Function and regulation of Platelet-Derived Growth Factor Receptor Alpha during development

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A thesis submitted to the University of London for the degree of Doctor of Philosophy
To my parents

Endless love
ABSTRACT

Platelet-Derived Growth Factor Receptor Alpha (PDGFRα) plays a vital role in the development of vertebrate embryos, since mice lacking this protein die at mid-gestation. The PDGFRα gene displays a complex time- and tissue-specific expression pattern during development, and participates in the development of many diverse tissues and organs. Among its many functions, PDGFRα is essential for the development of oligodendrocyte progenitors (OLPs), which originate from the ventral spinal cord in the central nervous system (CNS).

To gain more insight into the transcriptional regulation of the PDGFRα gene, I analyzed the relative promoter activities of a 6 kb upstream fragment of the murine PDGFRα promoter and a 2.2 kb human PDGFRα promoter by transient transfection assay in CG4 cells, an OLP cell line. I also mapped the transcription start site of PDGFRα in OLPs by primer extension and nuclease-S1 protection assay. These results suggest that distant cis-acting regulatory elements are required for PDGFRα expression in OLPs. To study PDGFRα regulation, particularly in OLPs, I generated a transgenic mouse model by pronuclear injection of a 380 kb yeast artificial chromosome (YAC) containing the entire human PDGFRα gene and flanking sequences. The human PDGFRα transgene was faithfully expressed in OLPs in the spinal cord, which is not observed with conventional transgenes containing up to 6 kb of 5′ flanking sequence, and also in many tissues outside of the CNS. There was also ectopic expression at sites that normally express c-kit and flk-1, which map close downstream of PDGFRα. Despite this, the YAC transgene rescued the profound craniofacial abnormalities and spina bifida in the PDGFRα null mutant mouse and prolonged survival until birth. In addition, PDGFRα null mice rescued with the YAC transgene died after birth from respiratory failure.

Furthermore, I also investigated the role of transcription factor Pax6 in specification of OLPs and motor neurons (MNs) in the ventral spinal cord. I found that OLPs originated...
in the ventral-most part of the *Pax6*-positive ventricular zone, which at earlier times generated somatic (*Isl2/Lim3*-positive) MNs. In *Pax6* mutant mice, the site of origin of OLPs was shifted dorsally and production of both OLPs and MNs was delayed by about a day. I suggest that OLPs and somatic MNs are derived from the same pool of precursors whose positional specification depends on Pax6. Neuron-glia fate switching might be a preprogrammed property of these precursors or a response to feedback from newly generated neurons. Oligodendrocytes developed normally in explants of *Isl1*(*-/-*) spinal cords, which lack MNs, arguing against feedback control. I suggest that the neuron-glia switch is an intrinsic developmental program in a specific subset of neural precursors.
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# ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>BCIP</td>
<td>5-bromo-4-chloro-3-indolyl-phosphate</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>BrdU</td>
<td>bromodeoxyuridine</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>DEPC</td>
<td>diethylpyrocarbonate</td>
</tr>
<tr>
<td>DIG</td>
<td>Digoxygenin</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>EBSS</td>
<td>Earle’s balanced salt solution</td>
</tr>
<tr>
<td>ECD</td>
<td>Extracellular domain</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>GC</td>
<td>Galactocerebroside</td>
</tr>
<tr>
<td>IN</td>
<td>Interneuron</td>
</tr>
<tr>
<td>kb</td>
<td>kilobase</td>
</tr>
<tr>
<td>KO</td>
<td>Knock out</td>
</tr>
<tr>
<td>LMP</td>
<td>Low melting point</td>
</tr>
<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>MBP</td>
<td>Myelin basic protein</td>
</tr>
<tr>
<td>MN</td>
<td>Motor neuron</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>NBT</td>
<td>Nitroblue tetrazolium salt</td>
</tr>
<tr>
<td>OL</td>
<td>Oligodendrocyte</td>
</tr>
<tr>
<td>OLP</td>
<td>Oligodendrocyte progenitor</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td>PDGFRα</td>
<td>platelet-derived growth factor receptor alpha</td>
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<tr>
<td>PFGE</td>
<td>Pulsed-field gel electrophoresis</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>---------</td>
<td>------------------------------------</td>
</tr>
<tr>
<td>PNS</td>
<td>Peripheral nervous system</td>
</tr>
<tr>
<td>Sey</td>
<td>Small eye</td>
</tr>
<tr>
<td>Shh</td>
<td>Sonic hedgehog</td>
</tr>
<tr>
<td>TEMED</td>
<td>Tetraethylmethylenediamine</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
</tr>
<tr>
<td>VZ</td>
<td>Ventricular zone</td>
</tr>
<tr>
<td>YAC</td>
<td>Yeast artificial chromosome</td>
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Chapter 1

Introduction
1.1. Initial formation of the neural structure: from the neural plate to the neural tube

Neurulation is one of the most widely studied morphogenetic processes during embryonic development. The entire nervous tissue develops from the ectoderm. Signals from the notochord are thought to induce the formation of neural plate from ectoderm. In mouse embryos, the neural plate is first distinguishable soon after Embryonic day 7.5 (E7.5). Subsequently, the neural plate changes shape dramatically. The neural plate deepens to form the neural groove and its lateral edges rise to form the neural folds. At this stage, in cross section, the neuroectoderm comes to resemble a "V" with the "hinge point" being the midline overlying the notochord. The cells of the medial hinge point will afterwards give rise to the floor plate. At about E8 to E8.5, the neural folds fuse along the dorsal midline at the level of the fourth and fifth somites. Then the neural tube is formed. Subsequently, fusion proceeds both rostrally and caudally in a zipper-like fashion from the cervical region of the neural tube. The anterior open end of the neural tube (anterior neuropore), is closed completely at about E9 (15-20-somite stage), whereas the posterior open end of the neural tube (posterior neuropore), remains open until E10 to E10.5 (32-somite stage).

After the neural tube has formed, the neuroepithelial cells continue to divide and the wall of the neural tube becomes even thicker. In the anterior region of the neural tube, the neuroepithelial cells rapidly divide and afterwards differentiate into primitive nerve cells or neuroblasts which form an easily recognized layer (subventricular zone). In the posterior region of the neural tube — the spinal cord, the neuroepithelial cells give rise directly to post-mitotic neuronal progenitors. In both cases, the immature neuronal progenitors migrate outwards to their final places along the processes of radial glia. They then differentiate and extend their axons to their target cells.

Lots of evidence suggests that the developing nervous system is a highly patterned tissue from a very early stage. At a gross level, there is obvious regional differentiation
along the anteroposterior neural tube — the forebrain, midbrain, hindbrain and spinal cord. It is thought that the dorsoventral polarity of the neural plate is also established early in development before closure of the neural tube. At the cellular level, the differentiation of specific neurons in the neural tube follows a stereotyped dorsoventral pattern. Motor neurons are located ventrally in the spinal cord, whereas commissural neurons and the neural crest cells arise in dorsal positions.

Once the neural folds are formed, the cells of the neural crest temporarily appear at the two lateral folds. After the neural folds have fused, the neural crest cells separate from the folds. This population of cells originates in the dorsal part of the neural tube. Afterwards, they form a continuous plate between the neuroepithelium and the surface ectoderm. In mouse embryos, neural crest cells first appear on the dorsal surface of the neural tube at the level corresponding to the third or fourth somite. Thereafter, they both emerge and migrate in a rostral-to-caudal sequence. Neural crest cells migrate along two pathways: a ventral pathway through the rostral half of the somites and a dorsolateral pathway between the dermamyotome and the epidermis. Once they arrive at their locations, neural crest cells differentiate into a wide variety of cell types, such as, bone and cartilage, melanocytes, glial cells including Schwann cells, and sensory neurons.

Over the past decade, the molecular mechanisms that induce the neural tissue, pattern the neural tube and control the specific cell types in the neural tube have been defined through genetic and biochemical approaches in both invertebrate and vertebrate. These studies have provided insight into the interaction of the extracellular signaling factors and intracellular proteins that direct cell fate. The rest of this Introduction focuses on recent progress in defining how neural plate is induced during early development, how the neural tube is patterned along the anteroposterior and dorsoventral axes and how diverse cell types in the neural tube are generated. Because the spinal cord is the simplest and most conserved region in the central nervous system (CNS), this introduction will focus on the spinal cord.
1.2. Induction of the neural plate: studies in BMP signaling in *Xenopus*

The development of the vertebrate nervous system requires induction of neural plate from undifferentiated ectodermal cells. Spemann and Mangold (1924) demonstrated that a small region of the *Xenopus* embryo, the organizer or dorsal lip, could direct neighbouring tissues to form dorsal mesodermal fates, such as notochord and muscle. The organizer grafted to the ventral side of another embryo can induce a complete secondary axis containing the central nervous system (Spemann and Mangold, 1924). This then raises the question of how the nervous system is induced.

Dissociation of blastula-stage ectoderm into single cells, which presumably prevents intercellular signaling, is sufficient to elicit the formation of neural tissue (Godsave and Slack, 1989; Sato and Sargent, 1989). This observation suggests the possibility that neural induction might result from the inactivation of a signaling pathway that represses neural fate. Members of a branch of the transforming growth factor-β (TGF-β) -like protein family, the bone morphogenic proteins (BMPs) were suggested to mediate this repressive signal. In *Xenopus* embryonic development, BMP4 is a strong ventralizing molecule that is able to overcome the dorsalizing effects of activin and of the Spemann Organizer itself (Dale et al., 1992; Jones et al., 1992). BMP7, which heterodimerizes with BMP4, is expressed widely in the gastrula and is also considered an important component of the ventralizing signal (Aono et al., 1995; Hawley et al., 1995). Besides the ventralizing activity of BMPs, an intact BMP signaling pathway is also required for the ectodermal patterning. In situ hybridization shows that *BMP4* is present in the entire animal cap at the start of gastrulation. At later stages, *BMP4* transcription disappears from the portion of the ectoderm that becomes the neural plate (Fainsod et al., 1994; Hemmati-Brivanlou and Thomsen, 1995).

*Xenopus* animal cap explants from the early gastrula develops as epidermis (ventral ectoderm). But ectoderm in dissociated cell culture during early gastrula stages forms
neural tissue (dorsal ectoderm). Interestingly, soluble BMP4 can induce epidermis in dispersed cells and suppress neural tissue formation (Wilson and Hemmati-Brivanlou, 1995). Microinjection of dominant-negative BMP receptor (Xu et al., 1995a), or dominant-negative ligands (Hawley et al., 1995) or BMP4 antisense RNA (Sasai et al., 1995) causes animal cap explants to develop as neural tissue. Thus, endogenous BMP4 present in the animal cap appears to repress neural development. Similar evidence has also been found in Drosophila. Drosophila embryonic dorsal-ventral patterning is accomplished partly through the action of Decapentaplegic (Dpp), which is a TGF-β member most closely related to vertebrate BMP2 and BMP4. The loss-of-function phenotype of dpp mutation involves expansion of the neurogenic ectoderm at the expense of epidermis (Irish and Gelbart, 1987), whereas, ectopic expression of dpp leads to the expansion of epidermis and reduction of the neurogenic ectoderm (Ferguson and Anderson, 1992a; Ferguson and Anderson, 1992b; Wharton et al., 1993).

Therefore, an attractive hypothesis is that the diffusible organizer signals might neuralize ectoderm and dorsalize mesoderm by antagonising the ventral BMP signaling pathway. Support for this idea has come from the demonstration that three secreted factors produced by Spemann’s organizer, noggin, follistatin and chordin, can act in this manner. Noggin is the first molecule shown to have the properties expected of an organizer signal (Smith and Harland, 1992). Noggin can bind to BMP4 and block its binding to cognate cell-surface receptors thus interrupting BMP4 signaling (Zimmerman et al., 1996). Chordin is a soluble protein secreted by organizer cells during gastrulation (Sasai et al., 1994). Chordin also induces neural tissue (Sasai et al., 1995) by specifically binding to mature BMP4 proteins and antagonising BMP4 signaling pathway (Piccolo et al., 1996; Sasai et al., 1995). Likewise, Follistatin is expressed by organizer cells, and binds to and inhibits BMPs (Hemmati-Brivanlou et al., 1994).

Taken together, BMPs signaling acts early in Xenopus embryogenesis to specify ventral fate within the mesoderm and ectoderm. BMP4 is an endogenous neural inhibitor and
epidermal inducer. Understanding of this pathway requires identification of more downstream components and investigation of combined function analysis.

1.3. Pattern formation of the neural tube: two independent signaling systems

Development of the vertebrate CNS is characterised by the formation of the neural tube with distinct anteroposterior (A-P) and dorsoventral (D-V) polarity. The signaling system which controls the A-P pattern establishes the formation of the forebrain, midbrain, hindbrain and spinal cord. The second signaling system patterns the neural plate along its mediolateral axis, which later becomes the dorsoventral axis of the neural tube.

1.3.1. Pattern formation along the anteroposterior axis of the neural tube

The A-P pattern formation was much studied by classical embryologists using a variety of approaches. The mechanisms that have been proposed for A-P formation can be classified into three models. The two inducer model suggests that archencephalic (anterior) and deuterencephalic (posterior) inducers promote the formation of anterior CNS and posterior CNS, respectively (Sasai and De Robertis, 1997). The second model is the two-step activation-transformation model. The neuroectoderm that is initially induced by neural inducers (first step: “activation” or “induction”) is specified as forebrain, and the more posterior structures (midbrain, hindbrain and spinal cord) are derived from the anterior neuroectoderm by the transforming signals (second step: “transformation”). The third model proposes that the activity of a single inducer gives rise to A-P neural pattern. The inducer induces the spinal cord in a low concentration whereas yields forebrain in a high concentration. Among these three A-P formation models, there is most experimental support for the two-step model (Saxen, 1989).
Recent studies have shown that noggin (Lamb et al., 1993), follistatin (Hemmati-Brivanlou et al., 1994) and chordin (Sasai et al., 1995), secreted proteins expressed in the organizer, are potential candidates for anterior neural inducers, since all of them induce neural tissue that is anterior in character. No direct neural inducer isolated so far has the ability to induce posterior neural tissue such as spinal cord. These observations are consistent with the concept of the primary neural inducing signal (activation) in the two-step model. Recently, three kinds of secreted protein factors have been suggested as candidate molecules for the posterior transformation signal (the second step).

1.3.1.1. FGF family and posterior neural induction

Fibroblast growth factor (FGF), previously studied as a mesoderm inducer, has also been proposed to be a neural inducer. Basic FGF (bFGF) is capable of specifically inducing dissociated ectoderm cells at the gastrula stage of *Xenopus* embryo to produce CNS neurons (Kengaku and Okamoto, 1993). In chick embryos, FGF-soaked beads can induce expression of the early neural markers in extraembryonic epiblast cells (Alvarez et al., 1998; Storey et al., 1998). Inhibiting FGF signaling by the dominant negative FGF receptor also prevents neural induction in animal cap explants (Launay et al., 1996).

There are several lines of evidence suggesting that FGF is also involved in the specification of anterior-posterior neuroectoderm. In *Xenopus* embryos, bFGF can induce posterior neural tissue from early gastrula stage ectoderm (Lamb and Harland, 1995). Explants treated with noggin and bFGF display the anterior-posterior character of neural tissue. bFGF can induce the expression of a hindbrain marker in prospective forebrain and caudalizes prospective hindbrain by inducing spinal cord (Cox and Hemmati-Brivanlou, 1995). These results illustrate that bFGF has a caudalizing effect on neural fate. Overexpression of XeFGF during gastrulation in *Xenopus* suppresses anterior head development and produces expanded posterior tissues derived from ectoderm (Isaacs et al., 1994). Similarly, overexpression of XeFGF also causes the up-regulation of a number of posteriorly expressed genes, such as *Xcad* and *HoxA7* in
Xenopus (Pownall et al., 1996). In chick embryos, FGF-soaked beads can specifically induce expression of *cash4* and *Sax-1*, which are markers of the early posterior nervous system, but not that of anterior neural markers (Henrique et al., 1997; Storey et al., 1998). Thus, it is possible to derive posterior neural fates from prospective anterior regions of neural tissue. These findings have provided strong evidence for the function of the second signal (transformation).

However, the two-step model is not fitted to the following findings. bFGF can mimic the organizer action by inducing Xenopus ectoderm cells in culture to express position-specific neural markers in a dosage-dependent manner, i.e., with lower doses eliciting more anterior markers and higher doses more posterior markers (Kengaku and Okamoto, 1995). Therefore, it seems that bFGF alone can drive both steps (induction and transformation) by establishing a concentration gradient. Moreover, in chick embryos, FGF signaling can induce expression of early posterior neural genes in the absence of anterior neural tissue (Storey et al., 1998).

1.3.1.2. Retinoid acid and anteroposterior neural patterning

Retinoid acid (RA) was first proposed to be involved in neural development when it was demonstrated that exogenously applied RA produces a concentration-dependent truncation of anterior and enhancement of posterior structures in *Xenopus* embryos (Durston et al., 1989; Sive et al., 1990). In *Xenopus* embryo at the neurula stage, RA concentration in the posterior end is 10 times higher than in the anterior end (Chen et al., 1994). This concentration gradient implys the positional induction of RA in anteroposterior axis formation. RA can mimic the action of endogenous signals involved in inducing posterior gene expression in the *Xenopus* nervous system (Sharpe, 1991). If animal caps expressing noggin are treated with RA, expression of anterior neural markers is lost while expression of posterior markers is maintained (Papalopulu and Kintner, 1996). When a constitutively active RA receptor or a dominant negative RA receptor is microinjected, embryos appear posteriorized and anteriorized, respectively (Blumberg et
al., 1997). These findings suggest that retinoid signaling is required for the expression of posterior neural markers and the correct spatial restriction of anterior markers.

1.3.1.3. Wnt signaling and posterior neural induction

The Wnt gene family encodes a group of cell signaling molecules that play important roles in vertebrate development (Parr et al., 1993). In Xenopus, Xwnt-3a is expressed in the neural ectoderm of the early neurula (Wolda et al., 1993). This expression indicates that Xwnt-3A may participate in patterning the neural axis. Coinjection of Xwnt-3a and noggin or follistatin mRNAs induces posterior neural markers in animal caps while Xwnt-3a alone cannot induce neural tissue (McGrew et al., 1995). These findings demonstrate that Wnts may synergize with noggin and follistatin to provide posteriorizing signals during development of the nervous system. Thus, Wnt-3A is another good candidate for a posterior transformation signal.

In summary, noggin, follistatin and chordin which are expressed in the organizer during early development are reasonable candidates for neural inducers working at the activation step of the two-step model. FGFs, RA and Wnt-3A seem to be the transforming signals to posteriorize neural tissues.

1.3.2. Pattern formation along the dorsoventral axis of the neural tube

1.3.2.1. Diversity and pattern in the ventral neural tube: Shh signaling pathway

Studies from experimental biology as well as from genetics have shown a central role of the notochord in the establishment of the D-V polarity of the vertebrate neural tube. In a notochord-less Xenopus embryo, the neural tube does not have a floor plate and the D-V arrangement is disrupted (Sasai and De Robertis, 1997). Tissue grafting assays in chick show that the notochord induces the floor plate at the midline of the neural plate and neural tube (Yamada et al., 1991). An ectopic notochord also causes ectopic motor
neuron differentiation and an increase in the total number of motor neurons. Floor plate
grafts can mimic the effect of the notochord. These results suggest that the notochord is
the source of two inductive signals: a local signal that induces floor plate differentiation
and a longer-range signal that induces other neural cells (Yamada et al., 1993).

An excellent candidate for this inducing molecule emanating from the notochord is the
secreted protein Sonic hedgehog (Shh) (Echelard et al., 1993; Krauss et al., 1993; Roelink
et al., 1995). Shh is a vertebrate homolog of the Drosophila segment polarity gene
hedgehog (hh) (Lee et al., 1992). In situ hybridization and immunostaining have shown
that Shh is expressed in the notochord and the floor plate in early embryonic
development (Echelard et al., 1993; Marti et al., 1995b). Ectopic Shh expression under
Wnt-1 enhancer activates floor plate gene HNF-3β expression in the brain (Echelard et
al., 1993). In addition, antibodies that inhibit Shh signaling in vitro block the ability of
the notochord and floor plate to induce ventral cell types (Ericson et al., 1996; Marti et
al., 1995a).

The direct role of Shh in normal embryonic patterning was investigated in Shh gene
mutant mouse (Chiang et al., 1996). Shh mutant mice were generated by targeted
disruption of Shh to produce a truncated protein. Homozygous Shh mutant embryos
show the absence of a morphologically distinct floor plate and rostral loss of notochord
tissue. These findings demonstrate that Shh is required for the induction of floor plate
and maintenance of notochord. In the Shh mutant neural tube, expression of Pax3, Pax6
and Paxl expands ventrally to the ventral midline. Thus, Shh plays a direct role in
dorsoventral patterning of the neural tube. Furthermore, Isl1 expressing motor neurons
are also absent from the Shh mutant neural tube. All these studies show that Shh is
necessary and sufficient for the induction of ventral cell types.

Shh protein induces ventral cell types in a concentration-dependent manner. In neural
explant culture, there is a dose-dependent increase in the induction of Isl1 expressing
cells in response to Shh (Marti et al., 1995a). Shh secreted from the notochord acts at a
low concentration threshold (0.4nM in vitro) to convert naive neural plate cells into ventralized progenitor cells. The loss of Pax3 and Pax7 expression in the medial region of the neural plate reflects this conversion process (Ericson et al., 1996; Tremblay et al., 1996). Ventralized progenitors at the midline of the neural plate respond to a high concentration of Shh with the generation of floor plate cells (Roelink et al., 1995). Moreover, Shh signaling induces ventralized progenitors to give rise to motor neurons at an intermediate Shh concentration threshold (≥1.2nM in vitro) (Ericson et al., 1996). At the onset of motor neuron differentiation in vertebrates, the notochord is displaced ventrally and is no longer close to the neural tube. Since Shh is also expressed in the floor plate cells once they have been induced (Echelard et al., 1993; Marti et al., 1995b), the Shh that is required to convert ventralized progenitors into motor neurons is most likely to derive from the floor plate (Ericson et al., 1996). However, ventralized progenitors generate Lim1/Lim2 interneurons rather than motor neurons when the late period of Shh signaling is blocked. These studies suggest that the identity and pattern of ventral cell types in the neural tube is controlled largely by Shh signaling, through actions at multiple concentration thresholds. It will be very interesting to investigate how small differences in extracellular Shh concentration generate distinct neural cell types.

1.3.2.2. Diversity and pattern in the dorsal neural tube

While there is a wealth of knowledge about the ventral signals that induce ventral cell types in the neural tube, little is known about the inductive events necessary for the patterning of dorsal cell types. Two possibilities have been suggested. First, the formation of dorsal cell types may represent a default state, whereby plate cells are predisposed to differentiate into dorsal cell types unless exposed to a ventralising signal from the notochord to repress the formation of dorsal cell types in ventral regions. Second, similar to the requirement of signals from notochord to form ventral structure, the formation of dorsal cell types requires signals from adjacent tissues, such as the epidermal ectoderm and the roof plate.
Pax3, Pax7 and Msx are expressed in the dorsal spinal cord. Dorsalin-1 (dsl-1), a novel BMP-like member of the TGF-β superfamily, is also expressed in the dorsal region of the spinal cord (Basler et al., 1993). Cultured ventral neural plate explants from stage 10 chick embryos do not show any Msx and dsl-1 expression and very low Pax3 expression. Thus, cells in the ventral neural plate explants that have been exposed to notochord-derived signals do not express definitive dorsal cell markers (Liem et al., 1995). Whereas, culture of intermediate and dorsal neural plate explants that have not been exposed to notochord-derived signals shows high Msx, dsl-1 and Pax3 expression. Certain genes characteristic of dorsal neural tube cells are acquired in the absence of ventralizing signals (Liem et al., 1995).

However, neural crest cell differentiation suggests the existence of dorsalizing signals from epidermal ectoderm. Neural crest cells have been shown to arise from the neural folds and subsequently migrate from the dorsal neural tube towards their target sites (Bronner-Fraser and Fraser, 1989; Bronner-Fraser and Fraser, 1988; Serbedzija et al., 1989). Grafting of the prospective neural plate in chick embryos into tissue culture does not show expression of HNK-1, a marker of neural crest cells. Thus, early neural plate alone is unable to form neural crest derivatives (Selleck and Bronner-Fraser, 1995). However, coculturing either early (stage 4-5 chick embryos) or later (stage 6-10 chick embryos) neural plate plus epidermis can induce the formation of the neural crest derivatives (Liem et al., 1995; Selleck and Bronner-Fraser, 1995).

It is very possible that the non-neural ectoderm is the source of a dorsalizing signal that induces the formation of dorsal cell types. Cultures of neural plate explants in the presence or absence of non-neural ectoderm have shown that neural plate or non-neural ectoderm alone fail to express Wnt-1 or Wnt-3a, markers of dorsal neural tube, whereas contact of neural plate and non-neural ectoderm induces Wnt-1 and Wnt-3a expression (Dickinson et al., 1995; Liem et al., 1995). Then, what molecules induce the formation of dorsal cell types? Since TGF-β-related factor Dsl-1 can induce neural crest cells (Basler
et al., 1993), Liem et al. (1995) have tested functions of other members of TGF-β family, especially BMP4 and BMP7, in dorsal induction. BMP4 and BMP7 are expressed in the lateral neural plate. Afterwards, BMP4 and BMP7 expression is lost from the majority of the epidermal ectoderm, except BMP4 expression in the region directly above the dorsal neural tube. BMP4 and BMP7 are also expressed within the caudal dorsal neural tube. Importantly, BMP4 or BMP7 induce the expression of dorsal markers, such as Pax3, Dsl-1, Msx, slug and HNK-1, in ventral neural plate explants. Thus, BMP4 and BMP7 can mimic the ability of epidermal ectoderm to induce the expression of markers of dorsal neural tube and to promote the differentiation of neural crest cells (Liem et al., 1995).

1.3.2.3. Common features in patterning dorsal and ventral neural tube

There are common features in pattern formation in dorsal and ventral neural tube. Both dorsal and ventral cell types are induced by signals secreted from non-neural tissues: the epidermal ectoderm and the notochord, respectively. Both dorsal and ventral signaling factors (BMPs and Shh) are initially expressed by non-neural tissues, then by cells at the midline of the neural tube (the roof plate and floor plate).

A morphogen is a diffusible molecule that establishes a gradient away from its source and triggers distinct cellular responses at different distances (Nellen et al., 1996; Reilly and Melton, 1996). Shh acts as a morphogen to induce the differentiation of ventral cell types. Are BMPs also morphogens? In Drosophila, the BMP homolog Decapentaplegic (Dpp) has been shown to act as a morphogen. Ectopic expression of dpp in wing disc cells induces the transcription of its target genes omb and spalt at the different distances from the source (Nellen et al., 1996). These results suggest that omb and spalt genes respond to different threshold concentration of Dpp.

