al., 1992; Orr-Urtreger et al., 1992; Schatteman et al., 1992; Schatteman et al., 1995; Soriano, 1997; Stephenson et al., 1991). *Ph* mutants also have defects in the heart septum. The heart septum is of cardiac neural crest origin and strongly expresses Pdgfra during formation of the septum. However, the septum defect has not been observed in the *Pdgfra* targeted mutants. It is likely that the *Ph* deletion affects more than one gene, since *Ph* mice have a pigmentation phenotype that does not exist in the *Pdgfra* null mutant (Nagle et al., 1994; Smith et al., 1991; Soriano, 1997).

Taken together, the phenotypes of *Pdgf-A* and *Pdgfra* mutants basically overlap, in that the affected tissues are mainly restricted to the Pdgfraα-positive tissues of mesodermal origin and to the non-neuronal neural crest derivatives. However, the *Pdgfra* null mutants exhibit a more severe phenotype than the *Pdgf-A* knock-out mice, including the cleft face, subepidermal blebs and bone defects (Soriano, 1997). In addition, *Pdgf-B* knockout mice display heart dilatation and an increased heart size, in contrast to the *Pdgf β-receptor knockout* phenotype (Leveen et al., 1994). These findings indicate that although Pdgf-A is the major ligand for Pdgfra, Pdgf-B and -C can also activate the α-receptor in distinct tissues (Boström et al., 1996).

**Autocrine PDGF stimulation in tumorigenesis**

In tumor development, paracrine and autocrine mechanisms probably act in a dysregulated fashion to promote tumor growth. Many human tumors express high levels of PDGF and their receptors, forming the basis for an autocrine PDGF stimulation (Fig. 2). This situation has mainly been described in gliomas and sarcomas (Guha et al., 1995; Hermanson et al., 1992; Smits et al., 1992). Whether there is an autocrine stimulation that leads to an autophosphorylation of the receptor and how important the autocrine PDGF stimulation is in tumorigenesis
are questions that need further elucidation. However, several modes of inhibition of such autocrine PDGF loops have led to effective inhibition of tumor cell growth \textit{in vitro} and \textit{in vivo} (Shamah et al., 1993; Uhrbom et al., 2000). In human glioma, PDGF-A and $\alpha$-receptor expression was increased in parallel with an increase in the degree of malignancy (Hermanson et al., 1996). A recent report has shown that autocrine PDGF-B signaling can significantly increase the rate of proliferation of glial precursors, and also that overexpression of PDGF-B will result in conversion of cultured astrocytes into glial precursors (Dai et al., 2001). Sustained autocrine PDGF stimulation also induced the formation of oligodendroglioma at a high rate in mice (Dai et al., 2001). These data suggest that autocrine PDGF can stimulate the proliferation of glial precursors and play an important role in gliomagenesis.

![Diagram of autocrine and paracrine PDGF signaling](image)

**Figure 2.** Schematic illustration of the hypothesis that autocrine PDGF-A stimulation \textit{in vivo} can increase Pdgf$\alpha$ activity.
Transcriptional regulation of the PDGFRα gene

As described above, expression of Pdgfra during embryogenesis is strictly regulated (Mercola et al., 1990; Orr-Urtreger and Lonai, 1992; Palmieri et al., 1992; Rappolee et al., 1988; Schatteman et al., 1992). However, the detailed regulatory mechanisms of gene expression are currently unknown. Although several consensus motifs for putative transcription factor binding sites have been found in the promoter sequences, such as for E2F, GATA, API, AP2, and PEA3, little is known about the functional role of the DNA binding proteins in the Pdgfra gene transcription regulation (Afink et al., 1995). However, in vitro studies have led to identification of some molecules that can regulate Pdgfra expression at mRNA and protein levels, including IL-1, TGF-β, and RA (Tsukamoto et al., 1991; Wang et al., 1990; Ward et al., 1996). IL-1 can increase the protein level of Pdgfra, but not of Pdgfrβ, in the normal osteoblast-like cell line MC3T3-E1 (Tsukamoto et al., 1991), but downregulates the α-receptor in human osteoblast-like cells (Xie et al., 1994). RA-induced differentiation of human and mouse embryonal carcinoma cells results in an induction of Pdgfra transcription (Wang et al., 1990). In mouse cells, it has been demonstrated that the transcription factor GATA4 plays the main role in the process (Wang and Song, 1996). In addition, it has recently been reported that the transcription factor Gli can up-regulate PDGFRA transcript expression in a BCC cell line (Xie et al., 2001; see also below).