In zebrafish, graded BMP4 induces intermediate mesoderm and non-neural ectoderm (Neave et al., 1997). In Xenopus neurogenesis, dissociated cell assays show that dorsal
fates are induced by noggin in a dose-dependent manner, with low doses inducing most strongly, whereas higher doses repressing dorsal fates (Knecht and Harland, 1997). Since BMP4 antagonizes the action of noggin, BMP4 may also act in a concentration-dependent manner. In fact, the highest concentrations of BMP4 induce epidermis (Wilson and Hemmati-Brivanlou, 1995), slightly lower concentrations induce neural crest cells (LaBonne and Bronner-Fraser, 1998) and dorsal neural fates (Knecht and Harland, 1997). In chicken, BMP4 induces a graded response in mediolateral patterning of somitic and lateral mesoderm (Tonegawa et al., 1997). All this evidence suggests that BMPs also act as morphogens in pattern formation. Shh and BMPs may induce ventral and dorsal cell types in a similar concentration-dependent manner.

Shh and BMPs act alone to induce ventral and dorsal cell fates in the neural tube. In addition, Shh and BMPs have antagonistic actions that specify intermediate cell fates (Basler et al., 1993; Liem et al., 1995). However, Shh and BMPs also cooperate to determine neural cell fates in the rostral diencephalon. In explants of rat intermediate neural plate, prechordal mesoderm can induce the expression of rostral diencephalic markers, BMP7 and Nkx2.1 (Dale et al., 1997). Like notochord, prechordal mesoderm expresses Shh but also BMP7. Notochord and caudal neural plate explants cultured with BMP7 generate rostral diencephalic ventral midline cells, whereas when BMP7 activity is blocked with an anti-BMP7 antibody, only floor plate cells are induced by notochord (Dale et al., 1997). Thus, the induction of rostral diencephalic ventral midline cells by prechordal mesoderm requires the actions of both Shh and BMP7.

1.3.2.4. Transcription factors and pattern formation of the neural tube

Besides Shh and BMPs, many transcription factors are involved in pattern formation in the neural tube. These transcription factors are expressed in discrete regions in the CNS. Analysis of mutants of these factors have provided critical insight into how they function in reginalization and cell-fate specification in the nervous system.
A. HNF-3p and floor plate development

Hepatocyte nuclear factor (HNF) was originally identified as a transcription factor for liver-specific genes (Lai et al., 1991). HNF-3p contains the Drosophila forkhead (fkh) domain which encodes a well-conserved 101 amino acid DNA-binding domain.

In mouse embryonic development, HNF-3p is expressed in the notochord first and then in the floor plate (Monaghan et al., 1993). Since HNF-3p expression precedes that of Shh in both the midline mesoderm and the ventral neural tube, the initial activation of Shh expression may be regulated by HNF-3p (Echelard et al., 1993). Ectopic expression of HNF-3p under the control of the midbrain/hindbrain specific En-2 promoter causes spatial expression of floor plate-specific genes (BMP-1 and HNF-3a) in transgenic brains (Sasaki and Hogan, 1994). These findings suggest that HNF-3p functions as a regulator of floor plate development. In HNF-3p null mutant embryos, no notochord or floor plate is ever formed (Ang and Rossant, 1994). Although neural tissue is formed, Shh expression is not detected in the neural tube. The D-V patterning of the neural tube is severely affected. The expression regions of Pax3 and Pax6 have also been extended ventrally. These results demonstrate that HNF-3p is a critical component in initiating the formation of the notochord.

In the Shh mutant embryos, the early expression of HNF-3p is initiated at a low level in the notochord. However, HNF-3p expression in the axial mesoderm is completely absent and its expression in the ventral neural tube is never initiated (Chiang et al., 1996). These findings show that Shh is not necessary for the initiation of HNF-3p expression. But once HNF-3p expression is initiated, Shh and HNF-3p expression become interdependent.

B. Pax gene family and the dorsoventral patterning of the neural tube
Through the homology to *Drosophila* genes which control early development, a large number of vertebrate genes have been identified, such as homoebox genes and paired box genes. The paired box was first identified in *Drosophila* segmentation genes (Bopp et al., 1986; Frigerio et al., 1986). After that, paired box genes were also isolated in the mouse (Deutsch et al., 1988). Until now, nine *Pax* genes have been identified, referred to as *Pax1* to *Pax9* (Mansouri et al., 1994).

The paired box is a 384 bp DNA sequence which encodes 128 amino acid DNA binding domain (Bopp et al., 1986). Other conserved sequence motifs are the octapeptide and the paired-type homeodomain which is 61 amino acids in length. Secondary structure predictions indicate that the paired domain contains three α-helices (Epstein et al., 1994). The structure of paired domain and homeodomain indicates that *Pax* proteins act as transcription factors that regulate gene expression (Chalepakis et al., 1991; Czerny et al., 1993; Epstein et al., 1994; Xu et al., 1995b; Zannini et al., 1992). *Pax* genes may be involved in the control of morphogenesis and pattern formation, since these genes are expressed in a spatially and temporally restricted pattern during embryonic development.

*Pax* genes show restricted expression regions along the dorsoventral neural tube during development. *Pax3* is expressed in the dorsal half of the neural tube and the roof plate (Goulding et al., 1991). *Pax7* is also expressed in the dorsal spinal cord except that it is not expressed in the most dorsal part of the neural tube (Jostes et al., 1990). *Pax6* is first expressed in the E8.5 mouse neural tube in a broad band of cells, being absent only from the most dorsal and ventral cells (Walther and Gruss, 1991). After that, the expression of *Pax6* is downregulated in the dorsal spinal cord. *Pax6* is highly expressed in the ventral spinal cord, but is not expressed in the floor plate and cells directly adjacent to the floorplate. Within the ventral spinal cord, there is a dorsal^{high}-ventral^{low} gradient of *Pax6* expression (Ericson et al., 1997). *Pax2* is expressed in two populations of interneurons in the lateral portion of the spinal cord (Burrill et al., 1997). Interneuron
markers *Enl* and *Evx1* are coexpressed with *Pax2* in a combinatorial manner to regulate interneuron identity.

Signals secreted from notochord regulate the dorsoventral expression pattern of *Pax* genes in the neural tube. In the chick embryos, after removing the notochord, the expression regions of *Pax3* and *Pax6* extend further ventrally (Goulding et al., 1993). On the other hand, a dorsal shift in expression domains of *Pax3* and *Pax6* occurs in regions of the spinal cord adjacent to the site where a second notochord has been placed. One of the candidate signals from the notochord is Shh (Echelard et al., 1993; Ericson et al., 1997; Krauss et al., 1993; Roelink et al., 1995). Graded Shh signaling is sufficient to establish graded *Pax6* expression in the neural tube (Ericson et al., 1997). However, *Pax6* expression is not initiated by Shh signaling. Using in vitro explant culture and grafting experiments, Pituell and colleagues (1999) showed that *Pax6* expression can be induced independently of the presence of Shh-expressing cells when neural plates are maintained in culture in the presence of paraxial mesoderm. *Pax6* expression disappears from the neural tube when a somite is replaced by presomitic mesoderm (Pitello et al., 1999). These results suggest that somites initiate and activate *Pax6* expression, but Shh plays a major role in regulating the level of *Pax6* expression.

The functions of *Pax* genes have been investigated in *Pax* gene mutant mice. The mutation of *Pax3* in *Splotch* mice leads to defects in neural crest derivatives and in limb muscle (Franz, 1990; Moase and Trasler, 1989). *Pax3* may play an important role in neural crest development. Similarly, homozygous *Pax7* mutant mice die shortly after weaning (Mansouri et al., 1996). They exhibit malformations in facial structures involving the maxilla and nose. *Pax7* also plays a vital role in neural crest development. On the other hand, Tremblay and colleagues (1996) produced transgenic mice expressing *Pax3* in the entire neural tube under the *Hoxb4* promoter region A enhancer. In this transgenic mouse, floor plate is absent in the neural tube and expression of Shh and *HNF-3β* is also missing in the floor plate area (Tremblay et al., 1996). These results show that Pax3 can inhibit floor plate differentiation.
*Pax6* mutant mice and rats display an eyeless phenotype in the homozygous condition (Hill et al., 1991; Matsuo et al., 1993). Homozygous *Small eye (Sey)* mice lack eyes and nasal cavities and die soon after birth. These findings show that Pax6 plays a vital role in eye development. Pax6 acts in eye development in a dosage dependent manner. Overexpressing *Pax6* in yeast artificial chromosome transgenic mice carrying the human *Pax6* locus causes abnormalities of the eye (Schedl et al., 1996). In the neural tube of *Pax6* mutant mice, Nkx2.2 expression region which is at most ventral part of the neural tube expands dorsally (Ericson et al., 1997). In the hindbrain, the loss of *Pax6* causes an apparent transformation of hypoglossal motor neurons (expressing Isl1 and Isl2, and transiently Lim3 and Gsh4) into vagal motor neurons (expressing Nkx2.2, Isl1 and Gsh4) (Ericson et al., 1997; Osumi et al., 1997). In the cervical spinal cord, the total number of motor neurons is decreased and somatic motor neurons are transformed into *Sim1* positive cell fate, which normally develop from the Pax6-negative domain adjacent to the floor plate (Ericson et al., 1997). These results demonstrate that the loss of Pax6 results in a dorsal-to-ventral transformation in the identity of progenitor cells. For the interneurons, there is a loss of a ventral population of *Pax2* expressing interneurons in the *Sey/Sey* spinal cord. *Enl* expressing interneurons are also not detected (Burrill et al., 1997). In addition to neurons, the site of origin of oligodendrocyte progenitors is also affected. In *Sey/Sey* neural tube, the origin of oligodendrocyte progenitors is shifted dorsally (Sun et al., 1998 and this thesis). The time of their appearance is also delayed up to a day.

**C. Gli gene family and Shh signaling pathway**

Despite the wealth of information regarding the induction of ventral cell fates in the neural tube by Shh, little is known about the intracellular signaling molecules that mediate these events.
In *Drosophila*, the actions of Hh have been shown to be transduced by the activity of four genes: *fuse* (*fu*), specifying a putative protein-serine/threonine kinase; *costal 2* (*cos2*), identified as encoding a distant relative of the kinesin motor proteins; *pka*, encoding protein kinase A; *cubitus interruptus* (*Ci*), a zinc finger transcription factor (Ruiz i Altaba, 1997).

Two candidate receptors for Hh have also been identified, encoded by genes known as *patched* (*ptc*), and *smoothened* (*smo*) (Alcedo et al., 1996; Hooper and Scott, 1989; Nakano et al., 1989; van den Heuvel and Ingham, 1996). The work of many labs has established the early events in the Hh signaling pathway. Normally, Ptc binds to and inhibits Smo function. Following Hh binding, Ptc is released from Smo, allowing the activity of Smo to be expressed. Acting through COS2, PKA and FU, this triggers Ci-mediated activation of Hh target genes (Aza-Blanc et al., 1997; Robbins et al., 1997; Sisson et al., 1997).

In vertebrates, three homologs of *Ci* have been identified as *Gli1* (Kinzler et al., 1988; Kinzler and Vogelstein, 1990), Gli2 and Gli3 (Hui et al., 1994; Ruppert et al., 1990; Walterhouse et al., 1993). In frog and mouse neural plate, Gli1 is expressed in midline cells at the time that they are induced to become floor plate, and in cells lateral to the midline (Hui et al., 1994; Lee et al., 1997; Sasaki et al., 1997). Gli2 is expressed throughout the neural plate with the exceptions of the midline and Gli3 is absent from the midline and shows a graded expression with highest levels laterally (Lee et al., 1997; Sasaki et al., 1997). Subsequently, the expression of Gli1, Gli2 and Gli3 becomes restricted to overlapping but distinct domains along the dorsoventral axis in the neural tube. Gli1 is expressed in the ventral most domain adjacent to the floor plate, Gli2 is restricted to ventral and intermediate regions, while Gli3 is only expressed dorsally (Hui et al., 1994; Lee et al., 1997; Sasaki et al., 1997). The similar roles of vertebrate Shh to *Drosophila* Hh, the homology of vertebrate Gli genes to *Drosophila* Ci gene and the expression patterns of Gli genes in the neural tube suggest that Gli genes may participate in the induction of neural patterning by Shh.
Gli1 proteins, but not Gli3, injected into frog embryos can induce ectopic expression of HNF-3β expression in floor plate. Gli1 binding site is required for the activity of the minimal floor plate enhancer of HNF-3β in vivo (Sasaki et al., 1997). These results demonstrate that Gli1 is involved in induction of floor plate (Lee et al., 1997). Ectopic expression of Gli1 in transgenic mice under the En-2 promoter-enhancer in the dorsal midbrain and hindbrain suppresses dorsal cell markers and induces ventral cell markers (Hynes et al., 1997). Moreover, Gli1 injected into frog embryos induces ectopic ventral neuronal differentiation (Lee et al., 1997). In addition, ectopic Shh induces Gli1 expression in both Xenopus and mice (Hynes et al., 1997; Lee et al., 1997). Shh also can directly up-regulate Gli1 expression in a cell line (Sasaki et al., 1997). Thus, Gli1 may act as a target and mediator of Shh signaling in floor plate and ventral neuronal differentiation.

However, in homozygous Gli2 null mutant mouse embryos, the floor plate does not form in the midbrain, hindbrain and spinal cord. Expression of HNF-3β is also not detected in these regions. But the notochord is present and it also expresses Shh (Ding et al., 1998; Matise et al., 1998). Two target genes of Shh signaling, Gli1 and Ptc are greatly downregulated in the mutant ventral spinal cord, whereas Gli3 expression is not affected (Ding et al., 1998; Matise et al., 1998). It seems that Gli2, but not Gli1, is the primary downstream activator of Shh target in the presumptive floor plate region. Diminished Shh signaling in the Gli2 mutant neural tube results in a ventral shift of Pax6 and Nkx2.2 expressing cells (Ding et al., 1998), but the dorsoventral pattern of cell types is arranged in a relative order similar to wild-type (Matise et al., 1998). Motor neuron differentiation is also normal. Furthermore, Gli1 mutant embryos posses a floor plate and do not show any obvious abnormalities in the spinal cord. Motor neurons are also generated in Gli1/Gli2 double homozygous mutant (Matise et al., 1998). It seems, therefore, that neither Gli1 nor Gli2 are required to generate ventral neuron types.
To the contrary, in frog embryos, only Gli1 can induce floor plate differentiation. Gli2 and Gli3 antagonise the effects of Gli1. The same regulation is also observed in the induction of ventral cell differentiation in forebrain (Ruiz i Altaba, 1998). Gli1 and Gli2 induce motor neuron differentiation, whereas Gli3 represses this induction. These results suggest that Gli1 and/or Gli2 mediate the Shh induction of motor neurons and Gli3 acts as a general repressor of Shh induction (Ruiz i Altaba, 1998). Thus it appears that the functions of Gli1 and Gli2 are not conserved among species.

1.4. Cell fates determination in neurogenesis

1.4.1. Specification of motor neurons and interneurons in the neural tube

There are three general classes of motor neurons at hindbrain and spinal cord levels: the branchiomotor neurons that innervate muscles from the branchial arches; somatic motor neurons that innervate skeletal muscles, and visceral motor neurons that innervate parasympathetic and sympathetic neurons. The somatic motor neurons are subdivided into two groups according to the position of their cell bodies and the location of the muscles they innervate. Motor neurons in the medial part of the medial motor column (MMC_M) project their axons to axial muscles that lie close to the vertebral column. Motor neurons in the lateral subdivision of the MMC (MMC_L) project their axons to body wall muscles. Motor neurons in the lateral motor column (LMC) innervate muscles in the limb. LMC can be subdivided into lateral LMC (LMC_L) and medial LMC (LMC_M) according to motor neurons in them project to dorsal limb muscles and ventral limb muscles, respectively.

The subclasses of motor neurons in the chick can be distinguished by the combinatorial expression of transcription factors of the Lim class (Tsuchida et al., 1994; Varela-Echavarria et al., 1996). Lim gene family members encode proteins with a homeodomain and a conserved tandemly-repeated cysteine-rich Lim domain (Freyd et al., 1990; Karlsson et al., 1990; Way and Chalfie, 1988). Motor neurons in the MMC_M coexpress
*Isl1, Isl2* and *Lim3*, while motor neurons in MMC express *Isl1* and *Isl2*. Motor neurons in LMC express *Isl1* and *Isl2*, while motor neurons in MMC express *Isl2* and *Lim1*, and transiently express *Isl1* (Tsuchida et al., 1994).

Graded Shh induces ventralised progenitors to give rise to motor neurons in the neural tube. However, the late commitment of progenitors to a somatic motor neuron fate in the spinal cord may suggest additional expression of genes that come on only during the final division cycle of motor neuron progenitors. By a PCR-based differential screen, a new homeobox gene *MNR2* has been identified in chick embryos (Tanabe et al., 1998). BrdU incorporation assay shows that *MNR2* is expressed in somatic motor neuron progenitors that also coexpress *Pax6* and *Lim3*. Ectopic expression of *MNR2* in the spinal cord using an avian retrovirus infection causes ectopic expression of *Isl1*, *Isl2*, HB9 and Lim3, even in the dorsal spinal cord where motor neurons never normally arise. Thus, MNR2 can induce the expression of somatic motor neuron markers and its expression in neural progenitors is sufficient to initiate motor differentiation. However, ectopic expression of *MNR2* in regions of the spinal cord where there is a high proportion of interneurons results in a ~50%-90% decrease in the number of cells that express interneuron markers (LH2, Brn3.0, En1 and chx3). This finding suggests that MNR2 may control ventral progenitors to give rise to either motor neurons or interneurons. Moreover, exposure of explants of neural plate to a Shh concentration at the threshold for motor neuron induction (1.2nM in vitro) leads to the generation of neurons that coexpress markers of motor neurons (*Isl1* and *Isl2*) and interneurons (*Lim1* and *Lim2*) (Ericson et al., 1996). It is most likely that there is a common progenitor for motor neurons and certain ventral interneurons. Furthermore, MNR2 appears to function upstream of a set of Lim transcription factors that cooperate to specify somatic motor neuron identity. Ectopic expression of *Isl1* in the spinal cord using virus infection does not cause ectopic expression of MNR2, Lim3, Isl2 or HB9. Similarly, misexpressing *Lim3* does not induce MNR2 or Isl2. However, Lim3 is also expressed by V2 interneurons. Misexpression of *Lim3* induces Chx10 expression. This implies the existence of a MNR2-independent pathway for the activation of Lim3 expression.
(Tanabe et al., 1998). Taken together, homeodomain transcription factor MNR2 is sufficient to direct somatic motor neuron cell fates in the vertebrate spinal cord, whereas Lim3 appears sufficient to specify V2 interneuron fates. It will be interesting to identify other factors that specify neuronal differentiation in the future.

1.4.2. Glial cell fate determination

Neurons and glia show great diversity with respect to position, morphology and physiological and biochemical properties. However, they can arise from common lineages in both the central nervous system and the peripheral nervous system (PNS). The relative simplicity of the *Drosophila* embryonic CNS makes it a suitable model system for the study of neuroglial lineages and the molecules that specify them.

In *Drosophila*, the origin of glia in the CNS is known from 3 types of progenitors. The longitudinal glia arise from the longitudinal glioblast (Jacobs et al., 1989). Longitudinal glia express *repo* which encodes a paired-like homeobox protein (Halter et al., 1995; Xiong et al., 1994). *Repo* is specifically expressed in most of the glia in embryonic CNS and PNS from an early stage. Longitudinal glia also express the homeodomain protein encoded by the *prospero (pros)* gene (Doe et al., 1991) and the P1 form of the *Ets* family of transcription factor encoded by the *pointed (pnt)* gene (Klaes et al., 1994). Midline glia arise from midline mesectodermal progenitors (Klambt et al., 1991). Midline glia neither express *repo* nor *pros*, but they express P2 form of the Pnt protein (Klaes et al., 1994). Other glia arise from neuroblasts (NBs) which act as common progenitors for glia and neurons (Udolph et al., 1993). For example, the NB1-1 divides asymmetrically into a ganglion mother cell (GMC) and another NB. After several divisions, NBs generate a series of GMCs. Each GMC divides to produce either neuron or glia.

An interesting question about the origin of neurons and glia has been raised: does a genetic switch exist that can turn presumptive neurons into glia or presumptive glia into neurons? This question has been answered by cloning of the *glial cells missing (gcm)* or
glide gene (Hosoya et al., 1995; Jones et al., 1995; Vincent et al., 1996). Drosophila GCM is a transcription factor with novel DNA binding domain that recognises the motif AT(G/A)CGGGT (Akiyama et al., 1996; Schreiber et al., 1997). In the CNS of loss-of-function gcm mutants, nearly all glia (except midline glia) are missing. Strikingly, the presumptive glial cells differentiate into neurons. To the contrary, after ectopic expression of gcm in presumptive neurons, many presumptive neurons are transformed into glia. In the PNS of loss-of-function gcm mutants, the presumptive glial cells are also transformed into neurons. Ectopic expression of gcm in presumptive neurons also causes transformation of presumptive neurons into glia (Hosoya et al., 1995; Jones et al., 1995).

The genetic network underlying glial cell development in Drosophila is also studied. At least three genes are known to be involved in glial cell differentiation. In repo putative null alleles mutant, glia progenitors originate normally and show normal migration behaviour. However, the differentiation of the presumptive glia seems to be severely affected (Halter et al., 1995). Pnt is also required for glial cell differentiation (Klaes et al., 1994). Ectopic expression of Pnt in the lateral CNS causes additional glial-like cells. In addition, tramtrack (ttk) encodes two different zinc-finger-type transcription factors, ttkp69 and ttkp88. Ttkp69 is expressed in all CNS glia. Ttkp69 may act to block the competence of cells to differentiate as neurons within the CNS (Giesen et al., 1997). Since the pnt-ttk double mutant phenotype (a fused commissure phenotype) is different from the gcm mutant phenotype and ectopic gcm expression is still capable to induce ectopic repo expression in pnt-ttk double mutants, the expression of repo, pnt and ttk is downstream of gcm (Giesen et al., 1997). Thus, GCM acts as a master regulator for glial development. GCM may control glial cell differentiation by concomitant activation of glial differentiation (function of pnt and repo) and suppression of neuronal differentiation (function of ttk).

Ectopic gcm also induces many aspects of the glial genetic program outside the nervous system. Expression of gcm in mesoderm in Drosophila embryos results in the
repression of mesoderm specific genes and in the loss of muscle morphology (Bernardoni et al., 1998). These findings suggest that gliogenesis does not require a ground neural state, and that gcm transcription must be tightly regulated in order to ensure correct embryonic development.

Mammalian homologs of gcm have been isolated in mouse (Akiyama et al., 1996; Altshuller et al., 1996) and rat (Kim et al., 1998). The conserved DNA-binding GCM motif, has been identified. However, the mammalian gcm is either expressed at very low levels during neuro- and gliogenesis, or not expressed in embryonic neural tissues (Altshuller et al., 1996; Kim et al., 1998). The function of gcm, at least those isoforms discovered to date, is not conserved from Drosophila to mammals. However, loss-of-function mutants of mammalian gcm have yet to be described and these will be important for our understanding of gcm function.

Glia and neurons can be derived from common lineages in vertebrates. In vertebrate retina, individual stem cells are multipotent and can generate both neurons and Muller glia (Walsh and Cepko, 1988; Wetts and Fraser, 1988). Reynolds and Weiss (1996) have identified a subset of cells from the embryonic striatum. These cells can form clonal spheres of nestin expressing cells. When the spheres are dissociated and grown in the absence of EGF, they differentiate into mixed colonies of neurons, astrocytes and oligodendrocytes (Reynolds et al., 1992; Reynolds and Weiss, 1992; Reynolds and Weiss, 1996). In the mammalian cerebral cortex, specified precursors that generate homogeneous clones of a single neural or glial cell type have been described (Grove et al., 1993; Luskin et al., 1993; Walsh and Cepko, 1992). However, multipotent precursor cells that can generate neurons, oligodendrocytes and astrocytes have also been identified in the cortex (Davis and Temple, 1994; Williams and Price, 1995). Recently, ependymal cells which are highly differentiated glial cells lining the lumenal surface of the adult ventricular zone have been demonstrated as neural stem cells which generate neurons, astrocytes and oligodendrocytes (Johansson et al., 1999). Moreover, some subventricular zone astrocytes have also been shown to be stem cells and to enter the
olfactory bulb to generate neurons (Doetsch et al., 1999). Whether these lineage studies identify dedicated progenitors or multipotent precursors presumably depends on how far back in the lineage tree are looked.

In mouse spinal cord, oligodendrocyte progenitor cells which express Platelet-Derived Growth Factor Receptor Alpha (PDGFRα) derive from the ventral ventricular zone as bilateral foci of cells (Noll and Miller, 1993; Ono et al., 1995; Pringle and Richardson, 1993; Yu et al., 1994). After that, they proliferate and migrate from the ventral spinal cord and finally spread through the whole spinal cord (Pringle and Richardson, 1993; Yu et al., 1994). The first oligodendrocyte progenitors appear in the ventricular zone on E12.5 in the mouse (E14.5 in the rat), shortly after the end of motor neuron production. Moreover, oligodendrocytes are induced in explant cultures of dorsal or intermediate neural tube at Shh concentration that overlap those required for motor neuron induction (Poncet et al., 1996; Pringle et al., 1996). These findings suggest that the same population of progenitor cells might give rise first to motor neurons and then to oligodendrocyte progenitors (Richardson et al., 1997). Support for this idea comes from the analysis of origins of oligodendrocyte progenitors and somatic motor neurons in the Pax6 mutant spinal cord (Sun et al., 1998 and this thesis). In Sey/Sey spinal cord, the appearance of oligodendrocyte progenitors is delayed by about a day. Interestingly, the appearance of Isl2 and Lim3 expressing motor neurons which are also generated in the ventral ventricular zone is also delayed by up to a day. These results indicate a close relationship between Isl2/Lim3 motor neurons and oligodendrocyte progenitors, and also suggest that both cell types may derived from common neuron-oligodendrocyte progenitor cells.

1.5. Aims of this thesis

To the gliogenesis in the spinal cord, an important question is to answer how glial precursors respond to ventral inducing signals from the notochord, such as Shh. To investigate this, I studied expression and function of PDGFRα gene in oligodendrocyte
progenitor (OLP) development. In this thesis, I mapped the transcriptional start site of PDGFRα in the CNS and found that PDGFRα transcribes from the same site in the CNS as in other tissues. To analyze the remote cis-acting regulatory elements that are required for PDGFRα expression in the CNS, I generated transgenic mice by pronuclear microinjection of a 380 kb yeast artificial chromosome (YAC) containing entire human PDGFRα gene. This YAC transgene is faithfully expressed in OLPs in the spinal cord and correctly expressed in most non-neural tissues. This YAC transgene can rescue the defect of oligodendrocyte development in PDGFRα null mutant mice. I also analyzed functions of PDGFRα in craniofacial and lung development. In addition, to investigate factors that affect OLP development, I mapped origins of OLPs in both wild-type and Pax6 mutant Small eye (Sey) mice spinal cords. A dorsal shift of OLP origin was observed in Sey/Sey spinal cord. This shift is accompanied with a delay of appearance of both OLPs and Isl2/Lim3 expressing somatic motor neurons in the ventral spinal cord.
Chapter 2

Materials and methods
General chemicals and reagents were all purchased from Sigma-Aldrich Co Ltd or BDH Chemicals Ltd unless otherwise stated, and were Molecular Biology grade where available.

Restriction and DNA/RNA modifying enzymes were all purchased from Promega Ltd or New England Biolabs (UK) Ltd unless otherwise stated.

Bacterial media and yeast media components were obtained from Difco Laboratories Ltd, or from the in-house facility.

All radio-chemicals were purchased from Amersham International.

All tissue culture media components were purchased from GIBCO BRL Life Technologies Company.

Sterilisation of solutions, where necessary and possible, was performed by autoclaving at 15 lb/sq.in. for 15 minutes. Heat-labile solutions were sterilised by filtration through a 0.22μm filter (Millipore). All solutions were stored at room temperature unless otherwise stated.
2.1. Bacteriology

2.1.1. Bacterial strains, growth and storage

All general cloning and sub-cloning was carried out using *Escherichia coli* (*E. Coli*) strain XL1-Blue (supE44 hsdR17 recA1 endA1 gyrA46 thi relA1 lac' F' [proAB lacP LacZΔM15 Tn10 (tet)])). Bacteria were grown at 37°C in Luria Broth (LB, 10g bacto-tryptone, 5g bacto-yeast extract and 10g NaCl per liter) or on LB-agar plates containing LB with 15g/L bacto-agar. Ampicillin was added to the LB or molten LB-agar (after cooling to 55°C) at a final concentration of 25-100µg/ml (100mg/ml stock in H₂O, 0.22µm filter-sterilised and stored in aliquots at -20°C). Liquid cultures were continually agitated in a rotating environmental shaker at 300 rpm.

For short term storage of bacterial strains and clones, they were stored on LB agar plates at 4°C for two or three weeks. For long term storage, 75% glycerol was added to overnight cultures to a final concentration of 15%. This mixture was stored in 1ml aliquots at -20°C or -80°C if necessary, for a few years.

2.1.2. Preparation and transformation of electrocompetent bacteria

1ml of an overnight liquid culture of *E. Coli* strain XL1-Blue (grown from a single colony) was transferred into 1L of LB. This culture was grown until it was in mid-log phase (an absorbance of ~0.6 at OD600nm), then chilled on ice and pelleted by centrifugation (4,000rpm, 4°C, 10 minutes). The bacterial pellet was gently resuspended in 1L of ice-cold H₂O, pelleted as before, resuspended in 500ml ice-cold H₂O and pelleted again. This pellet was then resuspended in 20ml ice-cold 10% (v/v) sterile glycerol, centrifuged as before, and finally resuspended in 2.5ml ice-cold 10% (v/v) sterile glycerol solution. Electrocompetent bacteria was stored in 50 or 100µl aliquots under liquid nitrogen after snap freezing in liquid nitrogen. These electrocompetent
bacteria can be transformed at frequencies >10^8 transformed colonies per microgram of supercoiled plasmid DNA.

For transformation of recombined plasmids into the electrocompetent cells, the bacteria aliquots were transferred on ice directly from liquid nitrogen, and thawed slowly before use. 20μl of competent cells was transferred to a chilled disposable electroporation cuvette (Bio-Rad Laboratories Ltd) which 2μl of the plasmid DNA (~100pg) had been preloaded. Competent cells were mixed with the DNA with a gel-loading tip. The mixture was then pulsed using a Gene Pulser electroporator equipped with a Pulse Controller (Bio-Rad Laboratories Ltd) set to 2,500V, 200Ω, 25μF. This resulted in a time constant of about 5.0 to 5.3 ms. The pulsed bacteria were transferred to 100μl prewarmed LB without ampicillin and incubated at 37°C for 30 minutes before plating on LB-agar-ampicillin plates.

When cloning into vectors where the multiple cloning site formed part of the β-galactosidase gene, a blue/white colour screen could be used to pick colonies containing vectors with inserts. In this case each plate was spread with 100μl of 1.25% (w/v) X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and 10mM IPTG (Isopropyl β-D-thiogalactopyranoside) and incubated at 37°C for 30 minutes before use. The X-gal stock was 2.5% (w/v) in dimethyl formamide and was kept at -20°C in dark. The IPTG stock was 0.1M in H2O and was stored at 4°C. Colonies containing religated vector are blue under these conditions, whereas colonies containing vectors with inserts remain white.

2.2. Molecular biology

2.2.1. Extraction of DNA/RNA with phenol/chloroform/iso-amyl alcohol
The phenol stock was extracted with Tris-HCl pH 7.5 for DNA and pH 4.0 for RNA. The extracted phenol was stored in aliquots at -20°C and kept at 4°C for routine use. The chloroform stock was made by mixing chloroform with iso-amyl alcohol (24:1, v/v) and kept at 4°C in dark. The DNA/RNA samples dissolved in TE (10mM Tris-HCl pH 8, 1mM EDTA pH 8) or Diethyl Pyrocarbonate (DEPC)-treated water were extracted with an equal volume of phenol/chloroform. The sample was then vortexed and centrifuged at 13,000rpm for 5 minutes. The upper aqueous phase was collected, re-extracted with an equal volume of chloroform, and then vortexed, spun and collected as before.

2.2.2. Precipitation of DNA/RNA with ethanol or isopropanol

0.1 volume of 3M NaOAc pH 5.2 was added to the DNA/RNA samples and then 2.5 volumes of cold ethanol (or 0.6 volumes of isopropanol) was added. The samples were vortexed, kept on ice or at -20°C for 10 to 20 minutes and then centrifuged at 4°C, 13,000rpm for 10 minutes to precipitate the DNA/RNA. If necessary (i.e. less than 1-2 µg or a very low concentration of DNA), 10µg of glycogen (from muscles molecular biology grade, Boehringer Mannheim) was added to the DNA as a carrier before the precipitation. The DNA/RNA pellet was washed with 500µl 70% (v/v) ethanol, centrifuged briefly at 13,000rpm, air dried and resuspended in an appropriate volume of TE or DEPC-treated water.

2.2.3. Agarose gel electrophoresis of DNA

GTG agarose (SeaKem) was used for all agarose gel electrophoresis, at 0.8-2% (w/v) depending on the size of the DNA. The agarose was dissolved in the appropriate volume of 1xTAE (0.04M Tris-acetate pH 7.5, 1mM EDTA) buffer by boiling in a microwave oven and cooled to <60°C. Ethidium bromide was added into the agarose to a final concentration of 0.5µg/ml. The agarose was poured into a suitable size Horizon electrophoresis system (Life Technologies Ltd) and the gel was set at room temperature.
10x DNA loading buffer containing 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF and 15% (w/v) Ficoll (Type 400, Pharmacia) was added to the DNA samples. The gel was run at 5V/cm. The DNA was visualised on an ultraviolet (UV) transilluminator at 302nm and recorded using a CCD camera and thermal printer.

2.2.4. Gel purification of DNA

The Geneclean II kit (Bio 101) was used to purify DNA bands from agarose gel. The DNA bands were cut out of the gel and collected in an Eppendorf tube. Three volumes of 6M sodium iodide was added, assuming a gel density of 1g/ml. The gel containing DNA bands was incubated at 55°C for more than 5 minutes until the gel had melted, then chilled on ice. 5µl of the supplied silica matrix was then added. The suspension was vortexed and incubated on ice for 5 minutes. After pelleting in a microcentrifuge, the matrix with bound DNA was washed 3 times in the supplied wash buffer and resuspended in an appropriate volume of TE. The DNA was eluted from the matrix at 55°C for 5 minutes. Then the matrix was spun again and the DNA-containing supernatant was recovered.

2.2.5. Small scale preparation of plasmid DNA by alkaline lysis (miniprep)

Bacterial colonies grown on a LB-agar plate were picked using sterile Gilson pipette tips, which were then ejected into sterile 25ml Universal tubes containing 5ml LB with ampicillin. Cultures were grown at 37°C, 250 or 300rpm overnight in a rotary shaker. Then 1.5ml of culture was pelleted at 13,000rpm for 30 seconds in an Eppendorf tube. After removal of the supernatant, the bacterial pellet was resuspended in 100µl of ice-cold ‘solution I’ (50mM glucose, 25mM Tris-HCl pH 8, 10mM EDTA) and incubated on ice for 5 minutes. Then 200µl of freshly prepared ‘solution II’ (1% SDS, 0.2N NaOH) was added and mixed by several gentle inversions of the tube. The mixture was kept on ice for another 5 minutes. 150µl of ‘solution III’ (3M potassium acetate, 2M acetic acid) was then added and mixed by shaking, and the tube again was incubated on
ice for 5 minutes, and then spun at 13,000rpm for 5 minutes in a microfuge. The supernatant was carefully removed and treated with pancreatic RNase (final concentration of 20μg/ml) at 37°C for 10 minutes. Then the supernatant was extracted with phenol/chloroform (as described in 2.2.1) and the DNA was precipitated with ethanol (as described in 2.2.2). The DNA was then air-dried and resuspended in 50μl TE, from which 2-5μl can be used per 30μl restriction enzyme digestion to check the plasmid DNA.

2.2.6. Large-scale preparation of plasmid DNA by caesium chloride equilibrium centrifugation (maxiprep)

Single bacterial colonies were inoculated into 5ml LB with ampicillin and grown at 37°C, 300rpm in a rotary shaker for 4-8 hours or until in mid-logarithmic phase during daytime. The growing culture was then added to 250ml LB with ampicillin in a 1 liter flask and grown overnight at 37°C, 300rpm in a rotary shaker. The bacteria were chilled on ice, pelleted at 3,000rpm at 4°C (Beckman J6-MV centrifuge), washed by resuspension in 100ml ice-cold STE (0.1M NaCl, 10mM Tris-HCl pH 8, 1mM EDTA) and pelleted again by centrifugation. The bacterial pellet can be kept at -20°C at this step if the following process can’t be done on that day. The pellet was then resuspended in 7ml ‘Solution I’ containing 5mg/ml lysozyme and the suspension was transferred to a 50ml Falcon tube. Then 14ml freshly prepared ‘Solution II’ was added and mixed by gentle inversion. After incubation at room temperature for 10 minutes, 7ml ‘Solution III’ was added and mixed by inversion. After another 10 minute incubation on ice, the suspension was centrifuged at 4,000rpm (no braking) for 15 minutes and the supernatant was transferred to a 50ml Falcon tube. 0.6 volumes isopropanol was added and the solution was centrifuged at 4°C, 4,000rpm for 15 minutes. The pellet was resuspended in 6ml TE, then 2ml 10M ammonium acetate was added and the solution was incubated on ice for 20 minutes in order to precipitate proteins. This mixture was then centrifuged in a 15ml Corex tube at 4°C, 10,000rpm for 10 minutes (Beckman J2-MI centrifuge) and the supernatant was collected. 16ml cold ethanol was added to the
supernatant and after incubating on ice for 5 minutes the solution was centrifuged in a 30ml Corex tube at 10,000rpm for 10 minutes at 4°C. The pellet was resuspended in 3.6ml TE. 0.4ml of 3M sodium acetate was added and the pellet was precipitated with 8ml ethanol.

The resultant DNA pellet was dried thoroughly in a SpeedVac for a couple of minutes, resuspended in 1ml TE containing 1.05g caesium chloride The solution was transferred to a 3.9ml Quick-seal tube (Beckman). 80μl ethidium bromide solution (10mg/ml) was added with a gel-loading tip, then the tube was filled with ‘topping solution’ (50ml TE, 52.5g CsCl, 4ml ethidium bromide solution (10mg/ml), 0.22μm filtered before use) and sealed. The tube was centrifuged at 100,000rpm in a bench-top ultracentrifuge (Optima, Beckman) at 20°C for a minimum of 4 hours with no braking. The lower supercoiled plasmid DNA band (visible as a red band under normal lighting) was collected through the side of the tube using a 2ml syringe and 21-gauge needle. The ethidium bromide was removed by repeated extractions with equal volumes of water-saturated 1-butanol (10ml 1-butanol, 0.6ml dH2O) until the aqueous phase appeared completely clear. The final aqueous phase was diluted to 4 times the original band volume with TE, precipitated with 2 volumes of ethanol and centrifuged at 10,000rpm for 15 minutes at 4°C. The pellet was resuspended in 450μl TE, 50μl 3M sodium acetate and re-precipitated with ethanol. After 2 washes in 70% (v/v) ethanol, the plasmid DNA pellet was resuspended in 500μl TE. The concentration and purity of the plasmid DNA was measured at 260nm and 280nm (OD260, OD280). The concentration can be calculated as [OD260 x dilution factor x 50 (DNA multiplication factor)] μg/ml, and the purity assessed by the ratio OD260/OD280, which for pure DNA is 1.7.

2.2.7. Isolation of genomic DNA

Mice tail-tips were incubated at 55°C overnight in 600μl ‘digestion buffer’ (50mM Tris-HCl pH 8, 100mM EDTA, 100mM sodium chloride, 1% (w/v) SDS) containing 100μg/ml proteinase K (Sigma). 6M ammonium acetate was added to a final
concentration of 1.5M. The sample was chilled on ice and centrifuged at 13,000rpm to precipitate proteins. Genomic DNA in the supernatant was precipitated with 0.6 volumes of cold isopropanol and washed with 70% ethanol. The DNA pellet was dissolved in 100-200μl TE depending on the size of the pellet.

2.2.8. **Total RNA extraction**

Total tissue or cell RNA was prepared by an adapted guanidinium isothiocyanate method. Tissue was freshly dissected and homogenised in 2 volumes solution D [4M guanidinium isothiocyanate, 25mM sodium citrate pH 7, 0.5% (v/v) sarkosyl and 0.72% (v/v) β-mercaptoethanol (added just before use)], while cultured cells (100 mm dish) were scraped in 600μl solution D. 0.1 volumes of 2M sodium acetate pH 4, one volume of phenol and 0.2 volumes of chloroform/isoamyl alcohol (49:1 (v/v)) were then added, shaking the sample vigorously. The sample was then incubated on ice for 15 minutes. After centrifugation at 13,000rpm for 20 minutes, the upper aqueous phase was removed, mixed with an equal volume of isopropanol, and precipitated at -20°C for at least 1 hour. The sample was centrifuged for 20 minutes at 13,000rpm and the resulting pellet was resuspended in 300μl of solution D. This was again isopropanol precipitated, resuspended in a suitable volume of DEPC-treated water, and stored at -70°C.

2.2.9. **Random-primer labelling of DNA probes with [α-32P]dCTP**

Template DNA digested with the restriction enzyme was purified as described in 2.2.4. 50-100ng of this DNA was denatured to 95°C for 5 minutes in 34μl dH2O, chilled on ice for a few minutes. The following reagents were added in this order:

- 10μl 5x labelling buffer (250mM Tris-HCl pH 8, 25mM MgCl2, 10mM DTT,
  1M HEPES pH 6.6, 26 OD260 units/ml random hexadeoxyribonucleotides)
- 2μl 25x dNTPs (500μM each of dATP, dTTP, dGTP)
- 4μl [α-32P]dCTP (10μCi/μl, Amersham)
0.5μl Large (Klenow) fragment of DNA polymerase I (5 units, Promega)
The reaction was incubated at room temperature for 2-4 hours, then passed over a Micro Bio-Spin 30 column (BioRad) to remove unincorporated radionucleotides, denatured by heating to 95°C for 5 minutes before adding to the hybridization buffer.

2.2.10. 5' termini labelling of oligonucleotides probes with [γ-32P]ATP

50-100ng of oligonucleotides were mixed with the following reagents:

- 1μl 10xT4 polynucleotide kinase buffer (400mM Tris-HCl pH 7.5, 100mM MgCl₂ and 50mM DTT)
- 4μl [γ-32P]ATP (10μci/μl)
- 3μl dH₂O
- 1μl T4 polynucleotide kinase (10 units, Promega)

The reaction was incubated at 37°C for 45 minutes, then inactivated at 65°C for 10 minutes, filtered over a Micro Bio-Spin 25 column (BioRad) to remove unincorporated radionucleotides, denatured by heating to 95°C for 5 minutes before adding to the hybridization buffer.

2.2.11. Southern blot analysis of genomic DNA

Genomic DNA samples (10μg) were digested overnight with a suitable restriction enzyme and electrophoresed on a TAE-agarose gel. After staining with ethidium bromide, the gel was soaked for 20 minutes in 0.4N NaOH, and then vacuum-blotted for 1 hour in 0.4N NaOH onto Zetaprobe nylon membrane (BioRad). The blot was neutralised in 2xSSC/0.2M Tris-HCl pH 7.4 for 10 seconds, baked at 80°C for 30 minutes and pre-hybridised at 65°C for 30 minutes in hybridisation buffer (1.5xSSPE, 10% PEG-8000, 7% SDS, 100μg/ml denatured, sonicated salmon sperm DNA). The radiolabeled denatured probe was then added and hybridisation continued overnight at 65°C. The blot was then washed in 2xSSC, 0.1% SDS at room temperature for 10
minutes, then in 0.1xSSC, 0.1% SDS at 65°C for 5 to 30 minutes depending on the sensitivity of signals. The blot was then exposed to Hyperfilm-MP (Amersham) overnight at -70°C with intensifying screens.

2.2.12. Northern blot analysis of RNA

For 100ml agarose gel, 0.8-1.0g agarose was dissolved in 72ml dH₂O and 10ml 10x MOPS buffer (0.2M MOPS, 80mM sodium acetate and 10mM EDTA pH 8). The agarose gel was cooled to about 60°C, mixed with 18ml formaldehyde, poured into a suitable eletrophoresis system and set in the hood. RNA sample was diluted in 5 fold volume of sample buffer (2ml sample buffer contains 1ml deionised formamide, 360µl formaldehyde, 200µl 10xMOPS and 440µl H₂O) and denatured at 65°C for 5 minutes. RNA sample was then loaded with loading buffer (50% glycerol, 1mM EDTA, 0.4% bromophenol blue and 0.4% xylene cyanol). The gel was run in 60-80v in 1xMOPS and then vacuum-blotted for 1 hour in 10xSSC onto Zetaprobe nylon membrane (BioRad). The blot was washed in 2xSSC/0.2M Tris-HCl pH 7.4 for 10 seconds, baked at 80°C for 30 minutes. The rRNA can be viewed by staining with methylene blue (0.02% methylene blue and 0.3M NaOAc pH 5.5). The stained band can be washed out in 0.1xSSC and 1% SDS solution. The hybridization was followed as described in 2.2.11.

2.2.13. Avoidance of RNase contamination

Since the good analysis of RNA depends on the quality of prepared RNA, it is important that all solutions and equipment used are RNase free. This was achieved by baking all glassware at 200°C, using fresh, sterile plasticware, and treating all solutions with 0.1% (v/v) DEPC before autoclaving. Solutions which could not be DEPC-treated (e.g. Tris) were made up using fresh chemicals in DEPC-treated water. Gel tanks and equipment were soaked in 3% H₂O₂ for 30 minutes and then rinsed in DEPC-treated water before use.
2.2.14. Primer extension

10μg total RNA was mixed with 1ng radiolabeled primer and reprecipitated with ethanol in a siliconized Eppendorf tube. The RNA pellet was dissolved in 25μl annealing buffer (1μl 125mM Na$_2$PO$_4$, 1μl 125mM EDTA and 23μl DEPC-treated water), heated to 90°C for 10 minutes. Then 2.1μl 1.2M NaCl was added at 90°C. The mixture was cooled slowly to 42°C. The following reagents were added in this order:

- 2.5μl 1M Tris-HCl pH 8.3
- 2.5μl 0.1M DTT
- 3.8μl 0.1M MgCl$_2$
- 5μl 10mM dNTP
- 0.5μl 1.2M NaCl
- 7.6μl DEPC-treated water
- 1μl AMV reverse transcriptase (10 units, Promega)

The reaction was incubated at 37°C for 30 minutes, then added 0.2μl 500mM EDTA to terminate the reaction. The reaction product was precipitated with ethanol and dissolved in 4μl TE. This primer extension reaction was analyzed on a sequencing gel.

Sequencing gel was prepared using ready-to-use 6% sequencing gel solution (Sequagel-6, National Diagnostics). For example, for 100ml of gel solution the following reagents were mixed together:

- 80ml Sequagel-6 concentrate
- 20ml 5x Sequagel-6 buffer
- 800μl 10% (w/v) ammonium persulphate

This mixture was quickly poured into an assembled 0.8mm vertical gel apparatus (Model S2, Life Technologies Ltd) and allowed to polymerise for one hour. After denaturing at 85°C for 5 minutes, samples were loaded onto the gel with loading buffer and run at 1,200-1,500V in 1xTBE (0.045M Tris-borate, 1mM EDTA) running buffer. After running the gel for the required length of time, the gel plates were separated and
the gel was fixed by soaking in 10% (v/v) methanol/10% (v/v) acetic acid for 30 minutes. The gel was then dried onto 3MM chromatography paper (Whatman) on a commercial gel dryer (Model 583, Bio-Rad) at 80°C for 1 hour. The gel was then exposed to autoradiographic film (Hyperfilm-MP, Amersham) in a cassette for overnight to visualise radiolabelled nucleic acids.

The primers used for primer extension are listed below:

T3 (rat PDGFRα exon2, antisense)

\[
5'-GGT\ AAT\ AAG\ AGC\ TGG\ GAG\ ACG\ -3'
\]

T8 (rat PDGFRα exon2, antisense)

\[
5'-GGA\ GGT\ CCC\ CAT\ CGC\ TCC\ TGA\ G-3'
\]

2.2.15. Nuclease S1 protection assay

100μg total RNA was mixed with 10ng radiolabeled probe and precipitated with ethanol in a siliconized Eppendorf tube. The RNA pallet was dissolved in 30μl hybridization buffer (40mM PIPES pH 6.4, 1mM EDTA pH 8.0, 0.4M NaCl and 80% formamide) and incubated at 85°C for 10 minutes. Mineral oil was added to the top of the reaction, the RNA and probe were hybridized at 55°C overnight. Then 300μl ice cold nuclease-S1 reaction buffer (0.28M NaCl, 0.05M NaOAc pH 4.5, 4.5mM ZnSO₄, 20μg/ml ssDNA and 15-100 units nuclease-S1) was added to RNA sample quickly. The reaction was incubated at 30°C for 30 minutes and chilled on ice afterward. 80μl ice cold nuclease-S1 stopping buffer (4M NH₄OAc, 50mM EDTA pH 8.0 and 50μg/ml tRNA). The reaction product was extracted with phenol, precipitated with ethanol and dissolved in 4μl TE. The reaction product was analyzed on a sequencing gel (as described in 2.2.14).

The primers using by PCR to synthesize probes for nuclease S1 protection assay are listed below:

T6 (rat PDGFRα exon1, sense)
5'-GTT GGA GCT TGA GGG AGT GAA AC-3'

T9 (rat PDGFRα exon2, antisense)

5'-CAT TCT CAT TTG GAA GGA TGG-3'

T12 (part of pBluescript II SK T7 primer, sense)

5'-CGT AAT ACG ACT CAC TAT AGG-3'

The PCR condition was: 95°C for 5 minutes, 30 cycles of 95°C 30 seconds, 55°C 30 seconds and 72°C 1 minute and then 72°C for 10 minutes.

2.2.16. Polymerase chain reaction (PCR)

A successful PCR depends on the primer design and optimization of reaction conditions. There are several pointers for good primer design: 1) Primers generally range in length from 15-30 bases and should contain 40-60% G+C. 2) To prevent self-annealing that leads to primer-dimer formation, primers should not be complementary. 3) As T is the least discriminating nucleotide at the 3'-position, none of the primers should have a 3' T. 4) To prevent mismatch tolerance of primers with consecutive Gs or Cs, each primer should have at least one A or T within the 3' most triplet. 5. Ideally, both primers should anneal at the same temperature.

The annealing temperature is dependent upon the primer with the lowest melting temperature. The Mg^{2+} concentration is a crucial factor affecting the PCR. Normally a final concentration of 1.5mM MgCl_2 is used in the PCR with 10x PCR buffer (500mM KCl, 100mM Tris-HCl pH 8.3, 1% (v/v) Triton X-100) (Promega). For a 50µl amplification reaction, 1.25units of Taq DNA polymerase, 1µl dNTP (10mM of each dNTP in stock, 0.2mM of each dNTP in the final concentration), 50pmol of each primer and a suitable concentration of DNA template were mixed. A standard PCR amplification condition can be: 94°C for 5 minutes, 30 cycles of 94°C 30 seconds, 60°C 30 seconds and 72°C 1 minute and then 72°C for 10 minutes. Positive controls and negative controls (i.e. complete reaction mixtures lacking template DNA) were always
set up simultaneously. The resulting reactions were run on a 2% agarose gel in 1xTAE buffer to check the PCR products.

2.2.17. Preparation of digoxigenin (DIG) -labelled antisense RNA probes for in situ hybridization

10μg template DNA in 100μl reaction volume was linearised with the suitable restriction enzyme for 2 or 3 hours, then 5μl of 10% (w/v) SDS and 1μl Proteinase K (20mg/ml) was added and incubation continued at 55°C for 15 minutes. The linearised DNA was extracted with phenol/chloroform and precipitated with ethanol. After washing with 70% ethanol, the DNA pellet was resuspended in 50μl DEPC-treated dH₂O and stored at -20°C. In vitro transcription reactions were set up at 37°C for 2 hours in the following order:

1μg linearized template DNA
5μl 5x transcription buffer (200mM Tris-HCl pH 7.9, 30mM MgCl₂, 10mM spermidine and 50mM NaCl)
7.5μl 100mM DTT
1μl RNasin (ribonuclease inhibitor, 20 units; Promega),
2.5μl 10xDIG RNA labeling mix (10mM of ATP, GTP, CTP, 6.5mM UTP and 3.5mM DIG-11-UTP, pH 7.5; Boehringer Mannheim)
1μl RNA polymerase (T3, T7 or SP6, 20 units, Promega)
3μl DEPC-treated dH₂O

Then 55μl DEPC-treated dH₂O and 20μl 10M NH₄OAc were added to the reaction mixture. The synthesized RNA probes were precipitated with ethanol and then dissolved in 100μl of 10mM DTT. 2μl of probes was checked to run on a 1% agarose gel in DEPC-treated 1xTAE buffer in order to confirm full length transcription. The remaining probes were stored at -70°C until use. The optimal dilution of each probe was determined by titration of the probe on control sections to achieve the maximum signal to noise ratio, and was usually between 1:500 and 1:1000.
The preparation of antisense RNA probes used in this thesis is listed in table 2.1.

2.3. Cell Biology

2.3.1. Cell culture of B104 neuroblastoma cells

B104 neuroblastoma cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% heat-inactivated fetal calf serum (FCS; Gibco-BRL) at 37°C in 5% CO₂/95% air and at 100% humidity. When cells were reaching confluence, cells were washed twice with 5ml Earle’s balanced salt solution (EBSS; Gibco-BRL), treated with trypsin at a 1:10 dilution, transferred to a 15ml Falcon tube, washed with some DMEM/10%FCS and precipitated at 1,200rpm for 5 minutes. Cell pellet was resuspended and split into 10-20 dishes (100mm) and then cultured in DMEM/10% FCS. When cells were >90% confluent, they were washed twice with 10ml EBSS and grown in 10ml N1 medium [200ml of DMEM with 1ml of N1 supplement (1.0mg/ml Transferrin, 3.2mg/ml Putrescine, 1.26μg/ml Progesterone and 1.04μg/ml Sodium Selenite) and 0.4ml of 2.5mg/ml insulin] for three days. This medium (called B104 conditional medium) was collected in 50ml Falcon tubes, spun at 2,000rpm for 5 minutes, filtered through a 0.8μm filter and stored at -20°C.