The full-length transcript of the PDGFRα gene is about 6.4 kb in humans and represents the sequence of exons 1-23. Several different sizes of PDGFRα transcripts in human and mouse tissues have been reported (Kraft et al., 1996; Lih et al., 1996; Mosselman et al., 1994; Mosselman et al., 1996) (Fig. 3). In the human embryonal carcinoma cell line Tera2, the undifferentiated Tera2 cells contain a 5.0 kb and a 1.5 kb PDGFRα transcript, while the RA-differentiated Tera2 cells express 6.4 and 3.0 kb PDGFRα transcripts. An alternative promoter located in intron 12 of the gene is found for generation of the 5.0 and 1.5 kb transcription (Kraft et al., 1996; Mosselman et al., 1994; Mosselman et al., 1996). In addition, an alternative promoter has been characterized within intron 1 in mouse fibroblasts (Lih et al., 1996). An alternative splicing TCCE domain at the end of the exon 16 has also been found to generate the 3 kb and 1.5 kb transcripts (Mosselman et al., 1994). Whether the alternative transcripts of the PDGFRα give rise to truncated Pdgfrα proteins and what the functional differences among the different transcripts are during development or tumorigenesis, still need to be established.

Taken together, these data indicate that the PDGFRα expression is
regulated by multiple transcription factors, and that the expression of Pdgfra in different tissues or cell types may require their specific regulatory elements as well as different transcription factors.

A 6 kb 5′ fragment of Pdgfra (Wang and Stiles, 1994), and a 2.2 kb and a 3.5 kb 5′ fragment of PDGFRA (Afink et al., 1995; Kawagishi et al., 1995) have been characterized. In vitro analysis of the promoter activity with series of 5′ deletions has shown that both positive and negative regulatory promoter elements are included in the 5′ flanking sequence (Afink et al., 1995; Kawagishi et al., 1995; Wang and Stiles, 1994).

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![Diagram of Pdgfra gene and transcripts](image)

**Figure 3.** A schematic illustration of the Pdgfra gene, transcripts and corresponding protein domains, based on characterization in man and mouse. (A) Promoter (P1), alternative promoters (P2 in humans and P3 in mouse) and alternative splice site (TCCE). (B) The sizes of the Pdgfra transcripts and the respective proteins.
**SHH SIGNALING PATHWAY**

**Shh-Ptc-Gli signaling pathway**

Hedgehog (hh) is a secreted signaling molecule that initially was found as a segment polarity gene in Drosophila. Hh precursor proteins undergo an autocatalytic cleavage into two mature portions: a 19 kDa N-terminal peptide and a 26-28 kDa C-terminal peptide (Hammerschmidt et al., 1997). The N-terminal peptide is highly conserved and is associated with the cell surface. It is modified by the addition of a cholesterol molecule, which might be involved in intracellular transport within epithelia (McMahon, 2000).

The hh pathway has been most well defined in Drosophila. The hh (N-terminal portion) binds to its receptor Patched (Ptc), a 12 transmembrane domain protein (Marigo et al., 1996; Stone et al., 1996) which releases the repression function of the other seven transmembrane domain receptor called smoothened (Smo) (Chen and Struhl, 1996). Smo does not interact physically with hh but is the receptor that activates a downstream transcriptional response to hh stimulation (Ingham, 1998; Ingham et al., 2000). Hh signaling regulates the transcription of target genes, including Ptc, decapentaplegic (dpp) and wingless (wg), and the signal is mainly mediated by the transcription factor Cubitus interruptus (Ci). Ci binds a serine/threonine kinase named fused (fu), the kinesin-like protein costal-2 (cos-2), and the Su(fu), a suppressor of fu, to form a Ci-Fu-Cos2-Su(fu) complex, which is connected with microtubules. Upon hh stimulation, the large cytoplasmic Ci-Fu-Cos2-Su(fu) protein complex dissociates from the microtubules, and the Ci transcriptional activator is translocated to the nucleus, leading to transcriptional activation of hh target genes. How activation of Smo can mediate dissociation of the Ci-Fu-Cos2-Su(fu) complex from microtubules is still not clear (Ingham, 1998; Robbins et al., 1997).

In vertebrates, the hh pathway is partly conserved. Drosophila has one hh, while at least three mammalian homologues of hh have been characterized, namely Sonic hedgehog (Shh), Desert hedgehog (Dhh) and Indian hedgehog (Ihh) (Hammerschmidt et al., 1997). Two Ptc homologues, Ptc-1 and Ptc-2 (Carpenter et al., 1998; Goodrich et al., 1996), and one Smo homologue (Akiyama et al., 1997; Stone et al., 1996) have been identified in the mouse and in man. The mammalian homologues of Ci comprise at least three homologous genes, named Gli-1, Gli-2 and Gli-3 (Park et al., 2000).

Shh signaling has been shown to have a large impact on the regulation of developmental events in the vertebrate embryo (Hammerschmidt et al., 1997;
Ingham, 1998; Villavicencio et al., 2000). For example, in the CNS, Shh from the notochord and floorplate can induce ventral cell fates. Shh induction of the floorplate and motor neurons depends on distinct concentration gradients, such that Shh induces floorplate at a higher and motor neurons at a lower concentration (Marti et al., 1995). Shh signaling establishes left-right body asymmetry (Zhang et al., 2001), is involved in the dorsal-ventral patterning of the somites (Chiang et al., 1996; Fan et al., 1995; Fan and Tessier-Lavigne, 1994), and polarizes anteroposterior limb development (Chiang et al., 1996). Shh also has an important role in the morphogenesis of many organs, including the eye, hair, tooth, gut, and lung (Dassule and McMahon, 1998; Hammerschmidt et al., 1997; Littingtung et al., 1998; Ramalho-Santos et al., 2000; Vortkamp et al., 1996).

Shh mutant mice exhibit severe developmental defects in many organs, including the brain and spinal cord, the axial skeleton and limbs. Loss of Shh function results in failure to induce and maintain the ventral midline structures, including the notochord, and the floorplate of the neural tube, and leads to cyclopia. Most of the craniofacial structures and also the sclerotome and axial bones are missing in Shh-/ mice and the distal limb structure is completely lost (Chiang et al., 1996).

Dhh is involved in the development of male germ cells (Bitgood et al., 1996) and development of the perineural sheath (Parmantier et al., 1999), while Ihh plays a central role in growth and differentiation of chondrocytes during the bone development (Karp et al., 2000; St-Jacques et al., 1999). Recent data on Shh and Ihh double null mice indicate that Shh and Ihh have redundant signaling functions in the establishment of left-right asymmetry, heart development, somite patterning and gut closure via a single Smo gene (Zhang et al., 2001).

Dysregulation of the Shh-Ptc-Gli pathway is the cause of several human diseases, including tumor formation (Villavicencio et al., 2000). Regarding tumorigenesis, mutations of PTCH-1, PTCH-2, and SMO have all been detected in basal cell carcinoma (BCC) and medulloblastoma. Overexpression of Gli1, but not Gli2 and Gli3, has also been found in BCC (Hahn et al., 1996; Hohnson et al., 1996; Xie et al., 1998). In addition, overexpression of Shh in skin causes BCC in mice (Dahmane et al., 1997), while Ptc null mutation in mice causes medulloblastoma (Goodrich et al., 1997). These data suggest that Shh signaling is involved in tumorigenesis, with PTCH playing a role as a tumor suppressor gene and SMO as a proto-oncogene.
Gli and Pdgfrα

Several findings give us reason to believe that Pdgfra might be regulated downstream of Gli and is involved in the Shh signaling pathway. First of all, the patterns of expression of Gli and Pdgfra are partly overlapping in the mesenchymal and neural crest cells (Hui et al., 1994; Orr-Urtreger et al., 1992; Orr-Urtreger and Lonai, 1992). Secondly, there are similar defects in Shh, Gli and Pdgfra mutants. For example both Gli2 and Pdgfra knockouts exhibit cleft palate, as well as vertebral body and intervertebral disc defects (Mo et al., 1997). Gastrointestinal and lung defects have also been found in Shh, Gli and Pdgfra mutants, although the defects in the Shh knockouts are much more severe (Litingtung et al., 1998; Ramalho-Santos et al., 2000; Soriano, 1997). The similarities may indicate that these genes are functionally related during development. Thirdly, a recently published report has also proven that Gli activates PDGFRA expression in a BCC cell line (Xie et al., 2001).

In addition, in oncogenesis, overexpression of both Pdgfra and Gli1 has been found in BCC (Xie et al., 2001). Taken together, these data point to the possibility that Pdgfra might be a target gene of Gli and may be involved in the hh signaling pathway, thereby playing an important role in tumorigenesis and in embryonic development.

Pax1 and Pdgfrα

Pax1 is a member of the Pax transcription factor family. A total of nine genes have been identified so far in the Pax family, and all of them contain a paired box that encodes a DNA-binding domain (Walther et al., 1991; Nol, 1993). Within the family, Pax1 and Pax9 are the only Pax genes that are expressed in the sclerotome. Pax1 is one of the earliest marker genes for the sclerotome; it is detected as early as at E8.5 in the somite, and is strongly expressed in the posterior, ventromedial components of the sclerotome (Peters et al., 1999; Wallin et al., 1994). Pax1 null mutants as well as naturally-occurring Pax1 mutant mice (undulated) all exhibit severe axial skeleton abnormalities. The axial bone defect in Pax1 mutants is found especially in the medial element of the vertebrae and includes spina bifida, while development of the lateral element of the vertebrae is controlled by the Pax9 gene (Peters et al., 1999; Wallin et al., 1994). Induction and maintenance of Pax1 expression in the sclerotome requires the Shh signal from the notochord (Fan and Tessier-Lavigne, 1994).