2.3.2. Cell culture of CG4 cells

100mm dishes were coated with 0.1mg/ml Poly-L-Ornithine [To prepare 1mg/ml stock, 100mg of Poly-L-Ornithine (Sigma) was dissolved in 100ml Boric Acid buffer (0.15M Boric Acid and 0.15M NaOH) and filtered through a 0.22μm filter] at room temperature for 60 minutes and washed twice with sterile water. Before use, dishes were preincubated in 8ml DMEM at 37°C. CG4 cells were plated out onto Poly-L-Ornithine coated dishes in 70% N1 medium (as described in 2.3.1) and 30% B104 conditional medium (as described in 2.3.1) in 10ml. The medium was changed every other day. To keep the CG4 cell line, cultured cells were washed twice with EBSS, trypsinized in
EBSS (as described in 2.3.1), spun with DMEM, resuspended in 1ml freezing medium (70% DMEM, 20% heat-inactivated FCS and 10% DMSO), frozen down at -70°C and then stored in liquid nitrogen. When culturing these cells, the frozen cells were thawed at 37°C rapidly, then transferred to a 15ml Falcon tube, spun with DMEM at 1,000rpm for 5 minutes and resuspended in 10ml of culture medium.

2.3.3. Transfection of CG4 cells by electroporation

Cultured CG4 cells were trypsinized in EBSS, washed once in Optimem and resuspended in 250μl Optimem (approximately 2.5x10⁶ cells per transfection). This cell suspension was mixed with 5μg plasmid DNA containing β-galactosidase reporter gene and transferred to a 0.4cm electroporation cuvette (Bio-Rad Laboratories Ltd). The mixture was then pulsed using a Gene Pulser electroporator equipped with a Pulse Controller (Bio-Rad Laboratories Ltd) set to 300V, ~Ω, 500μF. This resulted in a time constant of about 9 to 11 ms. Transfected cells were transferred to a 100mm dish and grown in the culture medium. The activity of β-galactosidase reporter gene was analyzed using a Galacto-light plus™ kit (Tropix) and detected in a luminometer (Anthos Labtec Instruments).

2.3.4. Spinal cord cell cultures

Embryos were removed from timed-mated females. The spinal cords were dissected away from surrounding tissue in Hepes-buffered minimal essential medium (MEMH) (ICN) and the meningeal membranes removed with tungsten needles. The tissue was transferred to 2 ml EBSS containing 0.0125% (w/v) trypsin (Boehringer Mannheim) and incubated at 37°C in 5% CO₂ for 30 minutes. The tissue was washed in DMEM containing 10% FCS to inhibit the trypsin, then transferred to fresh DMEM containing 10% FCS and 0.005% (w/v) DNase-I (Sigma). The tissue was immediately dissociated by gentle trituration with a Pasteur pipette. The resulting cell suspension was filtered through a 20 μm pore-diameter mesh and washed by centrifugation and resuspension in
DMEM containing 10% FCS. The number of live cells in a sample of the suspension was counted in a hemocytometer. Cells were then plated on poly-D-lysine-coated 13 mm diameter glass coverslips in a 50 µl droplet. The cells were allowed to attach for 30 minutes at 37°C. 350 µl of modified Bottenstein and Sato’s (1979) medium was added with 10 ng/ml PDGF-AA (Peprotech), and incubation continued at 37°C in 5% CO₂. Medium was changed every two days.

2.3.5. Immunolabelling of primary spinal cord cultures

Cells on coverslips were lightly fixed in 2% (w/v) paraformaldehyde in Phosphate-buffered saline (PBS, containing 8g NaCl, 0.2g KCl, 0.1g MgCl₂, 1.15g Na₂HPO₄ and 0.2g KH₂PO₄ per liter) for 5 minutes at room temperature. The coverslips were washed three times in PBS, incubated in monoclonal anti-galactocerebroside (GC) antibodies to visualize oligodendrocytes, which reacts with an unidentified antigen in addition to GC on the surface of oligodendrocytes for 30 minutes in a humid chamber at room temperature, and rinsed three times in PBS. The cells were then incubated in rhodamine-conjugated goat anti-mouse IgG (Pierce) diluted 1:100 in PBS for 30 minutes, post-fixed in 4% (w/v) paraformaldehyde in PBS and mounted under Citifluor (City University, UK). Labelled cells were viewed and photographed using a video microscope.

2.3.6. Spinal cord explant cultures

Embryos were placed in dispase (Boehringer; 1mg/ml) in DMEM and the spinal cord dissected free of surrounding tissues using tungsten needles. Individual spinal cords were further dissected into transverse segments approximately 0.1-0.2mm in length and cultured in three dimensional collagen gels as previously described (Tucker et al., 1996) in Bottenstein and Sato’s medium with 0.25% FCS. Collagen gels containing explants were cultured in 60mm-diameter plastic dishes with 4ml of medium on a rocking platform (6 cycles/minute) such that the gels were exposed above the surface of the culture medium for a quarter of each cycle. Half the culture medium was exchanged
every other day. Some gels were cultured for 48 hours before removing and immunolabeling the explants with anti-Isl1/2 antibodies to visualize motor neurons. The remainders were cultured for 11 days, until the equivalent of the day of birth, before staining with antibodies against the oligodendrocyte markers GC or myelin basic protein (MBP).

2.3.7. Immunolabelling of spinal cord explant cultures

Collagen gels containing spinal cord explants were removed and fixed in 4% (w/v) paraformaldehyde in PBS for 2 hours at room temperature, then washed 4 times for 15 minutes with PBS. Motor neurons were labeled with monoclonal antibody 4D5 (a gift from T. Jessell, Columbia University) that recognises both Isl1 and Isl2. The hybridoma supernatant was diluted 1:100 in PBS containing 0.1% (v/v) Triton X-100. Oligodendrocytes were labeled with monoclonal anti-GC antibodies or with anti-MBP rabbit serum (a gift from D. Colman, Mount Sinai School of Medicine, New York), diluted 1:1000 in PBS containing 0.1% (v/v) Triton X-100. All primary antibody incubations were overnight at 4°C followed by 4x15-minute washes with PBS. Secondary antibodies were rhodamine- or fluorescein-conjugated goat anti-rabbit or goat anti-mouse IgG (all from Pierce). Incubation was for 3 hours at room temperature followed by 4x15-minute washes in PBS before mounting in glycerol for fluorescence microscopy.

2.3.8. Preparation of APES-coated slides

Pre-washed microscope slides were coated with 2% (v/v) APES (3-aminopropyltriethoxysilane; Sigma) in acetone for 30 seconds, washed in 9% (v/v) industrial methylated spirits (IMS) for 30 seconds, washed in distilled water for 30 seconds, air dried under sterile conditions, baked at 200°C for 4 hours, and stored at room temperature for up to 3 months.
2.3.9. Preparation of tissue sections

Embryos were fixed by immersion in 4% (w/v) paraformaldehyde in PBS for 24 hours at 4°C. Embryos aged E14.5 and older were decapitated and their skin removed before immersion in fixative. After cryoprotecting in 0.5M sucrose in PBS for 24 hours at 4°C, embryos were immersed in OCT embedding compound (BDH), frozen on dry ice and stored at -70°C until required for sectioning. Frozen sections were cut (10- to 15-μm nominal thickness) on a cryostat and collected on 3-APES-coated glass microscope slides. Sections were air dried for 1-2 hours at room temperature and stored at -70°C in a dry box.

2.3.10. In situ hybridisation using digoxygenin (DIG) -labelled RNA probes

DIG-labelled antisense RNA probes were made as described in 2.2.17. These probes were diluted in hybridisation buffer (1x salts, 50% deionised formamide, 10% dextran sulphate, 1x Denhardt’s solution and 1mg/ml yeast RNA. 1xsalts was prepared from 10xsalts stock: 2M NaCl, 100mM Tris-HCl pH 7.2, 50mM NaH_2PO_4, 50mM Na_2HPO_4 and 50mM EDTA), denatured at 75°C for 5 minutes and 90μl of diluted probe was placed on each slide. The slides were coverslipped and hybridised overnight at 65°C in a sealed perspex box with 2 sheets of Whatman paper wetted with 1xsalts and 50% formamide. After hybridisation, slides were incubated in wash buffer (1xSSC, 50% formamide, 0.1% Tween-20) at 65°C in coplin jars for at least 45 minutes to let the cover slips fall off, then washed twice further in wash buffer at 65°C for 30 minutes each. The slides were then incubated 2x30 minutes at room temperature in MABT (100mM maleic acid pH 7.5, 150mM NaCl, 0.1% Tween-20). To label anti-DIG antibodies on sections, the slides were dried off around the sections using tissue paper, the sections circled with a Pap Pen (Agar Scientific). The slides were then transferred to a humidified chamber and incubated in blocking solution (MABT containing 2% blocking reagent (Boehringer Mannheim), 10% heat-inactivated sheep serum) for at least 1 hour at room temperature without a coverslip. The blocking solution was then
replaced with alkaline phosphatase-conjugated anti-digoxigenin antibody (Fab fragments; Boehringer Mannheim) diluted 1:1500 in blocking solution, and the incubation continued overnight at 4°C. To visualize hybrids, the slides were transferred to coplin jars and washed 5x20 minutes in MABT; then 2x10 minutes in freshly made prestaining buffer (100mM Tris-HCl pH 9, 100mM NaCl, 5mM MgCl₂). The prestaining buffer was then replaced with 30ml staining buffer (100mM Tris-HCl pH 9, 100mM NaCl, 5mM MgCl₂, 5% polyvinyl alcohol (Mw 70,000-100,000, Sigma) containing 50μl 5-bromo-4-chloro-3-indoly-l-phosphate (BCIP, Boehringer Mannheim) and 65μl nitroblue tetrazolium salt (NBT, Boehringer Mannheim) and incubated in the dark at 37°C until the signal reached a satisfactory intensity (usually 4 hours to overnight). The slides were then washed in distilled water for 30 minutes, dehydrated in increasing concentrations of ethanol (30, 60, 80, 95 and 100% for one minute each), cleared in xylene and mounted under cover slips in XAM mountant (BDH).

2.3.11. Combined BrdU immunolabeling and in situ hybridization

Pregnant female mice were injected intra-peritoneally with 50μg 5-bromo-2'-deoxyuridine (BrdU, 10mg/ml in PBS; Boehringer Mannheim) per gram body weight. Animals were sacrificed two hours after injection and embryos were fixed, sectioned and subjected to in situ hybridization (as described in 2.3.10). The in situ hybridization reaction was stopped by washing in PBS for 10 minutes at room temperature. After brief fixation in 4% (w/v) paraformaldehyde in PBS, the sections were fixed in 70% (v/v) ethanol for 20 minutes at -20°C, then treated at room temperature with 1% (v/v) Triton X-100 for 20 minutes, 6M HCl in 1% (v/v) Triton X-100 for 15 minutes, 0.1M Na₂B₄O₇ (pH 8.5) for 10 minutes and 50% normal goat serum in 1% Triton X-100 for 15 minutes. The sections were then incubated overnight at 4°C in anti-BrdU antibody (monoclonal BU209; Magaud et al., 1989) followed by Texas Red-conjugated goat anti-mouse IgG (Sigma). After a final post-fixation in 4% paraformaldehyde in PBS for 5 minutes at room temperature, the sections were mounted in Citifluor (City University, London) for microscopy. In situ hybridization images were photographed under bright-
field illumination and BrdU-labeling under fluorescence illumination. Both images were scanned and superimposed using Adobe Photoshop software and printed using a color inkjet printer.

2.3.12. Mapping PDGFRα⁺ oligodendrocyte progenitors in the dorsal-ventral axis

The site of origin of oligodendrocyte progenitor cells in the neuroepithelium was estimated from the positions of PDGFRα⁺ cells within the ventricular zone. For each cell, a perpendicular was dropped from the cell body to the ventricular surface, and the distance of the intercept from the ventral midline was measured, following the contour of the central canal. This was expressed as a proportion of the half-perimeter of the canal, to minimize the effects of distortions resulting from variable preparation procedures such as fixation, and slight variations in the angle of section relative to the longitudinal axis.

2.3.13. Whole mount in situ hybridization of mouse embryos

Whole mount in situ hybridization was performed as described by Wilkinson (1997). Embryos were dissected out in DEPC-treated PBS (Any cavities were opened to avoid the trapping of reagents.), fixed in 4% paraformaldehyde in PBS at 4°C overnight, then washed twice for 5 minutes each in PTW (PBS with 0.1% Tween-20 made in DEPC-treated H₂O) at 4°C, washed for 5 minutes each with 1:3, 1:1 and then 3:1 methanol/PTW, then twice with 100% methanol. Embryos can be kept in this methanol at -20°C for several months.

For hybridization, embryos were treated with 10g/ml proteinase K in PTW at room temperature for 5 to 15 minutes depending on the size of embryos, washed twice for 5 minutes each in PTW, refixed with 4% paraformaldehyde in PTW for 20 minutes, washed twice for 5 minutes each with PTW, then incubated in 1ml prehybridization buffer (50% formamide, 5xSSC pH 5, 50g/ml yeast RNA, 1% SDS and 50g/ml heparin)
at 65°C for 3 to 8 hours, replaced the prehybridization solution with 0.4ml hybridization buffer (the same as the prehybridization buffer) mixed with digoxigenin-labeled RNA probe and incubated at 65°C overnight. The embryos were then washed twice for 30 minutes each with solution 1 (50% formamide, 5xSSC pH 5 and 1% SDS) at 65°C, twice for 30 minutes each with solution 2 (50% formamide, 2xSSC pH 5) at 60°C, three times for 5 minutes each with TBST (10x TBST stock: 1.4M NaCl, 27mM KCl, 0.25M Tris-HCl pH7.5 and 1% Tween-20) at room temperature. The embryos were preblocked with 20% sheep serum in TBST for 60 to 90 minutes, rocked in 20% sheep serum in TBST with alkaline phosphatase-conjugated anti-digoxigenin antibody (Fab fragments; Boehringer Mannheim) in 1:1500 dilution at 4°C overnight, and then washed three times for 5 minutes each, then five times for 1 hour each with TBST. To visualize hybrids, the embryos were washed three times for 10 minutes each with fresh NTMT (100mM NaCl, 100mM Tris-HCl pH 9.5, 50mM MgCl₂ and 0.1% Tween-20), incubated with NTMT including 4.5μl NBT and 3.5μl BCIP per ml and rocked for 1 hour to overnight in the dark until the signal reached a satisfactory intensity. The embryos were washed twice for 10 minutes each with TBST, for 10 minutes with 100% methanol, then for 5 minutes each with 3:1, 1:1 and 1:3 methanol/PBT and then twice with PBT and kept in 2% paraformaldehyde in PBT at 4°C.

2.3.14. Skeletal preparations

Embryos were fixed and stained in 20% acetic/80% ethanol containing 0.015% alcian blue for 24 hours. Following dehydration in ethanol for 24 hours, embryos were cleared in 1% KOH for 12 hours and stained in 50mg/ml alizarin red in 1% KOH for 3 hours. Embryos were further cleared in a 4:1 mixture of 1% KOH and glycerol over several days until the embryos had equilibrated with the solution. Embryos were then transferred to solutions of 1:1 then 1:4 mixture of 1% KOH and glycerol, respectively, over a period of 2-3 weeks.
2.4. Yeast Artificial Chromosomes (YACs) and production of YAC transgenic mice

2.4.1. Yeast strains, media and storage

Four *Saccharomyces cerevisiae* strains, CEPH 449C2, CEPH 29E11 (Spritz et al., 1994), YLBW2 and YLBW3 (Hamer et al., 1995) were used. Yeasts were grown at 30°C in liquid media listed below (All were prepared in one liter.) or on agar plates containing liquid media with 20g/L bacto-agar. Ampicillin was added to the liquid or molten solid media (after cooling to 55°C) at a final concentration of 50-100µg/ml (100mg/ml stock in H₂O, 0.22µm filter-sterilised and stored in aliquots at -20°C). Liquid cultures were continually agitated in a rotating environmental shaker at 200-250 rpm at 30°C.

**YPD Medium:**
- 10g Bacto-yeast extract (Difco)
- 20g Bacto-peptone (Difco)
- 20g D-glucose

**YCD Medium:**
- 20g D-glucose
- 6.7g yeast nitrogen base without amino acids (Difco)
- 10g Casein hydrolysate, acid (Sigma)
- 570mg nutrients (50mg Lysine, 20mg Histidine, 40mg Arginine, 60mg Isoleucine, 60mg Leucine, 20mg Methionine, 50mg Phenylalanine, 200mg Threonine, 50mg Tyrosine and 20mg Adenine; Sigma)

For short term storage of yeast strains and clones, they were stored on agar plates at 4°C for two or three weeks. For long term storage, 75% glycerol was added to cultures to a final concentration of 15%. This mixture was stored in 1ml aliquots at -20°C or -80°C if necessary, for a few years.

2.4.2. Transfer of YACs into Window Strains
CEPH 449C2 and CEPH 29E11 YACs were transferred from their original host into an appropriate window strain using the Kar1-transfer procedure (Hugerat et al., 1994; Spencer et al., 1994). The mating type of 449C2 and 29E11 was MATα while the mating type of YLB W2 and YLB W3 was MATα. YAC 449C2, 29E11 and window strain YLB W2, YLB W3 were grown up seperately in 10ml YPD medium at 30°C, 200 rpm overnight. 5x10⁶ cells of each YAC strain and window strain were required for the following combination. YAC 449C2 and YLB W2 were mixed while YAC 29E11 and YLB W3 were mixed, and then collected at 13,000 rpm for 30 seconds. Pellets were resuspended in 1ml fresh YPD medium and cultured at 30°C, 200 rpm for 6 to 8 hours. These mating mixtures were plated in 0.1ml, 0.3ml and 0.5ml aliquots on YCD medium containing 3mg/L of cycloheximide (Cyh) and incubated at 30°C for 3-4 days. Positive colonies were picked for mating type analysis.

2.4.3. Rapid assessment of yeast mating type by PCR

The mating type of YACs transferred into window strains can be haploid and a/α diploid (Huxley et al., 1990). Primers present at the MAT locus were used to distinguish the mating type by PCR. MAT-1 (5'-AGT CAC ATC AAG ATC GTT TAT GG-3') corresponds to a sequence at the right of and directed towards the MAT locus. MAT-2 (5'-GCA CGG AAT ATG GGA CTA CTT CG-3') corresponds to a sequence within the α-specific DNA located at MATα. MAT-3 (5'-ACT CCA CTT CAA GTA AGA GTT TG-3') corresponds to a sequence within the a-specific DNA located at MATα. Haploid yeast generated a 404bp product (MATα) or 544bp product (MATα) while diploid yeast generated both.

Positive colony was picked by a yellow tip and transferred to 50µl H₂O in an Eppendorf tube. 1.5µl of 1:20 diluted β-mercaptoethonal was added. The yeast mixture was vortexed briefly and boiled for 5 minutes, incubated on ice for 1 minute, spun at 13,000 rpm for 2 minutes. The supernatant was transferred to a new tube. 5ul of
supernatant was used in a 25μl PCR reaction, containing 2.5μl 10x PCR reaction buffer (100mM Tris-HCl pH 8.3, 500mM KCl, 2.5mM MgCl₂), 0.2mM of each dNTP, 100ng MAT-1, MAT-2 and MAT-3 primers and 1 unit of Taq DNA polymerase (Promega). The reaction was under this condition: 94°C for 3 minutes, 30 cycles of 92°C 30 seconds, 58°C 60 seconds and 72°C 60 seconds, and then 72°C for 10 minutes. The PCR products were run on a 2% agarose gel and visualized by ethidium bromide staining.

2.4.4. Preparation of plugs containing YAC DNA from 50ml yeast cultures

Yeast colonies were picked by yellow tips to grow in 5ml YCD medium at 30°C, 200 rpm during daytime. This 5ml culture was transferred to 50ml YCD medium to grow at 30°C, 250 rpm for 2 days. The yeast culture was then spun at 3,000 rpm for 5 minutes. The pellet was washed in 15ml of 50mM EDTA (pH 8.0) twice, resuspended in 1.5ml of 50mM EDTA, mixed gently with cut off blue tips with 0.5ml SCE (1M sorbitol, 0.1M sodium citrate, 60mM EDTA), 2.5μl β-mercaptoethanol and 1mg zymolyase-100T (ICN) and 2.5ml of 1% low melting point (LMP) agarose (SeaPlaque, FMC) dissolved in 0.125M EDTA. 100μl aliquots of the mixture were pipetted using a cut off yellow tip into plug formers kept on ice, leaving for 10 minutes to allow the agarose to set to form plugs. The plugs were then transferred into 25ml of 0.45M EDTA, 10mM Tris-HCl (pH 8.0) and 7.5% β-mercaptoethanol, incubated at 37°C overnight, removed from the above solution and replaced with 0.5M EDTA, 1% N-lauroylsarcosirate and 1mg/ml Proteinase K, continually incubated at 50°C overnight and repeated it once, finally stored at 4°C in 0.5M EDTA, 1% N-lauroylsarcosirate and 10mM Tris-HCl (pH 9.5). These plugs can be used for Southern blots and as markers for YAC DNA purification.

2.4.5. Analysis of YAC DNA by Southern Blot
YACs were analyzed by Pulsed-field gel electrophoresis (PFGE) in 1% agarose gels in 0.25x TAE buffer. Plugs containing YAC DNA (as described in 2.4.4) were loaded into the wells in the gel and sealed with 1% LMP agarose in 0.25x TAE. CHEF (Clamped Homogeneous Electric Fields) gels were run at 200V for 30 hours with a 30 seconds switch time in a Pulsed Field Electrophoresis System (Bio-Rad). After the electrophoresis, the gel was stained with 1.0μg/μl ethidium bromide for 30 minutes with constant agitation, photographed using very short exposure to minimize exposure to UV radiation, then irradiated under UV for 10 seconds with a 254 nm light source, soaked in 0.4N NaOH for 15 minutes, and then vacuum blotted for 4 hours onto Zetaprobe nylon membrane (BioRad) in 0.4N NaOH by using a commercial vacuum blotter. The blot was neutralized briefly in 2xSSC, dried by blotting onto 3MM paper and baked for 30 minutes at 80°C. The following hybridization was described in 2.2.11.

2.4.6. Checking the integrity of YAC DNA by PCR

To check the integrity of YAC DNA, five pairs of primers were designed (see below). Two pairs of primers (YAC L1, YAC L2 and YAC R1, YAC R2) were used to detect the YAC vector arms. The PCR reaction condition was as following: 94°C for 3 minutes, 35 cycles of 92°C 30 seconds, 62°C 45 seconds and 72°C 45 seconds, then 72°C for 10 minutes. Another three pairs of primers specific for human PDGFRα exon 1 (H1 and H2), exon 20 (E20F and E20R) and 3'-untranslated region (3'-UTR) (H3 and H4) were used to detect the human PDGFRα gene in the YAC. The PCR reaction was under this condition: 94°C for 5 minutes, 33 cycles of 94°C 30 seconds, 53°C 45 seconds and 72°C 1 minute, then 72°C for 10 minutes.

YAC L1: 5'-CAC CCG TTC TCG GAG CAC TGT CCG ACC GC-3'
YAC L2: 5'-CCT TAA ACC AAC TTG GCT ACC GAG A-3'
YAC R1: 5'-ATA TAG GCG CCA GCA ACC GCA CCT GTG GCG-3'
YAC R2: 5'-GTA ATC TTG AGA TCG GGC GTT CGA-3'
H1: 5'-CTG GAC ACT GGG AGA TTC GGA G-3'
2.4.7. Preparation of high density yeast plugs containing YAC DNA for microinjection

High density yeast plugs were prepared as described by Schedle et al. (1996) and Huxley (1998). Single yeast colony carrying YAC DNA was picked and grown in 5 ml YCD medium during the day time. This 5 ml culture was inoculated into 500 ml YCD medium in a 2 liter flask and cultured at 30°C, 250 rpm for about 3 days to get 2-4x10^7 cells per ml culture. The yeast culture was spun at 4°C, 4,000 rpm for 5 minutes. The pellet was resuspended in 50 ml SE buffer (1M sorbitol, 20 mM EDTA pH 8.0) and then washed twice in SE buffer. After the last wash, the supernatant was carefully removed with a paper tower and the pellet was resuspended in 200 µl new SE buffer with a cut-off yellow tip. 0.5ml of aliquots of the cell suspension were transferred to 1.5 ml Eppendorf tubes and incubated at 37°C. 1% LMP agarose was dissolved in SE buffer containing 14mM β-mercaptoethanol and incubated at 42°C. Just before use, 10mg Zymolyase-100T (ICN Biomedicals Inc.) was dissolved in 2ml of the LMP agarose solution. 0.5ml of this solution was transferred into the 0.5ml yeast cell suspension and mixed thoroughly by pipetting up and down using a cut off blue tip. The mixture was incubated at 42°C at all times to avoid setting of the agarose. 100µl aliquots of the mixture were pipetted using a cut off yellow tip into plug formers kept on ice, leaving for 10 minutes to allow the agarose to set to form plugs. The set plugs were transferred to SE buffer containing 14mM β-mercaptoethanol and 1mg/ml Zymolyase, incubated at 37°C overnight, removed from the above solution and replaced with the new buffer containing 0.2M EDTA pH 8.0, 0.1M Tris-HCl pH 8.0, 0.5M NaCl, 0.1% SDS, 0.5M Na2EDTA, 0.1M Tris-HCl pH 8.0, 0.1% SDS, 0.5M
β-mercaptoethanol and 1mg/ml proteinase K under the volume of at least 0.5ml per plug. The plugs were then incubated in this buffer at 37°C overnight, washed in TE (pH 8.0) until no more bubbles can be seen, stored in 0.5M EDTA at 4°C until use.

2.4.8. Isolation of intact YAC DNA for microinjection

Intact YAC DNA was isolated by the two-step gel isolation procedure (Fig. 2.1). A 1% agarose gel was prepared in 0.25xTAE buffer by using a comb which several teeth have been sealed with a tape to obtain a preparative lane of approximately 5cm. The high density plugs (as described in 2.4.7) were washed for 4x15 minutes in TE (pH 8.0) with gentle shaking on a rocking platform at 4°C, then loaded next to one another into the preparative lane and sealed with 1% LMP agarose dissolved in 0.25xTAE buffer. Marker plugs were loaded parallelly to the high density plugs. The PFGE was run in a cool 0.25xTAE buffer at 200v for 30 hours with a 30 seconds switch time.

After the gel run, marker lanes were cut off on either side of the high density plugs including about 0.5cm of the preparative lane and stained in 0.25xTAE buffer containing 0.5μg/ml ethidium bromide (Fig. 2.1A). The positions of the YAC DNA band and endogenous yeast chromosomes indicated as marker 1 and marker 2 (above and below the YAC DNA bands) were marked under UV light using a scapel blade. The gel was reassembled. The part of preparative lane containing the YAC DNA, marker 1 and marker 2 was excised (Fig. 2.1A). The gel was then positioned on a minigel tray with the YAC DNA band in the middle. A 4% LMP agarose gel in 0.25xTAE buffer was casted around it. The minigel was run at a 90° angle to the PFGE run overnight at 4v/cm in 0.25xTAE circulating buffer (Fig. 2.1B). The two marker lanes above and below the YAC DNA were cut off and stained in ethidium bromide to localize the DNA (Fig. 2.1C). The concentrated YAC DNA from the corresponding position of the YAC DNA lane was excised and equilibrated on a rocking platform in 20ml TENPA buffer (10mM Tris-HCl pH 7.5, 1mM EDTA pH 8.0, 100mM NaCl, 30μM spermine and 70μM spermidine) for at least 1.5 hours. The gel slice was dried with a clean tissue, transferred
into a 1.5ml Eppendorf tube, melted for 3 minutes at 68°C, centrifuged for 10 seconds to bring down all of the molten agarose and incubated for an additional 5 minutes at 68°C. The Eppendorf tube was then transferred to 42°C for 5 minutes. 2 units of b-agarase (New England BioLabs) per 0.1ml of molten gel slice was added to the tube and incubated for further 3 hours at 42°C. The resulting DNA solution was dialysed for one hour on a floating dialysis membrane (Millipore, pore size 0.025μM) against microinjection buffer (10mM Tris-HCl pH 7.5, 0.1mM EDTA pH 8.0, 100mM NaCl, 30μM spermine and 70μM spermidine). The DNA concentration was checked by running 2μl of purified YAC DNA on a thin 0.8% agarose gel with very small slots. 1 DNA of known concentration was used as a standard. The integrity of the DNA was checked by running 20μl of the purified DNA on a PFGE gel.

2.4.9. Preparation of pipettes and electrodes for microinjection

To prepare handling pipettes, 1.0 mm diameter thin walled glass tubings (Clark GC100-10) were pulled over Bunsen flame, scored in middle of constriction with a diamond-point pencil and broken. Those with 150-180μm internal diameter were selected and polished lightly in the edge of flame.

Holding pipettes were made from 1.0mm diameter thin walled glass tubings (Clark GC100-10). The tubing was pulled over Bunsen flame, scored in middle of constriction with a diamond-point pencil and broken. Those with 100-120μm external diameter were selected, polished carefully using a microforge (Micro Instruments LTD) to constrict end to 15μm internal diameter and stored horizontally to protect pipette end.

To prepare oviduct transfer pipettes, 1.0mm diameter thin walled glass tubings (Clark GC100-10) were pulled over Bunsen flame, scored in middle of constriction with a diamond-point pencil and broken. Those with 120-150μm internal diameter were selected and polished lightly in the edge of flame.
Injection pipettes were made from 1.0mm thin walled glass capillary tubings with inner filament (Clark GC100TF-10). The glass capillary tubing was pulled with a micropipette puller (Model 773, Campden Instruments). The pulling program was: stage1: Heat intensity (40), Heating time (10 seconds), Pulling force (0), Pulling displacement (0); and stage2: Heat intensity (0), Heating time (0), Pulling force (75), Pulling displacement (24). The injection pipettes were stored horizontally to protect pipette end.

2.4.10. Production of YAC transgenic mice by microinjection

Oviducts were dissected from 4-6 superovulated plugged mice (matings from C57BL/6J x CBA/J). Try to avoid dissecting any uterus or ovary and avoid touching oviduct itself. The dissected oviducts were transferred to M2 medium (Sigma) in 35mm dish. Swollen ampulla was identified under microscope and a small nick was made on it with sharp forceps to release fertilised eggs which were in a single cluster accompanied by cumulus cells (Per oviduct normally yields 15 eggs.). Eggs were transferred with a hindling pipette to 3ml fresh M2 medium containing 50μl hyaluronidase (100mg/ml) and kept at room temperature for 10 minutes. Individual eggs were transferred with a handling pipette to fresh M2 medium and then to 20μl M16 (Sigma) drops covered with mineral oil. 20-30 eggs were kept in one M16 drop and incubated at 37°C in 5% CO₂/95% air and at 100% humidity.

Depression slide was cleaned and set up under a microscope. A big drop of M2 medium was load on the microwell on the slide to make an injection chamber. A holding pipette was positioned using the micromanipulator (Leitz) on the bottom of the microwell to the left of the field. 20-30 eggs were transferred from the incubator to the bottom of the field under the holding pipette. An injection pipette was filled with purified YAC DNA and placed in the injection holder of the micromanipulator (Leitz) and adjusted on the bottom of the well. The tip of the injection pipette was broken on the holding pipette.
The injection was proceeded in a standard way (Hogan et al., 1994) using a microinjector (IM300, Narishige). Injected eggs were transferred to fresh M2 medium, healthy eggs were selected and transferred to fresh M16 drop and stored at 37°C in the incubator.

Recipient pseudopregnant female mouse was anaesthetized with hypnorm/hypnovel cocktail for 20 to 40 minutes. The hypnorm/hypnovel (Janssen) cocktail was prepared with mixing 1 part hypnorm, 1 part hypnovel and 2 parts ddH₂O (Phoenix Pharmaceuticals LTD). The female mouse was injected intra-peritoneally with 120-140µl of this cocktail per 30 grams body weight. Injected eggs were transferred to fresh M2 medium. The oviduct transfer pipette was filled with M2 medium, then an air bubble, then injected 20-30 eggs in M2 medium, then another air bubble and then M2 medium. The ovary was released from the back of the mouse and the oocytes were transferred into the infundibulum of the oviduct. The skin was closed with two clips afterward. The mouse was allowed to recover on tissues underlamp for 1 hour.

2.4.11. Genotyping of transgenic mice

5mm tail clips were taken from possible founder animals at 3-4 weeks old. The genomic DNA was isolated from tail clips (as described in 2.2.7). Transgenic mice were identified by PCR and Southern blot analysis. Primers specific for the YAC vector arms and human PDGFRα exon1, exon20 and 3'-UTR were used to detect YAC construct and the human PDGFRα gene segment (as described in 2.4.6). Southern blots were hybridized with human PDGFRα extracellular domain (ECD) and 3'-UTR cDNA probes (as described in 2.2.11). Human genomic DNA and/or 449-W2 YAC DNA were used as positive controls in all PCR and Southern blot analyses. Transgenic lines were established by mating founders with wild type mice.

PDGFRα null mutant mice were genotyped by PCR using the following primers: 5'-CCC TTG TGG TCA TGC CAA AC-3', 5'-GCT TTT GCC TCC ATT ACA CTG
G-3’ and 5’-ACG AAG TTA TTA GGT CCC TCG AC-3’. The PCR produces 451 bp and 242 bp fragments for the wild type and mutant alleles, respectively (Soriano, 1997).

2.4.12. Copy number analysis of YAC transgenic mice

To determine the exact transgene copy number in each YAC transgenic line, a fragment in the 3’-UTR of the human PDGFRα gene was amplified (5’-CCC TTG TGG TCA TGC CAA AC-3’, and 5’-GCT TTT GCC TCC ATT ACA CTG G-3’, PCR conditions: 94°C for 4 minutes, 30 cycles of 94°C 30 seconds, 53°C 45 seconds and 72°C 1 minute, then 72°C for 10 minutes). PCR products were removed during the logarithmic phase of amplification (20, 22 and 24 cycles), digested with ClaI, which is present only in the mouse gene, and electrophoresed on a 2% agarose gel. Southern blots were hybridized with a labelled oligonucleotide (5’-ACG AAG TTA TTA GGT CCC TCG AC-3’). Signals were quantified on a phosphoImager (Bio-Rad) by volume integration of individual gel bands.

2.4.13. Staging of embryonic mice

Male transgenic mice were crossed with wild-type females and the females were checked every morning for vaginal plugs. Fertilisation was considered to have occurred at midnight and the day of discovery of the plug was termed E0 (Embryonic Day zero). Pregnant females were killed by cervical dislocation at the age required. The embryos were removed under sterile conditions and the developmental stage was confirmed and refined by morphology (Theiller, 1972).

2.4.14. Rescue analysis of PDGFRα null mutant mice

Heterozygous PDGFRα(+/−) mutant mice were crossed with hemizygous PDGFRα YAC transgenic mice and PDGFRα(+/−) offspring carrying the human PDGFRα YAC
transgene were identified by PCR (as described in 2.4.11). These offspring were sibling-mated to generate homozygous $PDGFR\alpha(-/-)YAC$ offspring (called KO-YAC mice). Alternatively, $PDGFR\alpha(+/+)YAC$ mice were crossed with $PDGFR\alpha(+/+)\text{mutant mice}$ to give KO-YAC mice. The former type of cross had the potential to generate KO-YAC mice with either one or two transgenic alleles. Pregnant females were killed and embryos were removed and staged as described in 2.4.13. Tail clips were cut from each embryo for genotyping. Bodies below the trunk were kept and spinal cords were dissectioned for spinal cord cell cultures (as described in 2.3.4) while bodies above the trunk were fixed for in situ hybridization (as described in 2.3.10). All spinal cord cell cultures and in situ hybridization were performed blind until the genotype was confirmed by PCR.
Fig. 2.1. Schematic drawing of the two-step gel isolation of YAC DNA for microinjection. (A) The first step gel isolation of YAC DNA by pulsed-field gel electrophoresis (PFGE). Yeast embedded in low-density in agarose plugs are used as marker lanes (M) on both sides of yeast embedded in high-density in agarose plugs, which has carried YAC. After preparative-PFGE, both sides of the gel are cut off and stained in ethidium bromide (hatched areas). The positions of YAC DNA and the endogenous yeast chromosomes indicated as marker 1 and marker 2 (above and below the YAC DNA band) are marked under UV-light using a scalpel blade. The gel is reassembled and the area of the gel containing the YAC (hatched box), marker 1 and marker 2 (black boxes) are excised. This gel slice is positioned on a new gel chamber and embedded in 4% low melting point (LMP) agarose. The YAC DNA is concentrated by the second step gel isolation which is run at a 90° angle to the PFGE run (B). Marker 1 and Marker 2 are cut off and stained in ethidium bromide (hatched areas) to localize the concentrated DNA (C). The area corresponding to the concentrated YAC DNA (hatched box) is excised and the YAC DNA is purified further as described in 2.4.8. (Modified from Figure 1 of Schedl et al., 1996).
Excise:
Marker 1
YAC
Marker 2

B

C

Excise
YAC DNA

78
**Table 2.1.** Preparation of antisense RNA probes for *in situ* hybridization

<table>
<thead>
<tr>
<th>Probes</th>
<th>Inserts</th>
<th>Vectors</th>
<th>Antisense transcript</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>PDGFRα</td>
<td>~1.6 kb <em>Eco</em> I ECD cDNA fragment</td>
<td>pBluescript KS cut with <em>Hind</em> III, transcribed with T7 RNA polymerase (T7pol)</td>
</tr>
<tr>
<td>Human</td>
<td>PDGFRα</td>
<td>~1.7 kb <em>Eco</em> I-<em>Not</em> I EST 3'-UTR cDNA fragment</td>
<td>pT7T3D-Pac (Pharmacia) cut with <em>Eco</em> I, transcribed with T3pol to generate ~1.5kb probe</td>
</tr>
<tr>
<td>Mouse</td>
<td><em>Pax6</em></td>
<td>~1.5 kb <em>Eco</em> I cDNA fragment</td>
<td>pBluescript SK cut with <em>Xba</em> I, transcribed with T7pol</td>
</tr>
<tr>
<td>Mouse</td>
<td>Nkx2.2</td>
<td>~2.1 kb cDNA full length</td>
<td>E61* cut with <em>Not</em> I, transcribed with T7pol</td>
</tr>
<tr>
<td>Rat</td>
<td><em>Isl1</em></td>
<td>~1.6 kb cDNA full length</td>
<td>pGEM3 cut with <em>Hind</em> III, transcribed with T7pol</td>
</tr>
<tr>
<td>Rat</td>
<td><em>Isl2</em></td>
<td>~1.6 kb cDNA full length</td>
<td>pBluescript II KS cut with <em>Hind</em> III, transcribed with T7pol</td>
</tr>
<tr>
<td>Mouse</td>
<td><em>Lim3</em></td>
<td>~2.5 kb cDNA full length</td>
<td>pBluescript SK cut with <em>Xba</em> I, transcribed with T7pol</td>
</tr>
<tr>
<td>Mouse</td>
<td><em>c-kit</em></td>
<td>~1.3 kb ECD PCR cDNA fragment</td>
<td>pGEM-T cut with <em>Not</em> I, transcribed with T7pol</td>
</tr>
<tr>
<td>Mouse</td>
<td><em>flk-1</em></td>
<td>~1.1 kb ECD PCR cDNA fragment</td>
<td>pGEM-T cut with <em>Not</em> I, transcribed with T7pol</td>
</tr>
</tbody>
</table>

Chapter 3

Investigating the relative promoter activities of $PDGFR\alpha$ gene fragments and 5’ end structure of $PDGFR\alpha$ mRNA
Introduction

Platelet-derived growth factor (PDGF) was first characterized as the major mitogenic component of serum for fibroblasts (Kohler and Lipton, 1974; Ross et al., 1974). Subsequent studies have shown that PDGF plays an important role in many different biological processes, including embryogenesis, wound-healing, atherosclerosis and neoplasia (reviewed by Ataliotas and Mercola, 1997). PDGF exists as a disulfide-bonded homo- or hetero-dimer of A- and/or B-chain polypeptides (PDGF-AA, -AB or -BB), encoded by two separate genes (Betsholtz et al., 1986; Chiu et al., 1984; Collins et al., 1985). PDGF isoforms are also more highly conserved among species, with *Xenopus* and mouse PDGF-A sharing 71% similarity at the coding sequence level, and with mouse and human PDGF-B sharing 89% similarity.

PDGF signaling is mediated by two distinct, high-affinity receptors termed PDGFRα and PDGFRβ (Claesson-Welsh et al., 1989; Matsui et al., 1989; Yarden et al., 1986). They are encoded by separate genes and belong to the superfamily of protein tyrosine kinase receptors. Like their ligands, the PDGF receptors are capable of dimerizing with each other as homo- or heterodimers (αα, ββ or αβ). PDGFRα can bind all three isoforms of PDGF with high affinity, whereas PDGFRβ is capable of binding PDGF-BB with high affinity and PDGF-AB with low-to-moderate affinity, but is unable to specifically associate with PDGF-AA. PDGFRα and PDGFRβ are transmembrane proteins. The extracellular domain comprises five immunoglobulin-like domains and the intracellular domain contains the catalytically active kinase. *PDGFRα* genes show 90-95% similarity in mouse, rat and human. *PDGFRβ* from human and mouse are 85% similar, whereas human *PDGFRα* and *PDGFRβ* share 47% similarity overall, most of the difference being in their extracellular domains.

The complementary expression patterns of PDGF-A and PDGFRα in *Xenopus* and mouse embryos have indicated a specific role for PDGF-A and PDGFRα in embryonic
development. At early gastrula stages in Xenopus embryos, PDGFRα is evenly expressed throughout mesodermal cells of the marginal zone, whereas PDGF-A is expressed in the inner layer of the animal pole ectoderm (Ataliotis et al., 1995; Jones et al., 1993). As gastrulation proceeds, PDGFRα expression is maintained in the mesoderm, whereas PDGF-A is in the adjacent ectoderm. A similar pattern has been observed in the mouse embryo. At embryonic day 7.5 (E7.5), PDGFRα is expressed in the mesoderm, with the exception of the primitive streak (Orr-Urtreger and Lonai, 1992), whereas PDGF-A is detected in adjacent ectodermal and endodermal layers (Orr-Urtreger et al., 1992; Orr-Urtreger and Lonai, 1992). These complementary expression patterns suggest paracrine stimulation of PDGFRα-expressing cells by PDGF-AA during development.

In the central nervous system (CNS), PDGF-A is produced by both neurons and astrocytes, while PDGFRα is expressed by oligodendrocyte progenitor (OLP) cells, suggesting that paracrine stimulation of PDGFRα is required for the development of the oligodendroglial lineage (Mudhar et al., 1993; Pringle and Richardson, 1993; Qiu and Ferguson, 1995; Yeh et al., 1991; Yu et al., 1994). In the retina, PDGF-A is expressed by neurons while PDGFRα is expressed by retinal astrocytes, again indicating a paracrine interaction involved in glial development (Fruttiger et al., 1996; Mudhar et al., 1993).

In the spinal cord, PDGFRα is expressed in the earliest-forming OLPs in the ventral ventricular zone near the floor plate (Noll and Miller, 1993; Ono et al., 1995; Pringle et al., 1998; Pringle and Richardson, 1993; Yu et al., 1994). Specification of these progenitor cells is controlled by signals, including Sonic hedgehog (Shh) from the notochord and floor plate at the ventral midline (Orentas et al., 1999; Poncet et al., 1996; Pringle et al., 1996). The PDGFRα gene can therefore be regarded as a downstream target of Shh signalling. This view is reinforced by the close proximity of Shh and PDGFRα expression in other parts of the embryo; for example in hair follicles and lung (Bellusci et al., 1997; Bostrom et al., 1996; Karlsson et al., 1999; Lindahl et al., 1997).
Therefore, to gain some insight into Shh signalling we decided to characterize Shh-responsive elements in the PDGFRα gene that are required for expression of PDGFRα in OLPs.

Upstream regulatory regions of PDGFRα have been isolated previously. A 6 kb upstream fragment of the murine PDGFRα promoter was isolated (Wang and Stiles, 1994). This promoter fragment is activated in retinoic acid-treated F9 teratocarcinoma stem cells. Analysis of promoter function by serial 5’ end deletion has identified a 93 bp subfragment containing basal promoter activity (Wang and Stiles, 1994). An approximately 2.2 kb human PDGFRα promoter fragment was also identified (Afink et al., 1995). The rat promoter has also been characterized in cultured vascular smooth muscle cells (Kitami et al., 1995). Comparison of the nucleotide sequences of the three promoters indicate a high degree of sequence conservation (Zhang et al., 1998). By using the 6 kb promoter for mouse PDGFRα to produce transgenic mice, the expression of the LacZ reporter protein was detected in cells originating from mesenchyme and ectoderm (the lens), but not in OLPs (Reinertsen et al., 1997). A 2.2 kb promoter for human PDGFRα is sufficient to drive PDGFRα expression in mesoderm- and neural crest-derived tissues, but also not in OLPs (Zhang et al., 1998).

There are at least two reasons why the cloned 5’ promoter fragments do not drive PDGFRα expression in OLPs in transgenic mice. 1) PDGFRα expression in OLPs might use the same basal promoter as other tissues but with distant cell-type-specific enhancer elements that lie outside the transgenes. 2) PDGFRα expression in OLPs might use a different promoter (different transcription start site) upstream of the identified start site. In NIH 3T3 cells, an alternative start site and exon1 (called 1A) has been identified in the first intron of the PDGFRα gene, indicating that 3T3 cells use a different promoter (Lih et al., 1996). This provides a precedent for the notion that OLPs might also use their own promoter. To investigate this possibility, I mapped the transcriptional start site of the PDGFRα gene in the CNS and outside the CNS by
primer extension and nuclease-S1 protection assay. These results indicated that the same transcription start site and exon1 are used in OLPs as in other cell types. Since I found that the promoter activities of the 6 kb mouse 5' fragment and the 2.2 kb human fragment were both extremely low in transfection assays in CG4 cells, the available data suggest that distant cis-acting regulatory elements cooperate with basal promoter sequences to drive transcription of PDGFRα in OLPs.

Results

1. Functional analysis of PDGFRα promoters in CG4 cells

To investigate the activities of PDGFRα promoters in OLPs, transient transfection analysis of PDGFRα promoters using different sizes of upstream fragments of the murine and human PDGFRα promoters was performed.

A 6 kb 5'-flanking region of the mouse PDGFRα promoter has been isolated and characterized (Wang and Stiles, 1994). The 6 kb regulatory region of the PDGFRα promoter either with or without part of the first intron sequence, named M1 and M2, respectively, was cloned upstream of the bacterial reporter β-galactosidase gene LacZ (Fig. 3.1A). A 2.2 kb upstream fragment of the human promoter has also been isolated (Afink et al., 1995). The 2.2 kb (H1) and a shorter 0.95 kb (H2) upstream regulatory fragment were cloned in front of a LacZ reporter gene (Fig. 3.1B). These four constructs were transfected into CG4 cells. A CMV-β-galactosidase plasmid was used as the positive control, while transfection without DNA was the negative control. The relative activities of the promoters were analyzed by a chemiluminescent assay to detect the activity of β-galactosidase reporter gene in a luminometer (Fig. 3.1C).

The 6 kb upstream regulatory fragment of mouse promoter either with or without the first intron produced a small amount β-galactosidase activity relative to the negative
control. The promoter without the first intron sequence (M2) showed a slightly higher activity than the promoter with the first intron (M1) (Fig. 3.1C). However, the activities of M1 and M2 were very low compared to the CMV-β-galactosidase control. The 2.2 kb (H1) and 0.95 kb (H2) human promoter fragments did not show any activity (Fig. 3.1C). Although the expected expression level of PDGFRα in vitro is not known, these results suggest that the cloned promoter fragments do not contain all the sequences required for correct expression of PDGFRα in OLPs.

2. Northern blot analysis of PDGFRα mRNA

PDGFRα is widely expressed in mesoderm in early embryonic development (Orr-Urtreger and Lonai, 1992). Its expression in the CNS is first detected in E12.5 mouse and E14 rat embryos (Noll and Miller, 1993; Ono et al., 1995; Pringle et al., 1998; Pringle and Richardson, 1993; Yu et al., 1994). To test the transcription of PDGFRα in the CNS and outside the CNS, total RNA from E14 rat embryos, in which PDGFRα is mostly expressed in mesodermal cells, and postnatal day 0 (P0) rat brains, in which PDGFRα is mostly expressed in OLPs, was extracted. Total RNA from CG4 cells was also extracted. CG4 cells are a permanent cell line of oligodendrocyte-type 2 astrocyte (O-2A) progenitor cells isolated from rat brain cultures (Louis et al., 1992).

Northern blots were performed to analyze the transcription of PDGFRα in E14 rat embryos, P0 rat brains and CG4 cells. The cDNA probe for the Northern blot analysis was a 1.7 kb EcoRI-NotI fragment containing most of the coding sequence for the extracellular domain of rat PDGFRα. Consistent with the previous description of a 6.8 kb transcript of the PDGFRα (Lee et al., 1990), a single mRNA band of about 7 kb was detected on blots of either E14 rat embryos, P0 rat brains or CG4 cells (Fig. 3.2).
3. Transcription initiation site of rat PDGFRα mRNA

To investigate whether the site of transcription initiation of rat PDGFRα in the OLPs is the same as that in mesodermal cells during development, the transcriptional start site of PDGFRα in the CNS (P0 brain) and outside the CNS (E14 embryo) was mapped by primer extension and nuclease S1 protection assay.

Primer extension was carried out using total RNA from P0 rat brains and E14 rat embryos and two oligonucleotides, T8 and T3, complementary to PDGFRα mRNA sequences in exon1 (Fig. 3.3). According to the published rat PDGFRα sequence (Lee et al., 1990), we expect to generate a 150 bp band using T8 and a 218 bp band using T3 by primer extension (Fig. 3.3A). Using primer T8, a 119 bp band from brain RNA and a 112 bp band from E14 embryo RNA were obtained (Fig. 3.2B). A weak band of around 145 bp was also observed in RNA from both brain and E14 embryo (open arrowhead in Fig. 3.3B). Using primer T3, a ~215 bp band from both brain RNA and E14 embryo RNA were detected (arrowhead in Fig. 3.2B). Several other bands were also observed; all the bands were the same size from brain RNA and E14 embryo RNA, suggesting that PDGFRα might transcribe from the same start site in the CNS and outside the CNS. However, these experiments have to be regarded as inconclusive.

To test the transcriptional start site of the PDGFRα further, nuclease S1 protection assays were performed. A 233 bp fragment (probe1) was amplified from the cloned rat PDGFRα cDNA by PCR using T9 and T6 (see 2.2.16). This was used to protect exon1 and 5’ fragment of exon2 (Fig. 3.4A). The fragment was labelled at the 5’ end, hybridized to total RNA from P0 brain, E14 embryo and CG4 cells, and digested with 100 units of nuclease S1 (Fig. 3.4B). This assay gave a ~230 bp protected fragment presumably covering exon1 and part of exon2. This suggests that the transcription of the PDGFRα in OLPs (P0 brain and CG4 cells) may be initiated from the same transcription site as in mesodermal cells (E14 embryo). A protected band of about 107
bp was also detected in RNA from P0 brain and CG4 cells. To analyze this further, I prepared a 321 bp PCR fragment (probe2) which extends 88 bp further than the 5' end of exon1 by using primers T12 and T9 (see 2.2.16 and Fig. 3.4A). This labelled fragment protected an approximately 233 bp fragment (Fig. 3.4C). Thus, nuclease S1 protection assay indicates that the site of transcription initiation of the PDGFRα is the same in the CNS and outside the CNS. Therefore, the reason the cloned PDGFRα promoter fragments do not function in OLPs in transgenic mice is not that OLPs use a special promoter and exon1, rather that there are OLP-specific distant enhancer elements that are not included in the existing cloned promoters.

Discussion

1. Up to 6 kb of 5’ sequence is not enough to drive expression of PDGFRα in OLPs

A 6 kb PDGFRα promoter fragment from mouse and 2.2 kb fragment from human have been characterized (Afink et al., 1995; Wang and Stiles, 1994). Transgenic mice produced by using the 6 kb and 2.2 kb PDGFRα promoter fragments display β-galactosidase reporter gene expression in mesodermal and neural crest derived cells, but not in OLPs in the CNS (Reinertsen et al., 1997; Zhang et al., 1998).

One possible explanation is that PDGFRα transcribes from the same transcriptional start site in OLPs as in other cell types, but with OLP-specific distant regulatory elements that are absent from the transgenes (Fig. 3.5A). The 6 kb upstream fragment of mouse promoter displays activity in retinoic acid treated F9 teratocarcinoma stem cells (Wang and Stiles, 1994). To test the activity of this promoter in OLPs, and to confirm the conclusions from transgenic experiments, I transfected the 6 kb promoter fragment into CG4 cells. To test whether there is enhancer or inhibitor sequence in the first intron, this promoter fragment with part of the first intron sequence was also transfected into CG4 cells. Both constructs displayed very low activities in CG4 cells.
compared with the CMV promoter (Fig. 3.1). If anything, the 6 kb 5'-flanking region without the first intron sequence showed slightly higher activity. It suggests that there is no OLP-specific enhancer sequence in this part of the first intron.

The activities of two shorter upstream fragments of human promoter, 2.2 kb and 0.95 kb, were also tested in CG4 cells. The 0.95 kb promoter fragment displays a relatively higher activity than the larger fragment in both human Tera-2 cells and mouse Swiss 3T3 cells (Afink et al., 1995). However, neither the 2.2 kb nor the 0.95 kb promoter fragments had significant activity in CG4 cells (Fig. 3.1C). These data indicate that the 6 kb upstream fragment from mouse and 2.2 kb and 0.95 kb fragments from human PDGFRα are not active in OLPs. These data are consistent with the failure of these promoter fragments to activate reporter gene expression in transgenic mice. This suggests that either PDGFRα transcribes using different promoter in OLPs or specific distant cis-acting regulatory elements are very likely required for PDGFRα expression in OLPs.

2. Transcription of PDGFRα is initiated from the same site in the CNS and outside the CNS

It seemed possible that OLPs use a special PDGFRα promoter, and this is why the existing PDGFRα transgenes are not active in transgenic mice (Fig. 3.5B). An alternative promoter fragment of PDGFRα has been found in NIH 3T3 cells (Lih et al., 1996). The PDGFRα transcribes from an alternative exon1A which is downstream of exon1 used in F9 teratocarcinoma stem cells (Lih et al., 1996; Wang and Stiles, 1994 and Fig. 3.5B).