The undulated mouse carries a point mutation in the paired box that affects
DNA binding of the Pax1 protein (Chalepakis et al., 1991). Both undulated mutant mice (Dietrich and Gruss, 1995) and Pax1 null mice exhibit abnormalities of vertebrae and intervertebral discs (Wilm et al., 1998). However, neither the homozygous undulated (un/un) nor the heterozygous Patched (Ph/+, see also above), exhibits a spina bifida phenotype. When crossing the undulated mutant with the Patch mutant, all the un/un, Ph/+ double mutant mice exhibit an additive phenotype, i.e., spina bifida in the lumbar region, indicating that a functional interaction may occur between the two gene products (Helwig et al., 1995). Since disruption of the Pdgfra gene does not disturb the pattern of expression of the Pax1 gene, Pdgfra might act downstream of Pax1 in the developing vertebrae (Soriano, 1997).

To test the functional interaction of Pax1 and Pdgfra genes, three different Pax proteins, i.e. wild type, undulated Pax1 (un-Pax1) and a mutant PAX1 from a spina bifida patient (sb-Pax1) (Hol et al., 1996), have been used to induce promoter activity in the human Tera-2 embryonic carcinoma and the U-2 OS osteosarcoma cell lines (Joosten et al., 1998). The results suggest that PDGFRA may be a target gene of Pax1 and the fact that Pax1 mutant proteins enhance PDGFRA promoter activity suggests that there may be a gain-of-function phenomenon in the neural tube defects (Joosten et al., 1998).
AIM OF THE INVESTIGATION

The general aim of this investigation was to gain further knowledge about the in vivo Pdgfra promoter regulation and, by using the characterized promoter, to create an in vivo autocrine PDGF situation, which mimicked the tumor development in human tumors.

The specific aims were as follows:

I. To determine **whether the isolated 2.2 kb PDGFRA 5′ flanking fragment can promote correct expression of the reporter gene in vivo.** Transgenic mouse lines carrying the 2.2 kb promoter fragment linked with a lacZ reporter gene were generated. The temporal-spatial pattern of expression of the lacZ gene was analyzed and compared with the expression of the endogenous Pdgfra mRNA.

II. To address the question **whether chronic autocrine PDGF stimulation induces embryonic defects during development.** This was done by creating an autocrine PDGF-A mouse model that mimics the tumor autocrine situation, i.e., with ectopic expression of PDGF-A in Pdgfra expressing cells. A PDGF-As cDNA driven by a 6 kb mouse Pdgfra 5′ flanking promoter fragment was used to produce transient transgenic embryos. The autocrine PDGF-A-induced defects were analyzed during embryonic development.

III. To elucidate the question **whether PDGFRα expression might be regulated by the Shh-Ptc-Gli pathway,** by comparing the mRNA expression patterns of Gli, Pax1, Shh, Pdgf-A and Pdgfra during mouse embryonic development.
MATERIALS AND METHODS

Generation of transgenic embryos
Two ways of generating transgenic mice were used in this work, the “classic” and the “transient” methods. With the classic method, transgenic founders are first produced, and subsequently, transgenic offspring of the founders are generated by crossing with wild type mice (Hogan et al., 1986). Analyses of the transgene expression and effects on the transgenic embryos are usually carried out on the next generation of the transgenic mice (F1 mice). Here we used the classic method to analyze the in vivo PDGFRA promoter function by testing the pattern of expression of the reporter gene lacZ in the offspring transgenic mice (see paper I).

With the transient transgenic method, analyses of the transgene expression and phenotypic effects are performed on the transgenic founder only. This is usually achieved by arresting the development of the implanted embryos at desired embryonic stages, and subsequently collecting embryos, identifying the transgenic embryos with a PCR method, and analyzing the transgene expression and the effects of the transgene on the embryos. The purpose of using the transient transgenic mouse is either to shorten the experimental period, or to analyze an embryonic lethal phenotype. The disadvantage of the transient method is that it is very difficult to get all the results, e.g., concerning transgenic determination, transgene expression, and function, from one founder embryo.

LacZ reporter gene
The simplest plasmid DNA transgene construct contains at least two parts, a promoter and a gene or cDNA controlled by the promoter. When the expected gene is used to illustrate an unknown promoter activity, it is called a reporter gene.

The bacteria lacZ gene encodes β-galactosidase, which can cleave a suitable substrate and generate a compound, which gives a blue color. Since the LacZ gene does not exist in most of the eukaryotes, it has been very useful as a reporter gene for testing patterns of transgene expression. Here we used the lacZ gene as a reporter gene under the control of PDGFRA 5′ flanking sequences. Only when the 5′ sequence contains promoter activity can the lacZ gene be transcribed and translated into β-galactosidase and give a tissue-specific blue color after being exposed to the substrate X-gal.
Transgene integration and expression
It is commonly believed that transgenes introduced by pronuclear injection are randomly integrated into the host genome at the one-cell stage. However, the question of where and when the transgene integration occurs in the host chromosome is still unanswered. It has been observed that the integration sites have a wide variety of locations in the host genome. In the limited studies on the sequence of the chromosome-transgene junctions, poor homology between chromosome and foreign DNA at the junction was found (Konopka, 1988), indicating that homologous recombination might not be a major event in the transgene integration. The number of copies of a transgene in the integration site vary from one copy to hundreds, and they usually line up in a repeated head to tail arrangement. Several studies have shown a high frequency of mosaicism in transgenic founders (about 15-62%), indicating that the transgene integration does not occur before the first mitotic S-phase (Brinster et al., 1985; Whitelaw et al., 1993).

Transgenic and non-transgenic cells develop together in mosaic embryos. The percentage of transgenic cells depends on how late the transgene integration has occurred, the later the integration the lower the percentage of transgenic cells. When both transgenic and non-transgenic cells occur as germ cells, this is then called a germline mosaicism. A germline mosaic founder will produce a smaller than expected population of transgenic offspring when crossed with a wild type mouse. In view of the high frequency of mosaicism and the chromosome integration effect, it may readily be speculated that the transgene expression patterns and levels vary to a great extent among the transgenic founders.

Ectopic expression of a transgene means that the transgene-expressing tissues go beyond the region of endogenous gene expression. When the endogenously expressing tissue lacks transgene expression, this is called silencing of expression or absence of expression. Both situations can be caused by the environment of the integration site, i.e., by regulatory elements locally surrounding the transgene.

Resorption of embryos and evaluation of dead embryos
Resorption of embryos occurs when embryos die at early stages of development, or a short time after implantation. When the embryos are collected, the resorbed embryos appear as small decidual masses and there is hardly any proper embryo to be seen inside the decidua. If the embryos die at a later time point, they usually
have a relatively smaller decidua and the embryo proper can be collected, although the dead embryonic tissue is usually pale and fragile.

In our work, the embryonic stage of dead embryos was determined by their morphologic appearance in comparison with known stages of embryonic development, e.g., development of limb buds, or of the eye.
RESULTS AND DISCUSSION

Specific expression of a human PDGFRA promoter/lacZ transgene in mice (Paper I)

To investigate the in vivo functionality of the human PDGFRα gene (PDGFRA), a total of 15 transgenic lines were established with 2.2 kb, 0.9 kb, and 0.4 kb PDGFRA promoter-lacZ constructs (Fig. 4), using the standard oocyte microinjection method. The transgenic founders were identified by both PCR and Southern blot analysis, using purified mouse tail DNA as template. The pattern of expression of lacZ in these mice was studied and compared with the endogenous Pdgfra mRNA expression as determined by whole mount in situ hybridization. The results showed an expression pattern of the lacZ transgene, that was grossly overlapping with the endogenous Pdgfra in tissues of mesenchymal and neural crest origin, by using the 2.2 kb and 0.9 kb promoter fragments. The 0.4 kb fragment did not promote tissue-specific expression of the LacZ gene.

![Diagram A](image)

![Diagram B](image)

Figure 4. Constructs used for generating transgenic mice. (A) 2.2 kb, 0.9 kb, and 0.4 kb PDGFRA 5’ fragments linked to a lacZ reporter gene. (B) Autocrine PDGF-As construct. INT: SV40 intron splice cassette; PolyA: SV40 Polyadenylation sequence cassette
The overlapping expression of lacZ and Pdgfra was studied in detail in the 2.2-07 transgenic mouse line, in which embryos were serially sectioned and analyzed from E7.5 to E12.5. Overlapping or partially overlapping expression of lacZ and Pdgfra mRNA was found in the sclerotome and condensing mesenchyme of other skeletal bone primordia. The neural crest cells and their derivatives also expressed lacZ, e.g., the mesenchyme of the first and second branchial arches, and the facial mesenchyme in the developing lips, nostrils, pharynx and eyelids. The heart septum, which is of cardiac neural crest origin, expressed both lacZ and Pdgfra mRNA. LacZ was expressed in the interstitial mesenchyme of different developing organs, for example, in the pancreatic primordium and urogenital sinus. Promoter activity was high in the lung in that lacZ was expressed in the whole interstitial lung mesenchyme. No lacZ gene expression was found in epithelial tissues, whereas “ectopic” expression was observed in the neuroepithelium and in certain neuronal cell types from about E11 to adulthood. Expression was unexpectedly absent in many organs and cell types, e.g., in OPs, mesenchyme of the developing gut, and the eye lens.