To test this possibility, I attempted to map the transcriptional start sites of PDGFRα in the CNS and outside the CNS by primer extension. This primer extension gave uninterpretable results since many extension products of widely differing size were
obtained. Nevertheless, the fact that the same bands were obtained in RNA from the CNS (P0 brain) and outside the CNS (E14 embryo) might suggest that the PDGFRα transcribes from the same site in OLPs and mesodermal cells.

The transcriptional start site of the PDGFRα was tested further using nuclease S1 protection assay. In rat olfactory epithelial cells, the PDGFRα transcribes from the start of an untranslated 126 bp exon1 (Lee et al., 1990). The translation initiation (ATG) codon is located at 12 bp downstream of the 5' end of exon2 (Fig. 3.4A). The sizes of protected fragments in the nuclease S1 protection assay are consistent with a similar structure at the 5' end of PDGFRα mRNA from OLPs in the CNS (Fig. 3.4).

However, this does not rule out the possibility that more than one exon1 (and promoter) is used in OLPs, and that the 5' ends detected in our nuclease S1 protection assays represent only a fraction of RNA from OLPs. In that case, we would expect only the exon2 sequences to be protected in this assay, resulting in a ~107 bp protected band with both probe1 and probe2 (Fig. 3.4). I did detect a band at approximately this size in some experiments, for example in RNA from brain and CG4 cells using probe1 (Fig. 3.4B). It could be the reason that PDGFRα also transcribes from the exon1A in OLPs as in NIH 3T3 cells or PDGFRα transcribes from an OLP-specific initiation site (Fig. 3.5B). Thus it remains possible that there is an alternative exon1, for example exon1B used in OLPs (Fig. 3.5B). However, the majority of transcripts would appear to contain the regular exon1.

Taken together, my results suggest that PDGFRα may transcribe from the same transcription start site in OLPs as in other cell types tested (excluding NIH 3T3s). It is likely that distant cis-acting enhancer elements are required for PDGFRα expression in OLPs. These regulatory elements can be further to the 5' side of exon1 or in 3' flanking sequence or in introns (Fig. 3.5A). To isolate these remote elements in the longer term, I
used large fragments including the whole *PDGFRα* gene in yeast artificial chromosomes (YACs) to produce transgenic mice. That work is described in chapter 4.
Fig. 3.1. Relative promoter activities of the *PDGFRα* in CG4 cells. (A) Constructs of 5' upstream fragment of the mouse *PDGFRα* promoter. The 6 kb 5'-flanking region of the mouse promoter either with or without part of the first intron sequence, named M1 and M2, respectively, was cloned in front of the LacZ reporter gene. (B) Constructs of 5' upstream fragment of the human *PDGFRα* promoter. The transcription initiation site is labelled as 0. The 2120 bp 5'-flanking region and 129 bp exon1 (totally about 2.2 kb, named H1), and 825 bp 5'-flanking region and 129 bp exon1 (totally about 0.95 kb, named H2) were cloned upstream of the LacZ reporter gene. (C) β-galactosidase assays of *PDGFRα* promoter constructs in CG4 cells. *PDGFRα* promoter constructs M1, M2, H1 and H2 were transfected into CG4 cells, the O-2A progenitor cells. CMV promoter was used as positive control, while the transfection without DNA was used as negative control. The activities of β-galactosidase reporter gene, which reflect the relative promoter activities, were analyzed using a Galacto-light plus™ kit (Tropix) and determined by a luminometer. The 6 kb upstream fragment of mouse promoter either with or without part of the first intron sequence generated same β-galactosidase activity, whereas the 2.2 kb and 0.95 kb upstream fragments of human promoter did not show any activity. The activity of the 6 kb promoter fragment was very low compared with that of the CMV promoter.
Part of the 1st intron

M1

M2

H = h C Z Z

Ml

LacZ

6 kb

6 kb

2.2 kb

0.95 kb

-2120

0

+129

-825

H1

H2

-200000

-100000

0

100000

200000

300000

400000

Relative light units

Relative promoter activity

CMV  Con  M1  M2  H1  H2
Fig. 3.2. Northern blot analysis of PDGFRα RNA from P0 rat brain, E14 rat embryo and CG4 cells. 15μg of total RNA were electrophoresed on a 1% agarose gel containing formaldehyde, transferred to nylon membrane and hybridized with a [α-32P]dCTP-labelled rat PDGFRα cDNA probe. This cDNA probe was a 1.7 kb EcoRI-NotI fragment containing most of the coding sequence for the extracellular domain of rat PDGFRα, and recognized a ~7 kb single band of mRNA either from P0 brain, E14 embryo or CG4 cells.
Fig. 3.3. Primer extension analysis of the PDGFRα transcription initiation site. (A) Diagram of oligonucleotides designed for primer extension analysis. Primer T8 (5'-GGA GGT CCC CAT CGC TCC TGA G-3') and T3 (5'-GGT AAT AAG AGC TGG CAG ACG-3') are complementary to mRNA sequences of the PDGFRα in the exon2. According to the transcriptional start site of the PDGFRα in rat olfactory epithelium, primer T8 will produce a 150 bp band, while primer T3 will produce a 218 bp band. (B) Primer extension. 10μg total RNA from P0 rat brain and E14 rat embryo were hybridized with 5'-end [γ-32P]ATP-labelled oligonucleotide T8 and T3, and reverse transcribed as described (see 2.2.14). The primer-extended products were analyzed on a 6% sequencing gel. A 119 bp band from brain RNA and a 112 bp band from E14 embryo RNA were detected using primer T8. Weak bands of ~145 bp were also detected (open arrowheads). Primer T3 produced ~215 bp bands (arrowhead) in RNA from both P0 brain and E14 embryo, but it also generated several other bands of unknown origin. Nevertheless, these bands were the same size in RNA from both brain and E14 embryo.
Fig. 3.4. Determining the 5' end structure of PDGFRα mRNA by nuclease S1 protection assay. (A) Diagram of probes used. The A of the translation start codon (ATG) is labelled as +1. PCR using primer T9 (5'-CAT TCT CAT TTG GAA GGA TGG-3') in exon2 and T6 (5'-GTT GGA GCT TGA GGG AGT GAA AC-3') at the 5'-end of exon1 amplifies a 233 bp fragment (probe1) which will protect exon1 and part of exon2. PCR using primer T9 and primer T12 (5'-CGT AAT ACG ACT CAC TAT AGG-3') amplifies a 321 bp fragment (probe2) which extends 88 bp from the 5'-end of exon1. (B) Nuclease S1 protection assay using probe1. 100μg total RNA from P0 rat brain, E14 rat embryo and CG4 cells were hybridized with 5'-end [γ-32P]ATP-labelled probe1, and digested with 100 units of nuclease S1. Yeast tRNA was used as negative control. The reaction products were analyzed on a 6% sequencing gel. The nuclease S1 protected fragments were about 230 bp in RNA from either P0 rat brain, E14 rat embryo and CG4 cells. A ~107 bp band was also observed in RNA from P0 brain and CG4 cells. Using probe2 (C), the nuclease S1 protected bands were also about 230 bp in RNA from P0 brain and E14 embryo. The transcription initiation site is at about -140 bp from the translation start codon (+1).
A

-138

-12

+1

ATG

T6

T9

T12

probe1

probe2

233 bp

321 bp

B

Brain

E14

CG4

rRNA

Probe1

C

Marker

Brain

E14

rRNA

Probe2

-230 bp

-107 bp

310 bp

234 bp

194 bp

118 bp
Fig. 3.5. Two possibilities that initiate specific expression of PDGFRα in OLPs. (A) OLP-specific enhancer elements are essential for PDGFRα expression in OLPs. As in other cell types, the PDGFRα transcribes from exon1 in OLPs. The translation start codon (ATG) is in exon2. Enhancers, for example En1 and/or En2, are required for PDGFRα expression in other cells, while OLP-specific enhancers (OLP-En1 and/or OLP-En2) are required for PDGFRα expression in OLPs. These OLP-specific enhancer elements can be at further 5' end from exon1 or in 3' flanking sequence or in intron sequences. (B) Expression of PDGFRα in OLPs uses different promoter. The PDGFRα transcribes from exon1 in F9 teratocarcinoma (Tera) stem cells. However, in NIH 3T3 cells, the PDGFRα undergoes an alternative splicing, i.e. transcribes from exon1A which is in the first intron. In OLPs, PDGFRα may transcribe from exon1B which can be at further 5' end from exon1. Transient transfection assay using up to 6 kb PDGFRα promoter fragment and 5' end structure analysis of PDGFRα mRNA using primer extension and nuclease S1 protection assay indicate that PDGFRα may transcribe in OLPs from the same initiation site as in other cells, but with OLP-specific regulatory elements.
A

OLP-En1 En1 En2 Exon1 OLP-En2 Exon2

OLP-En: OLP-specific enhancer
En: Enhancer

B

OLPs? Exon1B

Terastem cells Exon1

3T3 cells Exon1A

ATG Exon2
Chapter 4

YAC complementation of craniofacial and neural tube defects in

*PDGFRα* knockout mice*

*The work in this chapter is going to be submitted for publication.*
Introduction

Active PDGF is composed of dimers of A and B chains. All three dimeric isoforms of PDGF (AA, AB, BB) can bind to and activate PDGFRα in vitro. It is generally thought that, of these, PDGF-AA is the major ligand for PDGFRα in vivo (reviewed by Heldin et al., 1998: see Discussion). Both PDGF-A and PDGFRα display complex and dynamic expression patterns during embryonic development (reviewed by Ataliotis and Mercola, 1997). In mouse embryos, PDGF-A is expressed in the epithelium and endothelium while PDGFRα is expressed in the adjacent mesenchyme (Orr-Urtreger and Lonai, 1992). PDGFRα is also expressed in the cranial neural crest where it is thought to be involved in directed migration of crest into the branchial arches. In keeping with this, PDGFRα deficient mice [both the directed knockout and the spontaneous deletion mutant Patch (Ph)] have a variety of defects in crest-derived tissues including gross craniofacial abnormalities (Morrison-Graham et al., 1992; Orr-Urtreger et al., 1992; Schatteman et al., 1992; Soriano 1997). They also have neural tube defects (spina bifida) and die in early-to-mid gestation.

A proportion of PDGF-A null mice dies in mid-gestation but others survive to birth and beyond. Of these, most die within the next week or two but a few can survive as long as six weeks. The eventual cause of death is thought to be a failure of lung alveogenesis although there are several other obvious defects including reduced growth and vigour, malformation of the gut and hypomyelination in the central nervous system (CNS) (Bostrom et al., 1996; Fruttiger et al., 1999; Lindahl et al., 1997). These defects all seem to be caused by the failure of specific populations of PDGFRα-positive progenitor cells to proliferate and migrate properly (reviewed by Lindahl and Betsholtz, 1998). For example, the root cause of the myelin deficiency is a severe reduction in the number and distribution of PDGFRα-positive oligodendrocyte progenitors (OLPs) and a corresponding loss of myelin-forming oligodendrocytes (Fruttiger et al., 1999). Our particular interest in PDGF signalling stems from this critical role of PDGF-A and
PDGFRα in oligodendrocyte development. Many brain tumours (e.g. gliomas) feature up-regulation of PDGF and PDGFRα and some appear to be derived from OLP-like cells (reviewed by Westermark et al., 1995). Understanding normal glial cell development and pathology, as well as many other aspects of normal and abnormal development, requires that we understand the transcriptional regulation of PDGFRα.

In recent years, yeast artificial chromosomes (YACs), have been used to analyze distant regulatory sequences and to study gene function (Lamb and Gearhart, 1995; Peterson et al., 1997; Huxley, 1998). To analyze PDGFRα expression and function during development, I produced transgenic mice by pronuclear injection of a 380 kb YAC (CEPH 449C2; Spritz et al., 1994) containing human PDGFRα genomic DNA. I found that the human PDGFRα transgene was correctly expressed in OLPs in the CNS and in many mesoderm- and neural crest-derived tissues, though generally at a lower level than the endogenous gene. Thus, many cis-acting regulatory elements necessary for tissue-specific expression of PDGFRα during development seem to be contained within this 380 kb YAC, notably the element(s) for expression in OLPs. I showed that the human PDGFRα transgene complemented defective oligodendrocyte development in PDGFRα null mutant (KO) mice. Moreover, the neural crest and neural tube defects were rescued, and survival of the embryos was prolonged until birth. The ultimate cause of death was respiratory failure due to reduced lung growth and abnormal lung morphogenesis. Nevertheless, this YAC will act as a starting point for isolating cell type-specific PDGFRα regulatory elements such as that responsible for transcription in OLPs.

Results

1. Characterization and modification of human PDGFRα YACs
YACs CEPH 449C2 and CEPH 29E11 were originally isolated from the CEPH YAC library using primers specific for human \textit{PDGFR}\textalpha{} exon 2 and exon 20 (Spritz et al., 1994). I obtained these YACs from the Genome Technology Center, Leiden. To test the size and integrity of each YAC, I analyzed these YACs by pulsed-field gel electrophoresis (PFGE), indicating that 449C2 is ~380 kb and 29E11 is ~470 kb (Fig. 4.1A). The integrity of each YAC was tested further by Southern blots using probes specific for human \textit{PDGFR}\textalpha{} exon 1 and the 3’-untranslated region (UTR) (Fig. 4.1B). A single band was detected in each YAC using probes either in \textit{PDGFR}\textalpha{} exon 1 or 3’-UTR.

Because these YACs are in the same size range as the endogenous yeast chromosomes (Fig. 4.1A), and very pure YAC DNA is required for microinjection, 449C2 and 29E11 were transferred into two “window” yeast strains YLBW2 and YLBW3 with “windows” (yeast chromosome-free zones) in the size range 250-450 kb and 310-590 kb, respectively (Hamer et al., 1995). Three colonies of 449C2 transfer and four colonies of 29E11 transfer were picked. Haploid window yeast strains carrying 449C2 and 29E11 YACs were identified by PCR (Huxley et al., 1990) (Fig. 4.2). Only colony-1 was haploid in 449C2 transfer, while all four colonies in 29E11 transfer were haploid (Fig. 4.2). They displayed the same mating type as the window strains (\textit{MAT}\textalpha{}). The resulting YACs were named 449-W2 and 29-W3. Colony-1 in both 449C2 and 29E11 transfers was grown up for further analysis.

To characterize the transferred YACs in window strains, the original YACs, window strains and transferred YACs were analyzed by PFGE, indicating that YACs 449C2 and 29E11 have been successfully transferred into window strains from their original host strains without rearrangement (Figs. 4.3A, B). The integrity of transferred YACs was tested further by Southern blots using a probe specific for human \textit{PDGFR}\textalpha{} 3’-UTR (Fig. 4.3C). The same sized bands in original YACs and the transferred YACs indicated
that the transferred YACs were intact. These resulting YACs were used for
microinjection to produce YAC transgenic mice.

2. Generation of human PDGFRα YAC transgenic mice

Intact YAC DNA for microinjection was purified by the two-step gel isolation
procedure as described in 2.4.8 and Fig. 2.1. YAC DNA was first separated by PFGE.
To mark the position of the YAC DNA, two marker lanes (449C2) on both sides of the
gel were cut off and stained in ethidium bromide (Fig. 4.4A). The gel containing YAC,
marker1 and marker2 was subsequently run at a 90° angle to the PFGE run. The
concentrated YAC DNA was excised according to the location of two marker slices (Fig.
4.4B). By comparison with known amounts of λ DNA on the gel, the concentration of
purified YAC DNA was determined to be about 1ng/μl (Fig. 4.4C). Analysis of purified
YAC DNA by PFGE indicated that the YAC DNA was still intact (Fig. 4.4D).

Transgenic mice were produced by microinjection of gel-purified YAC DNA into
fertilized mouse oocytes harvested from C57BL/6J x CBA breeding (as described in
2.4.10). DNA from tail tips of the 3-4 week old mice was analyzed by PCR using
primers specific for YAC vector arms and human PDGFRα exon 1, exon 20 and 3’-UTR
(as described in 2.4.11). After injection of 449-W2 YAC DNA, four transgenics were
detected among 43 new-borns (Fig. 4.5 and Table 4.1). Southern blots using human
PDGFRα cDNA probes in extracellular domain (ECD) and 3’-UTR were used to
confirm the genotypes (Fig. 4.6 and Table 4.1). Founders 3-3, 5A1 and 8A4 all appeared
to contain full-length YACs by PCR and Southern blots (Fig. 4.5 and Fig 4.6). They
were bred with wild-type mice to establish lines. In transgenic 7-2, only the 3’-UTR
segment was detected by PCR and Southern blots (Fig. 4.5 and Fig 4.6). Following
injection of 29-W3 YAC DNA, no transgenics were detected among nine new-borns.
3. Determination of transgene copy number

To determine the numbers of integrated YAC transgenes, quantitative PCR was carried out using primers corresponding to matching sequences within the mouse and human PDGFRα 3′-UTRs (see details in 2.4.12). Southern blots of the mouse and human PCR products were quantified with a phosphoimager. In line 3-3, the human PCR product was about 11 times more abundant than the mouse product, suggesting that around 22 copies of human PDGFRα had integrated per diploid mouse genome (Fig. 4.7). There were around 10 copies of the YAC in line 5A1 and 2 copies in line 8A4.

4. Expression of the human PDGFRα transgene in non-neural tissues

I looked at the transgene expression patterns in these three transgenic lines by in situ hybridization with a probe to the human PDGFRα 3′-UTR. Under my experimental conditions, this probe did not cross-hybridize detectably with the endogenous mouse gene. Transgene expression was similar and wide-ranging in lines 3-3 and 5A1 (see below and Table 4.2). In line 8A4 transgene expression was not detected anywhere except the anterior epithelium of the eye lens and the posterior lens fibres, so this line was not studied further.

PDGFRα is expressed widely in the mesenchyme during early development (Orr-Urtreger et al. 1992; Schatteman et al. 1992). At E10.5, whole mount in situ hybridization showed that the PDGFRα YAC transgene, like the endogenous gene, was expressed in branchial arch mesenchyme, which is mainly of neural crest origin (white arrowheads in Fig. 4.8A, B). At E12.5, in situ hybridization on sections of wild type mice confirmed that PDGFRα is expressed in cranial neural crest mesenchyme (Morrison-Graham et al. 1992; Orr-Urtreger et al. 1992). Transgene expression was also detected in the mesenchyme of upper and lower jaws at this age, but the in situ signals were very weak compared with those of the endogenous PDGFRα (not shown). Neural
crest-derived tissues also contribute to heart formation (Orr-Urtreger et al. 1992; Schatteman et al. 1992). At E12.5, endogeneous PDGFRα was expressed in the aortic valve and aortico-pulmonary spiral septum (Fig. 4.8E). However, expression of the human PDGFRα transgene was not detected in the heart (Fig. 4.8F). From E14.5, transgene expression was also detected in the interstitial mesenchyme of the thymus (not shown) and in the interstitial lung tissue (Fig. 4.13) where endogenous PDGFRα is also expressed.

Endogenous PDGFRα was expressed in the somites at E10.5 and also in the limb buds (white arrows in Fig. 4.8A). At this age, expression of the human PDGFRα transgene was also detected in somites and, very weakly, in the limb buds (Fig. 4.8B). Note that the human transgene was also expressed in the dorsal neural tube at E10.5, in contrast to the endogenous gene (Figs. 4.8A, B and 4.10A, B). This and other ectopic sites of expression are discussed further below.

At E12.5, endogenous PDGFRα is expressed in a variety of mesenchymal tissues, particularly the sclerotome and dermatome (Fig. 4.8C). Expression of the human PDGFRα transgene was also observed in sclerotome (Fig. 4.8D) but was weaker and less extensive than the endogenous gene, being limited to the area around the procartilage primordia of the vertebrae and the cartilage that surrounds the notochord. Endogenous PDGFRα was expressed in mesenchymal cells surrounding the oesophagus and trachea where the human PDGFRα transgene was not expressed (Fig. 4.8C, D). Expression of the transgene was also observed in the developing limb mesenchyme, but the expression level was very low and the distribution was not as broad as that of endogenous PDGFRα (data not shown).

By E13.5, derivatives of the sclerotome have given rise to the cartilaginous framework of the spinal column and ribs. Endogenous PDGFRα is expressed in the perichondrium/periosteum of developing bones (Schatteman et al. 1992) (Fig. 4.8G, H).
The human *PDGFRα* transgene was also expressed at E13.5/E14.5 in the chondrifying bone primordia of limbs (not shown), vertebrae (Fig. 4.8I) and ribs (Fig. 4.8J). Expression was observed in the perichondrium/periosteum and cartilage. However, the transgene was also expressed in the chondrocytic core of the cartilage at E14.5 where *PDGFRα* is not normally expressed (arrows in Fig. 4.8I, J).

In the developing eye lens, both endogenous and transgenic *PDGFRα* were expressed in the anterior lens epithelium (Mudhar et al., 1993; Orr-Urtreger et al. 1992; Schatteman et al. 1992) (Fig. 4.8K, L). However, the human transgene was also expressed in the posterior lens fibres, where endogenous *PDGFRα* expression is normally extinguished (Fig. 4.8L).

To summarize, the human *PDGFRα* transgene was expressed at many of the normal sites of *PDGFRα* expression outside of the nervous system, though generally at a low level compared to the normal gene (Table 4.2 and Fig. 4.8). However, there were also significant differences between the endogenous and human gene expression patterns; there were places where the endogenous gene was expressed but not the human transgene, and vice versa. I did not notice any differences between transgenic lines 3-3 and 5A1, suggesting that the spatial disparities between transgene and endogenous gene expression are not a function of the site of integration.

5. The human *PDGFRα* transgene is faithfully expressed in oligodendrocyte progenitors in the CNS

To analyze transgene expression in the CNS, I focused on line 3-3. In transverse sections of wild-type mouse spinal cord, *PDGFRα*+ cells first appear transiently in the dorsal-most ventricular zone (VZ) before E12, but this expression dies away by E13 (E14 in the rat; Pringle and Richardson, 1993). There is another site of expression in the ventral VZ, starting on E12.5 in the mouse (Pringle et al., 1996; Calver et al., 1998) (Fig. 103).
4.9A). These ventral PDGFRα* cells, which initially form two longitudinal ribbons of cells through the spinal cord and brainstem, are nascent oligodendrocyte progenitors (OLPs). Soon after they appear, these cells proliferate and migrate from the ventral VZ in response to PDGF-AA (Fruttiger et al., 1999) (Fig. 4.9A-C), becoming evenly distributed throughout the cross-section of the cord by the time of birth (Fig. 4.9D). The human PDGFRα transgene was also expressed in OLPs in the ventral VZ on E12.5 (arrows in Fig. 4.9E), and continued to be expressed in these cells in parallel with the endogenous mouse PDGFRα until birth (Fig. 4.9F-H). Unlike many sites outside the CNS, the level of expression of the transgene in OLPs was comparable to that of the endogenous gene.

However, on E12.5, human PDGFRα was also ectopically expressed in a large sub-population of cells just outside the dorsal VZ (Fig. 4.9E). This pattern of PDGFRα expression is not seen in wild-type spinal cord (Fig. 4.9A). This ectopic expression of the transgene was very strong on E12.5 but subsided by E13.5 (Fig. 4.9F) and disappeared completely by E15.5 (Fig. 4.9G). From their distribution and expression pattern, I presume that the ectopic human PDGFRα-expressing cells are progenitors of dorsal neurons.

6. Ectopic expression of the PDGFRα transgene partly mimics c-kit and flk-1

In many tissues the human PDGFRα transgene was correctly expressed, though at a lower than normal level. However, there were also ectopic sites of expression — in the dorsal spinal cord, in the walls of major blood vessels and at the midline of the tongue, for example. Since PDGFRα is closely linked on human chromosome 4 and mouse chromosome 5 to the related receptor tyrosine kinases c-kit and flk-1 (reviewed by Ataliotis and Mercola, 1997), and since YAC 449C2 contains regulatory sequences upstream of c-kit (Spritz et al. 1994), I reasoned that ectopic expression of the YAC
transgene might reflect normal sites of c-kit and possibly even flk-1 expression. To test this, I looked at c-kit and flk-1 expression in wild type embryos by in situ hybridization.

c-kit was expressed in cells outside the VZ in the dorsal and ventral spinal cord at E10.5 (Fig. 4.10C). At E12.5 and E13.5 expression was restricted to the dorsal cord (Fig. 4.10E, G). This normal expression pattern of c-kit resembled the ectopic expression pattern of the human PDGFRα transgene (compare Fig 4.10B, C and Figs 4.9E, 4.10E and Figs. 4.9F, 4.10G), though transgene expression appeared stronger. The human PDGFRα transgene was also expressed in two lateral clusters of unidentified cells in the E10.5 ventral spinal cord (arrowheads in Fig. 4.10B) and two lateral regions directly above the dorsal neural tube (arrows in Fig. 4.10B), which are neither sites of endogenous PDGFRα expression nor of c-kit expression. flk-1 expression was detected as punctate labelling of blood vessels throughout the embryonic spinal cord (Fig. 4.10D, F, H). Thus, ectopic expression of the human PDGFRα YAC in the spinal cord mimics (imperfectly) the normal expression pattern of c-kit but not that of flk-1.

Endogenous PDGFRα is not expressed in blood vessels during early development — for example the vertebral arteries (Fig. 4.10I). However, the human PDGFRα transgene was expressed in the walls of these arteries (Fig. 4.10J, M). Similar expression in blood vessels was observed with a probe for flk-1 (Fig. 4.10L, N) but not c-kit (Fig. 4.10K). Thus the ectopic expression of the human PDGFRα transgene in blood vessels corresponds to sites of expression of flk-1 but not c-kit.

7. The human PDGFRα YAC rescues craniofacial and skeletal defects in PDGFRα null mutant mice

PDGFRα null mice have severe developmental defects, particularly in cranial neural crest-derived structures, and die before E16 (Soriano, 1997). To test whether the human PDGFRα YAC transgene can rescue these defects, I set up appropriate crosses to
introduce the YAC transgene onto the PDGFRα null background (see details in 2.4.14). I call these KO-YAC mice.

At E12.5, PDGFRα null mice displayed a cleft face and a wavy spinal cord, as described previously (Soriano, 1997) (compare Fig. 4.11A, B). The frontonasal and mandibular processes had failed to fuse at the midline. Many mutant embryos died by E14.5; those that survived later than this were smaller than their wild type littermates, had a cleft face and showed signs of internal bleeding at various sites (not shown). Neural tube abnormalities were also observed — the neural tube was not completely closed (spina bifida) and it had an abnormal shape.

Skeletal preparations of E16.5 PDGFRα null embryos clearly showed that the anterior skull plates were severely reduced in size and failed to fuse at the midline (compare Fig. 4.11D, E). The path of the spinal column was distorted (Fig. 4.11H) and the cervical and upper thoracic vertebrae had an abnormal shape (compare Fig. 4.11J, K), having failed to close on the dorsal side as in homozygous Ph mutant mice (Payne et al., 1997). These preparations also illustrate the marked decrease in size of the E16.5 PDGFRα null embryos compared to wild type (compare Fig. 4.11G, H and 4.11J, K). I did not recover any live PDGFRα null embryos older than E17.5.