We concluded that the isolated 2.2 kb human PDGFRA 5′ flanking fragment contains most but not all of the regulatory elements required for correct temporal-spatial expression of the Pdgfra gene during embryogenesis. The results also demonstrate that transcriptional regulation is the main mechanism for regulating Pdgfra mRNA expression levels in vivo and that the major part of the regulation takes place at the promoter level.

**Forced autocrine PDGFA stimulation in mice (Paper II)**

The next step in this project was to generate “autocrine PDGF” transgenic mice, and to study the effect of forced autocrine PDGF-A stimulation on mouse embryogenesis. For this purpose, the human PDGF-A short chain cDNA was cloned into a vector, containing a 6 kb mouse Pdgfra 5′ flanking fragment, and a SV40 intron splice cassette and a polyA cassette, to generate a construct named 6mPα-As. Thus, the PDGF-A transgene is supposed to be expressed in the Pdgfra-expressing cells, to form a forced autocrine PDGF-A stimulation. The 6 kb Pdgfra fragment had previously been shown to display in vivo promoter activity predominantly in mesoderm and neural crest cell derivatives in mouse embryos (Reinertsen et al., 1997). We chose the 6 kb mouse Pdgfra fragment as promoter because it promised to give more similar transgene expression patterns and levels among the transgenic founders than the 2.2 kb human PDGFRA fragment. In addition, we also tested the PDGF-A RNA and protein expression from this
construct by Northern and Western blot analysis, respectively, in 6mPα-As transfected NIH3T3 cells.

In this work we only generated “transient” transgenic mice with 6mPα-As DNA and analyzed embryos at E11.5, E12.5 and E14.5. A total of 126 embryos were collected and genotyped by PCR. Twenty-six transgenic founders were collected and the human PDGF-A transgene expression was subsequently determined in the transgenic embryos by in situ hybridization on sections, using a human PDGF-A cRNA probe. We found that the expression pattern of the human PDGF-A transgene basically fitted the lacZ expression pattern of the 6mPα-lacZ transgenic mice, although there were clear variations in the transgene expression level and tissue distribution among the transgenic founders. We also noted a dotted PDGF-A expression pattern, indicating a somatic mosaicism, in some of the transgenic founder embryos.

Four dead embryos were found within the transgenic group. Among them, two had detectable transgene mRNA expression, while the other two had no mRNA because of the long period between death and analysis, during which the RNA had been degraded. All of the four embryos exhibited a severely deformed phenotype, particularly in the head region, for example with defects in midline fusion, cranial facial mesenchyme deformities, overgrowth of nasofrontal, maxillary and mandibular processes, and a small body size.

To evaluate the general survival rate of the 6mPα-As transgenic embryos, we also generated transient transgenic embryos with a 2.2 kb human PDGFRA promoter-lacZ (2.2hPα-lacZ) construct and a 6 kb mouse Pdgfalpha promoter-lacZ (6mPα-lacZ) construct as control groups. The number of absorbed and retrieved embryos, and also the transgenic ratio and transgene expression ratio, were compared among the groups. The number of dead embryos and the embryo body weight and size were also compared between transgenic founders and their wild type littermates. We did not find more absorbed embryos in the 6mPα-As group than in the control groups, but the percent of dead E11.5-E14.5 embryos was larger in the PDGF-A transgene group than in the control group, indicating that the “autocrine mice” might start to die at the stage of mid-gestation but not at earlier stages.

**Discussion of the Papers I and II**

In conclusion, our results show that the mouse 6 kb Pdgfalpha promoter is able to drive the human PDGF-A transgene expression in a selection of Pdgfalpha-expressing tissues. The generated autocrine PDGF-A stimulation resulted in a disturbance of
body size determination, malformation of the cranial facial mesenchyme, and disturbance of the midline fusion process in cephalic and facial regions during embryogenesis.