In contrast, KO-YAC mice at E14.5, E16.5 and E17.5 were alive and completely normal with respect to external and skeletal morphology (Fig. 4.11C, F, I, L). There were no signs of bleeding and the heart appeared to be beating normally. The KO-YAC embryos survived and were born normally, but died shortly after birth.
8. PDGFRα null mice rescued with the transgene die after birth from respiratory failure

I have recovered four KO-YAC pups that had died after birth. These animals were normal in appearance but slightly smaller than their wild-type littermates. On postmortem examination, we found no obvious internal abnormalities except that the lungs were markedly smaller than normal (Fig. 4.12). It seems likely that this obvious lung defect is the ultimate cause of death at birth.

I further examined the developing lungs of wild type (+/+) or PDGFRα (+/-), YAC transgenic (wt-YAC), KO-YAC and PDGFRα null mutant (KO) mice by in situ hybridization with mouse and/or human PDGFRα probes. In E15.5 lungs, the endogenous mouse PDGFRα was expressed in mesenchymal cells surrounding epithelial tubules (Fig. 4.13A, D). Due to the incomplete deletion of the PDGFRα gene (Soriano, 1997), PDGFRα mRNA was also detected in KO lungs with a similar expression pattern in wild type lungs but a lower signal level (Fig. 4.13C, F). In addition, the numbers of epithelial tubules were noticeably reduced in KO lungs (compare Fig. 4.13 A and C). Expression of the human YAC transgene was observed in mesenchymal in wt-YAC lungs but at a generally low level (Fig. 4.13B). At high magnification, it was evident that fewer of the strongly-labelled cells that contact the outer surfaces of the epithelial tubules were detected with the human PDGFRα probe than with the mouse probe on consecutive sections (Fig. 4.13E and not shown). This suggests that the cells in question (presumptive alveolar smooth muscle cell progenitors) are present in the wt-YAC lungs but do not express the transgene normally.

By E17.5, the sacular stage of lung development, endogenous PDGFRα was expressed in scattered manner in mesenchymal cells (Fig. 4.13G and arrows in Fig. 4.13K). Fewer weakly PDGFRα+ cells were detected in wt-YAC lungs with the human PDGFRα probe (Fig. 4.13H and arrows in Fig. 4.13L). At this stage, sac-like structures, the future
alveoli, have formed from the terminal tubules through continued branching (Fig. 4.13G, H). However, this progress was not so obvious in KO-YAC and KO lungs (Fig. 4.13I, J). Higher magnification of these lungs also displayed thicker mesenchyme (arrowheads in Fig. 4.13M, N). These observations suggest that alveoli were not forming properly in KO and KO-YAC lungs.

At P0, just after birth, the sac-like structures have expanded to a greater degree, characterized by a thinning mesenchyme (Fig. 4.13O, P). Similar to the endogenous PDGFRα, human PDGFRα transgene was also weakly expressed in the thin walls of the terminal sacs (Fig. 4.13O, P). However, although terminal sacs were formed in KO-YAC lungs, sac-like structures did not expand (Fig. 4.13Q). The thinning of the mesenchyme also did not happen at this stage (arrowheads in Fig. 4.13Q). This could result from the failure of the lungs to inflate after birth.

In wild-type lungs there is a class of migratory alveolar myofibroblast precursors that express PDGFRα. In PDGF-A null mice these cells do not proliferate or migrate properly; as a result alveogenesis is impaired and the animals die of respiratory failure (Bostrom et al., 1996; Lindahl et al., 1997). In situ hybridization showed that transgene-derived human PDGFRα was expressed in alveolar smooth muscle cell progenitors but at a low level. The thinning of the mesenchyme during expansion of the terminal tubules did not occur in the KO-YAC lungs. It seems that these PDGFRα-positive alveolar smooth muscle cell progenitors were present in the KO-YAC lungs but they did not proliferate and/or migrate properly. The small size of the KO-YAC lungs at birth suggests that they failed to inflate, possibly because of delayed or defective lung development and that this is the probable cause of death. According to this interpretation, the phenotypes of the PDGFRα KO and PDGF-A mutant lungs are essentially similar, except that the PDGFRα KO phenotype seems to be more severe since no mice survived after birth. Perhaps it is more severe because another ligand can partially substitute for PDGF-A in the PDGF-A mutant lungs (see discussion).
9. The human PDGFRα transgene rescues oligodendrocyte development in PDGFRα null mice

PDGF signalling is essential for proper proliferation and migration of OLPs and consequently for development of differentiated oligodendrocytes (OLs). This is known because PDGF-A knockout mice have many fewer PDGFRα+ OLPs and OLs than normal and are severely hypo-myelinated as a result (Fruttiger et al., 1999). It seemed likely that OL development should be severely affected in PDGFRα knockout mice too, although this had not previously been tested because the PDGFRα null mice die before OLs normally start to appear. I tested this prediction by in situ hybridization and spinal cord cell culture, and at the same time asked whether the human PDGFRα transgene could rescue the normal function of endogenous PDGFRα in OL development in vitro.

In transverse sections of E14.5 wild-type mouse spinal cord, PDGFRα+ cells divide and migrate from the ventral VZ and almost spread throughout the whole spinal cord (Fig. 4.14A). The human PDGFRα transgene was also expressed in OLPs in the same pattern as the endogenous mouse PDGFRα (Fig. 4.14B). At the same age, compared to wild-type and YAC transgenic mice, the spinal cord of PDGFRα null mutant mouse had an abnormal shape (compare Fig. 4.14A, B and C). While PDGFRα mRNA could still be easily detected in non-neural tissues, for example the bone primordia of vertebrae (arrowheads in Fig. 4.14C), only a weak PDGFRα expression was observed in the ventral VZ of the PDGFRα null mutant spinal cord (arrows in Fig. 4.14C). The PDGFRα+ cells did not seem to proliferate and migrate from the VZ (Fig. 4.14C). No PDGFRα expression was observed in the spinal cord of E17.5 PDGFRα null embryos, while its expression was normal in the spinal cords of PDGFRα (+/-), wt-YAC and KO-YAC mice (Fig. 4.14D-G). By E17.5, OLPs start to differentiate into oligodendrocytes
(OLs) which can be detected with probe for the myelin basic protein (MBP) gene. Similar to PDGFRα (+/-), wt-YAC and KO-YAC mice, MBP+ cells were also detected in the spinal cord of the PDGFRα KO (Fig. 4.14H-K). Thus, OLs are generated in the PDGFRα null spinal cord, but they are abnormally placed (Fig. 4.14K). At P0, PDGFRα and MBP expression appeared normal in KO-YAC spinal cord, comparing to those in PDGFRα (+/-) spinal cords (Fig. 4.14L-O).

Since live PDGFRα null embryos older than E17.5 had not ever been found, it is not possible to follow OL development after this age. I therefore took a cell culture approach. I cultured spinal cord cells from E14.5 wild-type, PDGFRα KO and KO-YAC mice for several days until the equivalent of the day of birth (E19), then fixed and immunolabelled with anti-galactocerebroside (GC) to visualize differentiated OLs. Many fewer GC+ oligodendrocytes developed in cultures of PDGFRα null spinal cord than in wild-type cultures (Fig. 4.15). However, there were no significant differences in the numbers of OLs that developed in KO-YAC and wild-type cultures (Fig. 4.15A-D, G). In one representative experiment, 630 ± 62 GC+ cells per coverslip were detected in wild-type cultures, 612 ± 9 GC+ cells per coverslip were detected in KO-YAC cultures, while only 57 ± 6 GC+ cells per coverslip were detected in homozygous PDGFRα null cultures (means ± range, duplicate coverslips, one embryo of each genotype) (Fig. 4.15G). Therefore, OL development is severely deficient in null mutant mouse as expected, but this deficiency is effectively rescued by the human PDGFRα YAC.

Discussion

I have produced transgenic mice carrying the complete human PDGFRα structural gene with 5'- and 3'- flanking sequences, by pronuclear injection of a 380 kb YAC. Before birth, the human transgene was correctly expressed in many tissues, including mesodermal and neural crest derivatives, as well as oligodendrocyte progenitors (OLPs) in the CNS. However, the YAC transgene was not expressed at all sites of normal
PDGFRα expression and there was also significant ectopic expression. Therefore, this YAC contains cis-acting DNA elements necessary for much but not all of the normal developmental regulation of PDGFRα.

The profound craniofacial defects and spina bifida observed in mice with a targeted PDGFRα null mutation were completely rescued by the YAC. Nevertheless, the animals still died at birth, almost certainly from respiratory failure due to defective lung development. PDGF-A knockout mice die after birth from alveogenesis failure because PDGFRα-positive alveolar myofibroblast precursors do not proliferate and migrate properly in the developing lung. It is possible that the YAC is not expressed correctly by myofibroblast precursors which consequently do not proliferate and migrate as in the PDGF-A knockout.

1. The 380 kb YAC contains regulatory elements specific for PDGFRα expression in oligodendrocyte progenitors

Our long-term aim in generating the YAC transgenics is to identify regulatory elements that are responsible for PDGFRα transcription in OLPs. In the spinal cord, PDGFRα+ OLPs are first specified in the ventral ventricular zone under the influence of Sonic hedgehog (Shh) protein from the ventral midline (Orentas and Miller, 1996; Orentas et al., 1999; Poncet et al., 1996; Pringle et al., 1996). Identifying the OLP-specific regulatory elements of PDGFRα should therefore provide insights into the DNA targets of Shh signalling. In addition, it will provide us with a means of directing expression of conventional transgenes to OLPs. Human PDGFRα was expressed appropriately in OLPs in our YAC transgenics and the presence of the YAC rescued oligodendrocyte development in cultures of spinal cord cells taken from mice lacking endogenous PDGFRα (KO-YAC mice). Therefore, the OLP-specific element(s) driving PDGFRα expression is functionally intact in the YAC transgene.
In contrast, plasmid-based transgenes containing 2.2 kb or 6 kb of upstream sequences from the human or mouse PDGFRα genes were not expressed in OLPs (Reinertsen et al., 1997; Zhang et al., 1998), suggesting that the OLP-specific element(s) is located further upstream or else at the 3' side of the gene or within an intron. Our 380 kb YAC contains 65 kb human PDGFRα genomic DNA (Kawagishi et al., 1995) and about 10 kb of YAC vector arms. Therefore, the total length of 5'- and 3'- flanking regions is about 300 kb, but the position of PDGFRα within the YAC is not established. Preliminary mapping indicates that the PDGFRα gene is close to one end of the insert so that it contains less than 6 kb of upstream sequence (personal communication from Gijs Afink and Monica Nister, Uppsala University, Sweden). This implies that the OLP-specific elements probably lie within the gene or downstream of it.

2. Regulatory elements that normally regulate c-kit or flk-1 promoters are within this 380 kb YAC

Ectopic expression of the PDGFRα transgene matched some aspects of the normal expression patterns of c-kit and flk-1 (also known as KDR), two closely related receptor tyrosine kinases that cluster downstream of PDGFRα on human chromosome 4. This suggests strongly that there are regulatory elements (enhancers) in the YAC transgene that normally communicate with the c-kit or flk-1 promoters, but are mis-directed towards the PDGFRα promoter in our transgenic mice. It is expected that c-kit regulatory sequences should be in the YAC because Spritz et al. (1994) found that c-kit exon 1 (but not exon 21) is present. It is more surprising that flk-1 regulatory sequences might also be present in the region between PDGFRα and c-kit. If this is so, and if any of the multiple copies of the YAC in our transgenic lines (3-3 and 5A1) are arranged head-to tail, then the c-kit and flk-1 elements would be brought in close proximity to the PDGFRα promoter. This might allow the re-positioned c-kit and flk-1 regulatory elements to compete with genuine PDGFRα elements for the PDGFRα promoter. A similar thing happens in reverse in the Patch (Ph) mouse, which carries a 200 kb deletion
Moreover, transgene expression was only detected in the anterior epithelium of the eye lens and the posterior lens fibers in line 8A4. One possibility that causes the lens-specific expression could be the rearrangement of this 380kb YAC in line 8A4. Another possibility is the copy number of the transgene in mouse genome. Since there are only two copies of YAC transgene in line 8A4, the transgene interaction may be necessary for its proper expression. These can be investigated by mapping the transgene in mouse genome in the future.
including the \( PDGFR\alpha \) locus and part of the intergenic region between \( PDGFR\alpha \) and \( c\text{-}kit \). One effect of the \( Ph \) mutation is to bring \( PDGFR\alpha \) 5' regulatory sequences close to the \( c\text{-}kit \) promoter (Stephenson et al., 1991; Wehrle-Haller et al., 1996), resulting in ectopic expression of \( c\text{-}kit \) in places that normally express \( PDGFR\alpha \) (Duttlinger et al., 1995; Wehrle-Haller et al., 1996). This sort of interference by mis-placed regulatory elements could help explain not only the ectopic \( PDGFR\alpha \) expression that we observe in our transgenics but also the generally low level of expression. Therefore, removing sequences downstream of \( PDGFR\alpha \) in our YAC might improve both the fidelity and strength of transgene expression. Of course, there are alternative explanations for the inaccurate, weak expression of the \( PDGFR\alpha \) transgene; for example, the endogenous mouse transcriptional apparatus might mis-read human \( PDGFR\alpha \) regulatory signals, or there might be enhancers and/or silencer elements missing from the YAC transgene.

I was unable to detect transgene expression in some normal sites of \( PDGFR\alpha \) expression, notably in the heart. Normally, \( PDGFR\alpha \) is expressed from E9 in the pericardium and the endocardial cushions of the heart, and later in the forming atrial and ventricular valves and in the immediately abutting myocardium (Morrison-Graham et al., 1992; Orr-Urtreger and Lonai, 1992; Orr-Urtreger et al., 1992; Schatteman et al., 1992). In homozygous \( Ph \) mutants the heart fails to septate, suggesting that \( PDGFR\alpha \) is crucial for heart development. However no heart defect was found in the targeted \( PDGFR\alpha \) null mutant (Soriano, 1997). This discrepancy could be due to different genetic backgrounds of the mutations in question, or might point to insufficiency of a gene other than \( PDGFR\alpha \) in the \( Ph \) mice (e.g. \( c\text{-}kit \)). It will be interesting to see if the \( PDGFR\alpha \) YAC can complement defective heart development in \( Ph \) mutant mice. Our preliminary data suggest that it does not.
3. PDGFRα signalling is essential for mouse early development

Note that PDGF-A null mice do not develop the craniofacial defects and spina bifida of the PDGFRα nulls, suggesting that PDGF-BB might be an important ligand for PDGFRα in vivo. Against this, the phenotypes of PDGF-B and PDGFRβ null mice are indistinguishable (both die at birth with malformed kidneys and leaky blood vessels), implying that PDGF-BB activates exclusively PDGFRβ in vivo (Leveen et al., 1994; Soriano, 1994). Moreover, PDGF-A/PDGFR-B double-null mice do not recapitulate the PDGFRα null phenotype (personal communication from C. Betsholtz, University of Goteborg, Sweden).

Alternative explanations for the more severe phenotype of PDGFRα compared to PDGF-A knockouts include (1) PDGF-AA or PDGF-AB might normally be supplied in utero by the mother. However, cells that express PDGF-A and PDGFRα are closely apposed in the embryo, suggesting that PDGF signalling is normally in local effect. (2)There might be some ligand-independent activation of PDGFRα in vivo. One of the candidate molecules to activate PDGFRα could be the transmembrane chondroitin sulfate proteoglycan NG2. In the CNS, NG2 and PDGFRα have been found to be co-expressed by OLPs (Nishiyama et al., 1996a). The proliferation of OLPs in response to PDGF can be blocked by antibodies against NG2 (Nishiyama et al., 1996b). Similarly, the ability of rat aortic smooth muscle cells to divide and to migrate in response to PDGF-AA, but not to PDGF-BB, can be both inhibited by antibodies against NG2 (Grako and Stallcup, 1995). Furthermore, dissociated aortic smooth muscle cells from NG2 deficient mice fail to proliferate or migrate in response to PDGF-AA (Grako et al., 1999). These observations imply an important role for NG2, at least in potentiating activation of the PDGFRα by PDGF-AA. (3) There might be additional undiscovered ligand(s) for PDGFRα. In fact, the existence of a third PDGF isoform (PDGF-C) that
activates PDGFRα has been discovered recently (personal communication from C. Betsholtz, University of Goteborg, Sweden).

In conclusion, my YAC transgene recapitulates some but not all of the endogenous expression pattern and does not fully rescue the PDGFRα null mouse probably because it fails to be expressed properly in certain lung cells, leading to respiratory failure at birth. Nevertheless, my demonstration that the craniofacial defects of the knockout can be rescued by a wild type PDGFRα transgene opens the way to a mutational analysis of PDGFRα in vivo. This is an important application of YAC transgenic technology in general.
Fig. 4.1. Analysis of CEPH 449C2 and CEPH 29E11 YACs carrying human PDGFRα.

(A) Pulsed-field gel electrophoresis (PFGE) analysis of YACs 449C2 and 29E11. The chromosomes of the wild-type yeast strain (control lane) and yeast strains carrying 449C2 and 29E11 YACs were separated by PFGE (1% agarose gel, 0.5xTBE buffer, 200V for 30 hours at 4°C with a 60-100 second switch time) and detected by ethidium bromide staining. The size of each YAC was determined by that of the chromosomes of the control yeast strain, indicating that YAC 449C2 is ~380 kb and 29E11 is ~470 kb (arrowheads). (B) Analysis of the integrity of YACs by Southern blots. Each YAC was separated by PFGE (1% agarose gel, 0.5xTBE buffer, 200V for 30 hours at 4°C with a 30 second switch time), blotted onto nylon membrane and hybridized with [γ-32P]ATP-labelled oligonucleotides specific for human PDGFRα exon 1 (H1: 5'-CTG GAC ACT GGG AGA TTC GGA G-3') and 3'-untranslated region (UTR) (H3: 5'-GTG ATG TCC TTA AAA TGT GGT-3'). A 380 kb band in 449C2 and a 470 kb band in 29E11 were detected using probes either in PDGFRα exon 1 or 3'-UTR.
Fig. 4.2. Determination of mating type in window yeast strains carrying 449C2 and 29E11 YACs by PCR. The mating type of original yeast strains carrying YAC 449C2 and 29E11 is MATα, while the mating type of window strains is MATα. Three primers (MAT-1: 5'-AGT CAC ATC AAG ATC GTT TAT GG-3'; MAT-2: 5'-GCA CGG AAT ATG GGA CTA CTT CG-3'; MAT-3: 5'-ACT CCA CTT CAA GTA AGA GTT TG-3') were used to detect the mating type by PCR (94°C for 3 minutes, 30 cycles of 92°C 30 seconds, 58°C 60 seconds and 72°C 60 seconds, then 72°C for 10 minutes). Colony-1 in 449C2 transfer was haploid (mating type α), whereas colony-2 and -3 were diploid. Four colonies in 29E11 transfer were haploid (mating type α). They displayed the same mating type as their host window strains (mating type α). The resulting YACs were named 449-W2 and 29-W3 as indicated.
Fig. 4.3. Analysis of 449C2 and 29E11 YACs transferred into window strains by PFGE. (A) Transfer of 449C2 YAC (~380 kb) that comigrates with endogenous yeast chromosome (~365 kb) into window strain YLBW2. YLBW2 contains a “window” (endogenous yeast chromosome-free zone) in the size range (250-450 kb) which allows the free migration of 449C2 YAC (arrowhead) within this window. Similarly, 29E11 YAC (~470 kb) that comigrates with endogenous yeast chromosome (~450 kb) was transferred into window strain YLBW3 which contains a “window” in the size range 310-590 kb (B). (C) Analysis of the integrity of transferred YACs by Southern blots. The original YACs 449C2 and 29E11 and transferred YACs 449-W2 and 29-W3 were separated by PFGE (1% agarose gel, 0.5xTBE buffer, 200V for 30 hours at 4°C with a 30 second switch time), blotted onto nylon membrane and hybridized with [γ-32P]ATP-labelled oligonucleotides specific for human PDGFRα 3'-UTR (H3: 5'-GTG ATG TCC TTA AAA TGT GGT-3'). 380 kb bands in 449C2 and 449-W2, and 470 kb bands in 29E11 and 29-W2 were detected using this probe.
Fig. 4.4. Two-step gel purification of intact YAC DNA for microinjection. (A) The first step gel isolation of YAC DNA by PFGE. Yeast carrying YAC 449C2 was used as maker lanes on both side of yeast embedded in high-density in agarose plugs, which had carried YAC 449-W2. After preparative-PFGE, both sides of the gel were cut off and stained in ethidium bromide (see details in 2.4.8). The region of the gel containing the YAC (arrowhead) and the endogenous yeast chromosomes indicated as marker 1 and marker 2 (above and below the YAC DNA band) was excised for the second step gel isolation. The YAC DNA was concentrated by the second step gel isolation which was run at a 90° angle to the PFGE run (B) (see details in 2.4.8). Arrows in (A) and (B) indicate the direction of each gel run. The concentrated YAC DNA was excised according to the location of marker 1 and marker 2. The concentration of purified YAC DNA was determined using \( \lambda \) DNA of known concentrations of 10ng, 5 ng and 2.5 ng indicated in (C). The integrity of purified YAC DNA was checked further by PFGE (D). A 380 kb sharp band, the same size as the 449C2 YAC, was detected, indicating that the purified YAC DNA was intact.
Fig. 4.5. Genotyping of the human PDGFRα YAC transgenic mice by PCR. DNA from tail tips of the 3-4 weeks old mice was analyzed by PCR. The following primers were used to verify the presence of the YAC ends (the PCR condition: 94°C for 3 minutes, 35 cycles of 92°C 30 seconds, 62°C 45 seconds and 72°C 45 seconds, then 72°C for 10 minutes) and the PDGFRα insert (the PCR condition: 94°C for 5 minutes, 33 cycles of 94°C 30 seconds, 53°C 45 seconds and 72°C 1 minute, then 72°C for 10 minutes): YAC vector left arm (L1: 5'-CAC CCG TTC TCG GAG CAC TGT CCG ACC GC-3' and L2: 5'-CCT TAA ACC AAC TTG GCT ACC GAG A-3'); vector right arm (R1: 5'-ATA TAG GCG CCA GCA ACC GCA CCT GTG GCG-3' and R2: 5'-GTA ATC TTG AGA TCG GGC GTT CGA-3'); human PDGFRα exon 1 (H1: 5'-CTG GAC ACT GGG AGA TTC GGA G-3' and H2: 5'-CGA TGT TAT TCC GCA ATG AAT G-3'); 3'-UTR (H3: 5'-GTG ATG TCC TTA AAA TGT GGT-3' and H4: 5'-GTA ATA CAT TTT GTA TTG GTA G-3'); exon 20 (E20F: 5'-TGG CAC CCC TTA CCC CGG CA-3' and E20R: 5'-ACT TCA CTG GTA GCG TGG T-3'). Human genomic DNA (lane 5) and purified 449C2 YAC DNA (lane 6) were used as positive controls. After injection of 449-W2 YAC DNA, four founders were detected among 43 new-borns. Founders 3-3 (lane 1), 5A1 (lane 2) and 8A4 (lane 4) all appeared to contain full-length YACs as indicated. In founder 7-2 (lane 3), only the 3'-UTR segment was detected using primers H3 and H4.
Fig. 4.6. Genotyping of the human PDGFRα YAC transgenic mice by Southern blots. DNA from tail tips of the 3-4 weeks old mice was digested with EcoRI at 37°C overnight, electrophoresed on a 0.8% agarose gel, transferred onto nylon membrane and hybridized with [α-32P]dCTP-labelled human PDGFRα cDNA probes in extracellular domain (ECD) and 3'-UTR. The cDNA probe in ECD was a ~1.1 kb EcoRI-NcoI fragment and the probe in 3'-UTR was a ~1.5 kb EcoRI-NotI fragment. Wild-type mouse genomic DNA and human genomic DNA were used as controls. A single band was detected in DNA from founders 3-3, 5A1, 8A4 and 7-2, as well as in human DNA, using probe in 3'-UTR. Two bands were observed only in DNA from founders 3-3, 5A1 and 8A4 but not founder 7-2 using probe in ECD, indicating that YAC in founder 7-2 was not intact.
Human
Fig. 4.7. Determining the numbers of integrated YAC transgenes in transgenic lines.
DNA from transgenic mice was subjected to quantitative PCR analysis (see 2.4.12). The ratio of the signals from the human transgene signal compared to the endogenous mouse gene is shown below the autoradiogram. About 22 copies of human PDGFRα had integrated into the diploid mouse genome in line 3-3, about 10 copies in line 5A1 and 2 copies in line 8A4.
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Fig. 4.8. Expression of endogenous mouse PDGFRα and human transgene-derived PDGFRα outside the CNS. At E10.5, whole-mount in situ hybridization showed that the endogenous PDGFRα (A) and the human PDGFRα transgene (B) were both expressed in branchial arch mesenchyme (white arrowheads), limb buds (white arrows) and somites (arrows). The human transgene was also ectopically expressed in the dorsal neural tube (arrowheads in B). At E12.5, in situ hybridization on transverse sections through embryos indicated that both endogenous mouse PDGFRα and human PDGFRα are expressed in the sclerotome and dermatome (C, D), but that expression of the transgene was generally weaker and less extensive. Endogenous mouse PDGFRα but not human PDGFRα was expressed in the aortic valve and aortico-pulmonary spiral septum of the heart (E, F). At E14.5, the human PDGFRα transgene (I, J), like the endogenous gene (G, H), was expressed in the periosteum/perichondrium of developing vertebrae (G, I) and ribs (H, J). However, the transgene was also expressed in the chondrocytic core of the cartilage (arrows in I and J) where endogenous PDGFRα is not expressed. In situ hybridization to cross sections through E12.5 eyes showed that endogenous PDGFRα (K), as well as the transgene (L), were expressed in the anterior lens epithelium, but the human transgene was also aberrantly expressed in posterior lens fibres (L).
Fig. 4.9. Expression of endogenous and transgene-derived PDGFRα transcripts in embryonic spinal cord. Transverse sections through cervical spinal cords of wild-type (A-D) and transgenic (E-H) mice at the indicated ages were hybridized with DIG-labelled mouse and human PDGFRα probes, respectively (see 2.3.10). PDGFRα⁺ cells first appeared in the ventral ventricular zone (VZ) in the E12.5 wild-type spinal cord. The human PDGFRα⁺ cells were also detected in the ventral VZ on E12.5 (arrows in E). However, strong ectopic expression of human PDGFRα was also observed in two columns of cells outside the dorsal VZ (E). Soon after they appear, both mouse and human PDGFRα⁺ cells proliferated and moved away from the ventral VZ (B, C, F, G), and the ectopic dorsal expression of the transgene had declined (F) and disappeared by E15.5 (G). By the time of birth these PDGFRα⁺ cells had spread more-or-less evenly throughout the cross-section of the cord (D, H). Scale bar: 200μm.
Fig. 4.10. Ectopic expression of the PDGFRα transgene partly mimics the normal expression of c-kit and flk-1. Transverse sections through the cervical spinal cords of E10.5 (A-D), E12.5 (E, F) and E13.5 (G, H) embryos were subjected to in situ hybridization with DIG-labelled probes for mouse PDGFRα (A), human PDGFRα (B), c-kit (C, E, G) or flk-1 (D, F, H). At E10.5, endogenous PDGFRα was expressed in the mesenchyme outside the CNS but not in the spinal cord (A). However, the human PDGFRα transgene was ectopically expressed in cells in the dorsal and ventral spinal cord, just outside the VZ (B). The transgene was also erroneously expressed outside the dorsal spinal cord, in presumptive neural crest (arrows in B) and in two lateral clusters of cells in the ventral spinal cord (arrowheads in B). At E10.5 c-kit was expressed faintly in cells just outside the dorsal and ventral VZ (C). At E12.5 (E) and E13.5 (G), c-kit expression was restricted to the dorsal cord. The ectopic expression of human PDGFRα partly mimics endogenous c-kit (compare B and C, Figs. 4.9E, 4.10E and Figs. 4.9F, 4.10G). flk-1 was expressed in a punctate pattern in blood vessels throughout the spinal cords (D, F, H). Neither mouse PDGFRα nor mouse c-kit were expressed in the walls of vertebral arteries (I, K), whereas human PDGFRα and mouse flk-1 were expressed there (J, L, and M, N). Scale bar: 200μm for A-H, 80μm for I-N.
Fig. 4.11. Craniofacial and skeletal defects in homozygous PDGFRα null (KO) mice were rescued by the human PDGFRα YAC. At E16.5 KO embryos displayed a cleft face and internal bleeding (compare A, B). Skeletal preparations of KO embryos of the same age indicated that the mandibular bone (mb), nasal bone (nb) and frontal bone (fb) had failed to fuse in the midline (compare D, E). From the lateral and back view of the skeletons of intact E16.5 embryos, KO embryos are clearly smaller than wild type and have severe arching of the spinal column and abnormal vertebrae (compare G, H and J, K). The human PDGFRα YAC rescued all these craniofacial defects in KO-YAC mice (C, F, I, L). Note that the KO-YAC mouse shown is one day older than the others (E17.5).
$PDGFR_{\alpha}$

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Fig. 4.12. Comparison of PDGFRα (+/-), wt-YAC and KO-YAC lungs at P0. Lungs were dissected from newborn PDGFRα (+/-), wt-YAC and KO-YAC mice. The size of KO-YAC lungs is markedly smaller than those of PDGFRα (+/-) or wt-YAC lungs.
Fig. 4.13. Abnormal lung development in KO-YAC mice. Sections through wild type (+/+) PDGFRα (+/-), YAC transgenic (wt-YAC), null (KO) and KO-YAC embryos were subjected to in situ hybridization with probes to mouse PDGFRα (A, C, D, F, G, J, K, N, O) and human PDGFRα (B, E, H, I, L, M, P, Q). In E15.5 KO lung, endogenous mouse PDGFRα was expressed in mesenchymal cells surrounding epithelial tubules as in wild type lungs (A, D), but with a lower signal level (C, F). Human PDGFRα transgene was also expressed in mesenchymal cells but at a low level (B, E).