Previous *in vivo* studies using the 6 kb mouse *Pdgfra* promoter have shown similar lacZ expression pattern as the 2.2 *PDGFRA*, i.e. the mouse 6 kb promoter fragment can direct lacZ expression in most Pdgfra-expressing cells of mesoderm and cranial neural crest origin in transgenic mice. However, the fragment failed to promote lacZ expression in the OPs, the cardiac neural crest cells, and most of the mesenchyme in the developing gut. “Ectopic” lacZ expression was found in neuronal cells in the cerebrum and dorsal root ganglia, which are negative for the endogenous Pdgfra mRNA (Reinertsen et al., 1997). These *in vivo* data together with the results of *in vitro* promoter studies suggest that both negative and positive regulatory elements are present in the isolated promoter, and in this respect the mouse and human fragments give the same results.

The PDGF-A transgene expression pattern also proved that the 6 kb promoter sequence is unable to promote *Pdgfra* transcription in OPs, cardiac neural crest cells, or visceral mesenchymal tissue in the lung, digestive tract and diaphragm. Thus, the autocrine PDGF-A stimulated tissues are mainly restricted to the craniofacial neural crest and the skeletal bone primordia. The pattern of expression of PDGF-As mRNA showed a discrepancy similar to that noted previously between the lacZ and endogenous Pdgfra expression, indicating that neither the 6 kb mouse nor the 2.2 kb human *PDGFRA* promoter fragments contain all the regulatory elements that are required for the proper expression of Pdgfra. The lacking regulatory elements can be located either upstream or downstream of the 2.2 kb promoter sequences. However, a human YAC transgene containing an approximately 65 kb *PDGFRA* gene, including a 3.5 kb promoter and 300 kb downstream sequences, was faithfully expressed in OPs, indicating that the OP-regulatory element(s) is located downstream of the promoter sequence (Sun et al., 2000).

Expression in OPs can also be a result of using an alternative promoter (Kraft et al., 1996; Mosselman et al., 1994) although it has not been proven that alternative transcripts, other than 6.4 kb, can be found in those tissues. The finding of “ectopic” lacZ expression in the brain neuronal cells may be explained either by the possibility that repression elements for Pdgfra expression in the neuronal cells were not included in the promoter fragment, or by an effect of the integration site in the mouse genome. However, it may also reflect a true promoter
activity, and explanation for the discrepancy might be involvement of different regulatory mechanisms in the lacZ protein and Pdgfra mRNA expression, and that the endogenous mRNA signal is too weak to be detected, but is still functional.

The midline fusion phenotype, for example with defects in neural tube closure, cleft face or palate, and spina bifida, can be induced either by knockout of Pdgfra (Soriano, 1997) or by an ectopic autocrine mode of PDGF-A stimulation. This indicates that the normal PDGF dosage and paracrine PDGF stimulation are important for proper pattern formation in the craniofacial and cephalic neural tube regions and in the face.

The variation among transgene mRNA expression levels may be the major reason why the autocrine PDGF-As embryos had different phenotypes, varying from normal to embryonic lethality. It has been shown that there is a rather high frequency of mosaicism among the transgenic founders as a result of the occurrence of the transgene integration later than at the one-cell stage (Whitelaw et al., 1993). In this study, we also found some transgenic embryos with a mixture of transgene-expressing and the non-transgene-expressing cells in the tissues, and those embryos usually appeared normal, while embryos with diffuse PDGF-A transgene expression often were the ones with a severely abnormal phenotype. These data indicate that the dotted expression represents transgenic mosaicism and may consequently have relatively low transgene expression. The mosaicism may therefore be an important reason for the phenotypic variation in the autocrine PDGF-As transgenic embryos. However, the transgenic founder mosaicism can be avoided by investigating the offspring of the founder.

**Future studies**

The construct we used here does not include any reporter gene, and the transgene expression is based on the in situ hybridization results on sections. This method for analysis of transgene expression is both time-consuming and difficult to get to reflect the whole embryo. A new construct with a reporter gene would therefore be required to allow quick screening of gene expression, especially for the purpose of generating transgenic lines.

The numbers of autocrine PDGF-As embryos observed are still small, especially of those with severe phenotypic abnormalities. To be able to obtain more detailed and complete data for phenotypic analysis, we still need to produce more transient transgenic founders with transgene expression. It would also be interesting to determine the levels of proliferation and apoptosis among the
defective tissues in order to gain a better understanding of the mechanism underlying the phenotype.

To reduce the variation of transgene expression, a better way is to produce several transgenic lines. The offspring of the transgenic founder should be devoid of mosaicism, and with a line it should be possible to analyze the phenotype in different embryonic developmental stages. In addition, one might be able to find a dosage effect, by comparison of the phenotypes between homozygotes and heterozygotes within the same transgenic line. However, the basic prerequisite for producing transgenic lines is that the transgenic founders are able to survive normally. An inducible transgene could circumvent that problem.

Is *Pdgfra* a target gene in the Shh signaling pathway? (Paper III)

To investigate the possible involvement of the Shh signaling pathway in the regulation of *Pdgfra* expression *in vivo*, we compared the patterns of expression of *Pdgfra*, *Gli*, *Gli2*, *Gli3*, *Ptc* and *Pax1* genes during mouse embryogenesis using the *in situ* hybridization method. Embryos from E9.5 to E14.5 were collected, fixed, embedded and sectioned. *In situ* hybridization on sections and whole mount mouse embryos was performed with Digoxigenin-labeled cRNA probes. The results showed overlapping, complementary and divergent expression when different tissues and stages were examined. Initially overlapping and subsequently complementary expression patterns of Gli and *Pdgfra* were seen in the developing skeletal bones. Generally overlapping expression of both Gli and *Pdgfra* was found in some epithelial-mesenchymal interaction regions, e.g., in the digestive tract and skin follicles. Divergent expression of *Pdgfra* and Gli was seen mainly in the heart and CNS. Comparisons of the expression patterns of Gli, Ptc, Shh, Pdgf, and *Pdgfra* in different developing organs are exemplified in Figure 5. The data suggest that Gli might be involved in the regulation of *Pdgfra* expression during bone and foregut development. Gli-mediated down-regulation of *Pdgfra* activity in the chondrification of bone was also indicated, while *Pdgfrα* is expressed independently of Gli in the heart and CNS.

Discussion of the Paper III

The main conclusion drawn from this study is that *Pdgfra* expression may be regulated by the Shh-Ptc-Gli signaling pathway during early bone development and in the developing gastrointestinal tract. The regulatory mechanisms of *Pdgfra* expression may vary in different tissues and at different developmental stages. If the overlapping expression is considered to point to the possibility of up-regulation, while complementary expression is regarded as indicating possible
down-regulation, the \textit{Pdgf}r\textalpha gene activity would rather have been down-regulated by Gli and Pax1 during chondrification of bone. The expression patterns of Gli, Pax1 and \textit{Pdgf}ra generally overlap at the earliest stages of axial bone development. In addition, a well-overlapping expression of Gli and \textit{Pdgf}ra was also observed in the developing digestive tract, indicating an up-regulation of \textit{Pdgf}ra expression by Gli in this area.

Figure 5. Comparison of the expression patterns of Shh, \textit{Pdgf}A, Ptc, Gli1 and \textit{Pdgf}ra in the developing foregut, tooth buds and whisker follicles
The divergent expression of Pdgfra and Gli observed in the cardiac neural crest and OPs indicated that Gli probably is not involved in regulating Pdgfra expression in these areas, but it does not exclude the possibility that other hh signal downstream elements may play a role in the regulation of Pdgfra expression in these tissues. Since the mRNA distributions of Gli, Gli2, and Gli3 are partly overlapping during embryonic development, and the Gli double mutants have shown a functional redundancy (Hui et al., 1994; Mo et al., 1997), it is therefore possible that Gli2 and Gli3 may also contribute to regulate Pdgfra expression.

**SUMMARY**

The 2.2 kb PDGFRA 5′ flanking sequence is a basic promoter. It is able to drive lacZ reporter gene expression in Pdgfra-expressing tissues of mesoderm and non-neural crest origin, e.g., in skeletal bone primordia and facial mesenchyme. However, absence of lacZ expression in OPs, cardiac neural crest cells and mesenchyme of internal organs, and “ectopic” expression of lacZ in brain neuronal cells were observed, indicating that the 2.2 kb fragment does not contain all the regulatory elements for normal PDGFRA gene expression.

Forced autocrine PDGF-As stimulation is embryonic lethal. The major tissue defects were found within the Pdgfra-expressing areas in the head region, and included midline fusion defects and abnormal development of the facial structures. The pattern of expression of the PDGF-As transgene follows that of the reporter gene with the same promoter. The data indicate that normal PDGF dosage, as well as paracrine expression of PDGF, is important for normal embryonic development. However, the absence of some promoter regulatory elements results that the expression of transgene does not cover all endogenous Pdgfra-expressing tissues. A wide variation in expression levels and phenotypes was also found among the PDGF-As transgenic founder embryos.

The Shh-Ptc-Gli signaling may regulate Pdgfra expression in some tissues, e.g., in early bone development and in the developing gastrointestinal tract. In the heart and CNS on the other hand, Gli may not be involved in the regulation of Pdgfra expression. Our results also indicate that the regulatory mechanisms of Pdgfra expression may vary in different tissues or cell types.
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