At E17.5, scattered PDGFRα+ cells were detected in PDGFRα (+/-) lungs (G and arrows in K), but fewer human PDGFRα+ cells were detected in wt-YAC and KO-YAC lungs (arrows in L, M). At this stage, sac-like structures, the future alveoli, have formed through continued branching (G, H). This process was not obvious in KO-YAC and KO lungs (I, J, M, N). Higher magnification also showed generally thicker mesenchyme (arrowheads in M, N). At P0, the sac-like structures had expanded to a greater degree in (+/-) and wt-YAC lungs, characterized by a thinning mesenchyme (O, P). The thinning of the mesenchyme did not happen in KO-YAC lungs at this stage (arrowheads in Q).

Fig. 4.14. *PDGFRα* and *MBP* expression in spinal cords of wild type (+/+), *PDGFRα* (+/-), YAC transgenic (wt-YAC), *PDGFRα* null (KO) and KO-YAC mice. At E14.5, both endogenous and human *PDGFRα*+ cells proliferate and migrate from the ventral spinal cord and almost spread throughout the whole spinal cord (A, B). However, at the same age, *PDGFRα* null mutant mouse has an abnormal shape and only fewer *PDGFRα*+ cells were detected in *PDGFRα* null spinal cord (arrows in C). By E17.5, endogenous and human *PDGFRα*+ cells distribute evenly in spinal cords of *PDGFRα* (+/-) (D), wt-YAC (E) and KO-YAC (F). *PDGFRα*+ cell was not observed in KO spinal cord (G). Expression of *myelin basic protein* (MBP) was detected in all cords (H–J), except that the distribution of MBP+ cells in KO spinal cord is abnormal (K). At P0, *PDGFRα*+ (L, M) and MBP+ (N, O) cells were observed in spinal cords of *PDGFRα* (+/-) (L, N) and KO-YAC (M, O) mouse. Scale bars: 200μm.
Fig. 4.15. Spinal cord cell cultures of E14.5 wild type, PDGFRα null (KO) and KO-YAC embryos. Spinal cord cells were cultured for several days until the equivalent of the day of birth (E19), then fixed, immunolabelled with anti-galactocerebroside (GC) and photographed under fluorescence (A, C, E) and phase contrast optics (B, D, F). Many fewer GC+ oligodendrocytes developed in cultures of PDGFRα null spinal cord than in wild-type and KO-YAC cultures. (G) In one representative experiment, 630 ± 62 GC+ cells per coverslip were detected in wild-type cultures, 612 ± 9 GC+ cells per coverslip in KO-YAC cultures, while only 57 ± 6 GC+ cells per coverslip were detected in homozygous PDGFRα null cultures (means ± range, duplicate coverslips, one embryo of each genotype). Scale bar: 50μm.
Table 4.1. Summary of genotyping of human *PDGFRα* YAC transgenic mice

<table>
<thead>
<tr>
<th>Mice (F1)</th>
<th>YAC arm (left)</th>
<th>Exon1</th>
<th>Exon20</th>
<th>UTR</th>
<th>YAC arm (right)</th>
<th>Southern Blot</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>L1/L2 (250bp)</td>
<td>H1/H2 (286bp)</td>
<td>E20F/R (99bp)</td>
<td>H3/H4 (347bp)</td>
<td>R1/R2 (150bp)</td>
<td>ECD</td>
</tr>
<tr>
<td>3-3 (M)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>5A1 (M)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>7-2 (M)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>8A4 (F)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

After injection of 449-W2 YAC DNA, four founders (3-3, 5A1, 7-2 and 8A4) were detected among 43 newborns. The efficiency of transgenesis is about 9%. Three founders (3-3, 5A1 and 8A4) contain full length human *PDGFRα* YAC.
Table 4.2. Expression of endogenous mouse *PDGFRα* and the human *PDGFRα* YAC transgene outside the CNS

<table>
<thead>
<tr>
<th>Tissues</th>
<th>Endogenous <em>PDGFRα</em></th>
<th>Human <em>PDGFRα</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Dorsal mesoderm</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sclerotome</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>Dermatome (dermis)</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Myotome</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Lateral mesoderm</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Perichondrium/periosteum</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Chondrocytic core</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Mesenchyme (connective tissues)</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>Heart</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>Blood vessels</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>Limb</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>Tongue</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td><strong>Notochord</strong></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Mesenchyme of endoderm</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trachea</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>Esophagus</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>Lung</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Thymus</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td><strong>Ectoderm</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lens epithelium</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Branchial arches</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>Dorsal root ganglia</td>
<td>+</td>
<td>++</td>
</tr>
</tbody>
</table>
Expression of human *PDGFRα* YAC transgene in E12.5 transgenic mice (strains 3-3 and 5A1) compared with the expression of endogenous mouse *PDGFRα* in wild type mice of the same age. Numbers of the "+" reflect the expression level of *PDGFRα* gene in transgenic and wild type mice.
Chapter 5

Pax6 influences the time and site of origin of oligodendrocyte progenitors in the ventral spinal cord*

Introduction

During development of the vertebrate central nervous system (CNS), distinct neuronal and glial cell types are produced at different dorsoventral levels of the neural tube. The initial dorsoventral polarity of the tube is established through a combination of dorsalizing and ventralizing signals from the dorsal and ventral midlines, respectively. The main ventralizing signal is the product of the Sonic hedgehog (Shh) gene, the vertebrate homologue of the Drosophila patterning gene hedgehog. From an early stage of development, Shh secretion by the notochord and floor plate generates a ventral-to-dorsal (high-low) concentration gradient in the adjacent neural tube. Initially, this acts to suppress dorsal markers in the ventral neural tube. Then, when a ventral identity has been established, graded Shh signals provide positional cues that direct the development of specific neuronal and glial cell types.

Ventral neurons are derived from dividing neuroepithelial precursors that reside at the ventricular surface of the basal plate. These precursors generate post-mitotic progenitor cells that migrate radially and differentiate into motor neurons (MNs) or interneurons (INs), which can be classified by their combinatorial expression of LIM-homeodomain transcription factors and other proteins (Burrill et al., 1997; Ericson et al., 1997; Matise and Joyner, 1997; Tsuchida et al., 1994; Varela-Echavarria et al., 1996). The precursors of these different types of neurons are specified at different positions along the dorsal-ventral axis, defined by the local concentrations of Shh and other factors. On top of this, signals from the flanking paraxial and surface mesoderm confer regional specificity of MN subtype along the anterior-posterior axis (Ensini et al., 1998; Muhr et al., 1997).

The intracellular effects of Shh signaling are mediated in part by the transcription factor Pax6 (Burrill et al., 1997; Ericson et al., 1997; Goulding et al., 1993; Osumi et al., 1997). Shh represses Pax6, so that Pax6 expression is high where Shh is low and vice versa. As a result, the ventral half of the neural tube is subdivided into a Pax6-negative domain abutting the floor plate and a more dorsal Pax6-positive domain that displays a gradient
of Pax6 expression, increasing ventral-to-dorsal (Ericson et al., 1997; Osumi et al., 1997). Pax6 in turn represses expression of the transcription factor Nkx2.2, which is therefore restricted to the Pax6-negative domain (Ericson et al., 1997). In the cervical spinal cord, neuroepithelial precursors in the ventral part of the Pax6-positive domain generate somatic MNs of the median motor column (MMC), which express LIM-domain proteins Isll, Isl2, Lim3 (also known as Lhx3) and Gsh4 (Lhx4) (Ericson et al., 1997; Li et al., 1994; Seidah et al., 1994; Tsuchida et al., 1994; Zhadanov et al., 1995). Precursors from more dorsal parts of the Pax6-positive domain generate several classes of ventral INs that express different combinations of Pax2, Enl, Evx1, Lim1, Lim3, Gsh4 and Chx10, (Burrill et al., 1997; Ericson et al., 1997). Precursors from the Pax6-negative, Nkx2.2-positive domain generate a subset of MNs that express Isl1 but not Isl2, and a population of cells that express Sim1, the vertebrate homologue of the Drosophila gene single-minded (Ericson et al., 1997, Fan et al., 1996).

Inactivating point mutations in Pax6 underlie multiple eye and brain defects in the Small eye (Sey) mouse and rSey rat (Hill et al., 1991; Matsuo et al., 1993; Stoykova et al., 1996; Warren and Price, 1997). In homozygous mutants the ventral Nkx2.2-positive domain expands dorsally into what would normally be Pax6 territory. Consequently, Isl2/Lim3-expressing MNs are wholly or partly respecified as more ventral cell types (Ericson et al., 1997; Osumi et al., 1997; Varela-Echavarria et al., 1996). In the brainstem this results in loss of hypoglossal and abducens MNs (and corresponding cranial nerves) and a gain of vagal MNs, while in the spinal cord there seems to be an increase in the number of Sim1-expressing cells at the expense of somatic MNs (Ericson et al., 1997; Osumi et al., 1997; Tsuchida et al., 1994; Varela-Echavarria et al., 1996). Pax6-deficient animals also lack En1/Pax2-expressing INs in both brainstem and spinal cord (Burrill et al., 1997; Ericson et al., 1997). On the other hand, in the homozygous Nkx2.2 mutant spinal cord, the Pax6-positive domain has not changed (Briscoe et al., 1999). However, there is a great reduction of V3 neurons derived from the Nkx2.2 domain, which coexpress Sim1 and Ngn3 genes. The loss of Nkx2.2 results in the respecification of V3 neurons into somatic MNs (Briscoe et al., 1999).
Glial cells are also derived from neuroepithelial precursors in the ventral half of the spinal cord. Oligodendrocytes (OLs), the myelin-forming cells of the CNS, develop from migratory, proliferating oligodendrocyte progenitors (OLPs) that descend from a small number of precursors in the ventral ventricular zone (VZ) near the floor plate (Nishiyama et al., 1996; Noll and Miller, 1993; Ono et al., 1995; Pringle and Richardson, 1993; Pringle et al., 1998; Timsit et al., 1995; Yu et al., 1994: reviewed by Miller, 1996; Richardson et al., 1995). The first OLPs appear in the VZ on embryonic day 14 in the rat (E12.5 in the mouse), shortly after the end of MN production (Altman and Bayer, 1984; Nornes and Das, 1974). Shh has been shown to induce the development of oligodendrocytes in explant cultures of dorsal or intermediate neural tube at Shh concentrations that overlap those required for MN induction (Orentas et al., 1999; Poncet et al., 1996; Pringle et al., 1996). We have suggested, therefore, that the same population of neuroepithelial precursors might give rise first to MNs, then to OLPs (Richardson et al., 1997).

Here I conducted experiments to compare the requirements of OLPs and ventral neurons for Pax6 in the mouse cervical spinal cord. I established that OLPs arise from the most ventral region of the Pax6-positive domain, which also is reported to give rise to Isl2/Lim3 MNs. In Sey/Sey mice the origin of OLPs was shifted dorsally and their appearance delayed by up to a day. The appearance of Isl2/Lim3 MNs was also delayed by about a day. Thus, Pax6 is not absolutely required to specify cell fate but it influences the time and pattern of fate decisions in the VZ. These results indicate a close developmental relationship between Isl2/Lim3 MNs and OLPs, suggesting that both cell types descend from the same set of neuroepithelial precursors. Fate switching might depend either on a cell-intrinsic programme in the precursors or feedback control from Isl2/Lim3 MNs. Against the latter hypothesis, we found that OLs develop normally in explant cultures of Isl1(-/-) spinal cords, which do not contain any MNs. Therefore, I propose that a specific subset of neuroepithelial precursors first generates Isl2/Lim3
MNs and then switches to OLPs, and that the neuron-glia fate switch is part of a stereotyped developmental program initiated by Shh together with Pax6.

**Results**

1. *Oligodendrocyte progenitors originate in the ventral-most part of the Pax6-positive domain*

In transverse sections of E12.5 mouse spinal cord, OLPs first appear as bilateral foci of PDGFRα-positive cells in the ventral VZ (Fig. 5.1B) (Calver et al., 1998; Hardy, 1997; Nishiyama et al., 1996; Pringle and Richardson, 1993; Pringle et al., 1998; Yu et al., 1994). I attempted to define the site of origin of OLPs with more precision by mapping the positions of a total of 28 PDGFRα+ OLPs in sections of E12.5 spinal cord, relative to the ventral and dorsal midlines (see 2.3.12). I found that the majority of OLPs clustered in a tightly defined domain of the VZ ranging from about 9% to about 13% of the way from the ventral toward the dorsal midline, measuring along the surface of the spinal cord lumen (Fig. 5.1D). The mean position was at 11% ± 2% (mean ± s.d., n=28), about 10 to 15 cell diameters from the edge of the floor plate. I also determined the location of the first OLPs relative to the Pax6- and Nkx2.2-positive domains by comparing adjacent sections of cervical spinal cord (C3-C5) hybridized *in situ* with probes for PDGFRα, Pax6 or Nkx2.2.

*pax6* is initially expressed in all regions of the neural tube, with the exception of the ventral midline (Gruss and Walther, 1992). By E9.5, when the first MNs begin to differentiate, its expression is down-regulated and can no longer be detected in the ventral-most region adjacent to the floor plate, while in the remainder of the basal plate *pax6* transcripts form a dorsal-ventral (high-low) concentration gradient (Ericson et al., 1997). This pattern persists in the E12.5 neural tube (Figs. 5.1A, 1E).
Nkx2.2 is a homeodomain protein related to the Drosophila NK-2 gene product (Price et al., 1992). From before E9.5 to at least E12.5 it is expressed in cells in the most ventral part of the neural tube adjacent to but not including the floor plate (Ericson et al., 1997; Shimamura et al., 1995; Figs. 5.1C, 1G). The dorsal expression limit of Nkx2.2 approximately coincides with the ventral limit of Pax6 (Ericson et al., 1997). This, together with the observation that Nkx2.2 expression spreads dorsally in the Small eye (Sey) mutant mouse, which lacks Pax6 function, suggests that Pax6 acts to repress Nkx2.2 expression (Ericson et al., 1997).

Direct comparison of PDGFRα with Pax6 and Nkx2.2 expression in adjacent sections indicates that OLPs are derived from the ventral-most region of the Pax6 expressing domain, possibly in a small region of overlap with Nkx2.2 (Figs. 5.1E-1H). Therefore, it is likely that the earliest OLPs express a low level of Pax6 and possibly also a low level of Nkx2.2. Isl2/Lim3-positive somatic MNs also are believed to come from the ventral-most part of the Pax6 domain (Ericson et al., 1997).

2. Appearance of oligodendrocyte progenitors is delayed in Sey/Sey mice

Having established that OLPs arise from within the Pax6-positive region of the VZ, I investigated the role of Pax6 in development of these cells by examining Sey mutant mice. In wild type spinal cord PDGFRα+ OLPs are first detected in the ventral VZ at E12.5 (Calver et al., 1998) (Figs. 5.1B, F). After that, they divided and migrated from the ventral VZ (Fig. 5.2A, panels A, C and E). In Sey/Sey spinal cords PDGFRα+ OLPs could not be detected until E13.5, one day later than normal (see Figs. 5.4B, 4E). Subsequently, they proliferated and eventually accumulated to normal steady-state numbers, but with a delayed time course relative to wild-type (Fig. 5.2A). I also counted the numbers of PDGFRα+ OLPs in transverse sections of wild-type, Sey/+ and Sey/Sey spinal cords at different embryonic ages (Fig. 5.2B). The time of appearance and subsequent proliferation of PDGFRα+ OLPs were similar in wild-type, Sey/+ spinal
cords, whereas they were retarded in Sey/Sey spinal cords. By E18.5, wild type and Sey spinal cords were indistinguishable (Fig. 5.2A, panels G, H and Fig. 5.2B).

3. Oligodendrocyte progenitor cell cycle dynamics in Sey/Sey embryos

A distinctive feature of OLP early development is their cell cycle kinetics. Their division cycle is initially very short—6 to 8 hours—but slows down to about 24 hours over a period of a few days before birth. In other cell populations Pax6 has been implicated in the control of cell proliferation (Warren and Price, 1997), so we asked whether OLP cell cycle dynamics were altered in Sey/Sey mice.

I compared cell division rates in mutant and wild type OLPs by bromodeoxyuridine (BrdU)-labeling in utero. At different embryonic ages I gave a single injection of BrdU into the mother, fixed the embryos two hours later and double-labeled spinal cord sections for BrdU and PDGFRα to identify OLPs that had been in S phase during the labeling period (Figs. 5.3A, B). Since the previous work in this lab has shown that all OLPs are actively engaged in the division cycle (Calver et al., 1998), the BrdU labeling index (proportion of OLPs that incorporates BrdU) correlates inversely with the average cell cycle time.

In wild type embryos the BrdU labeling index was over 60% at E12.5 but fell rapidly as cell numbers increased, reaching a stable low value of around 15% by E18.5 (Fig. 5.3C). This is in line with previous in vivo labeling experiments in this lab (Calver et al., 1998). In Sey/Sey littermates the BrdU labeling index also started high (around 60%) and declined to about 15% by E18.5 (Fig. 5.3C). I conclude that the cell cycle kinetics of OLPs are similar in Sey/Sey and wild type embryos, except that the Sey/Sey curve is displaced to later ages, as expected, since the first OLPs appear later in the mutant. Pax6 therefore appears to have at most a subtle effect on OLP proliferation.
4. The site of origin of Oligodendrocyte progenitors in the ventricular zone is shifted dorsally in Sey/Sey embryos

If Pax6 helps relay positional information from Shh to the cell nucleus, one would expect positional values to be altered in Sey/Sey mice. The fact that PDGFRα marks progenitor cells before they leave their site of origin in the neuroepithelium allowed us to test this prediction. I mapped the positions of emergent PDGFRα⁺ OLPs in the VZ of E13.5 Sey/Sey embryos. These cells were clearly displaced dorsally relative to wild type embryos — 25% ± 3% (mean ± s.d., n=11) of the distance from the ventral towards the dorsal midline, compared to 11% ± 2% in wild types (Fig. 5.4C; compare Fig. 5.1D and Figs. 5.4E, 4F). Although the cells appear up to a day later in Sey/Sey than in wild type, this is unlikely to explain the shift because I found that there was no significant change in the circumference of the spinal cord lumen between E12.5 and E13.5. I conclude that the lack of Pax6 results in a dorsal-ward shift in specification of OLPs.

5. Appearance of specific neuronal populations is delayed in Sey/Sey embryos

If the dorsal shift in precursor cell specification were a general consequence of Pax6 deficiency, we would expect ventral neurons to develop in Sey/Sey mice, but to originate from more dorsal parts of the VZ. Because the markers commonly used for MNs and INs are not expressed fully until after the cells leave the VZ, it was not possible to test this prediction. However, it seemed possible that a dorsal shift might be accompanied by a delay in their appearance, as for OLPs. I therefore followed the expression of three neuronal markers—Isll, Isl2, and Lim2—in cervical spinal cords of wild type and Sey mutant mice from E9.5 to E13.5. Somatic MNs of the MMC express all these marker genes, while one class of ventral INs expresses Lim3 but neither Isll nor Isl2 (Ericson et al., 1997). Other classes of spinal cord MNs express Isll but not Isl2.

In wild type spinal cord, expression of all three markers was detected at E9.5 (Figs. 5.5A-5C). In Sey/Sey embryos at the same morphological stage Isll labeling was similar
in intensity to wild type, though it extended further dorsally (Fig. 5.5D), but neither \textit{Isl2} nor \textit{Lim3} could be detected (Figs. 5.5E, 5F). A day later, at E10.5, \textit{Isl2} and \textit{Lim3} expression was evident in the mutant but fewer cells were labeled than in wild type, particularly for \textit{Isl2} (Fig. 5.6). \textit{Isl1} labeling, which was relatively normal at E9.5, marked significantly fewer cells in \textit{Sey/Sey} than in wild type at E10.5; in particular, there appeared to be a ventro-medial population of \textit{Isl1}-positive cells that were absent from the \textit{Sey/Sey} cord (Figs. 5.6A, 6D). Also, a group of presumptive sensory neurons in the dorsal cord was missing at E10.5 (Fig. 5.6D). At E11.5, there were still fewer \textit{Isl2} and \textit{Lim3} labeling cells in the mutant than in wild type (Fig. 5.7). A general reduction of \textit{Isl1}-positive cells in dorsal and ventral \textit{Sey/Sey} spinal cord was also observed (Figs. 5.7A, 7D). However, by E13.5 expression of all three markers appeared normal in the \textit{Sey/Sey} spinal cord, both in the number and distribution of labeled cells (Fig. 5.8).

6. \textit{Oligodendrocytes develop normally in vitro in the absence of motor neurons}

The observation that OLPs appear to be generated in the same part of the ventral VZ as somatic MNs (see above) suggests that the same set of neuroepithelial precursors might first generate MNs, then switch to production of OLPs. This neuron-glia switch might be part of an intrinsic developmental program in the neuroepithelial precursors. Alternatively, the switch might be triggered by feedback signals from the newly-formed MNs. To test the latter idea, we looked to see if OLs can develop in \textit{Isl1(-/-)} spinal cord, which lack all classes of MNs and at least one class of ventral (V1) INs (Pfaff et al., 1996). Because the \textit{Isl1(-/-)} mice die \textit{in utero} before the emergence of the OL lineage, we studied OL development in spinal cord explant cultures.

Explant cultures from the spinal cords of E9.5 embryos were established. The embryos were genotyped retrospectively by PCR and \textit{Isl1/2} immunolabeling as described in 2.3.7. After culturing until the equivalent of the day of birth (11 days in culture), the explants were immunolabeled with anti-galactocerebroside (GC) or anti-myelin basic protein (MBP) to visualize differentiated OLs. In five independent experiments on five
separate litters, we examined a total of 11 \textit{Isl1}(-/-), 26 \textit{Isl1}(+/-) and 6 (+/+) embryos. OLs developed in explants from every one of these embryos (Fig. 5.9). We conclude that signals from \textit{Isl1}-expressing MNs are unlikely to be required for normal development of OL lineage cells in the spinal cord.

**Discussion**

Neuroepithelial precursors in the ventral VZ of the spinal cord generate MNs and INs, and then a subset of precursors starts to produce OLPs. I have mapped the site of origin of OLPs in the spinal cord and found that OLPs originate in the ventral-most part of the \textit{Pax6}-positive VZ, which at earlier times generates somatic MNs. In \textit{Pax6}-deficient Small eye mice, the origin of OLPs is shifted dorsally and both OLPs and \textit{Isl2/Lim3} MNs are delayed. I suggest that somatic MNs and OLPs are generated sequentially from a common set of precursors whose position in the VZ is influenced by \textit{Pax6}.

1. **Motor neurons and oligodendrocyte progenitors may descend from the same neuroepithelial precursors in a cell-intrinsic program**

Development of ventral neurons and OLPs depends on Shh signaling in vivo and both MNs and OLPs are induced by the same concentrations of Shh in vitro (Pringle et al., 1996). This co-dependence on Shh, together with other evidence (see below), led us to suggest (Pringle et al., 1996; Richardson et al., 1997) that MNs and OLPs might descend from a common group of neuroepithelial MN-OL precursors, specification of which is dependent on Shh. If so, we expect that MNs and OLPs should originate from the same site(s) within the VZ. In support of this idea, I have established that OLPs arise in the ventral-most region of the \textit{Pax6}-positive domain of the spinal cord VZ, the same region that at earlier times is thought to give rise to somatic MNs of the MMC (Ericson et al., 1997; Tsuchida et al., 1994).
In *Sey/Sey* mice, which lack functional Pax6 protein, somatic MNs and some ventral INs in the spinal cord were found to be reduced in number, interpreted as partial respecification (Burrill et al., 1997; Ericson et al., 1997). To confirm and extend previous studies of neuronogenesis, I followed the expression of neuronal markers, *Isll, Isl2* and *Lim3*, in the cervical spinal cord. I found a delay in onset of expression of *Isl2* and *Lim3*. Onset of *Isll* expression occurred at the normal time, at around E9.5, although at E10.5 there were fewer *Isll*-positive cells in *Sey/Sey* embryos than their wild-type littermates. By E13.5, the intensities and patterns of expression of all the LIM transcripts were indistinguishable in *Sey/Sey* and wild-type spinal cords. Therefore, it appears that development of ventral neurons is retarded, but not blocked, by the loss of Pax6.

If, as I suggest, OLPs and MNs share a common lineage, one would predict that OLP development should also be disturbed in *Sey/Sey* mice. I tested this prediction and found that appearance of OLPs is delayed by about a day in the cervical spinal cord in *Sey/Sey*, but that they subsequently proliferate and catch up with their wild-type littermates before birth. The fact that both MNs and OLPs originate from the same part of the VZ, and development of both MNs and OLPs is retarded in *Sey/Sey* spinal cord is compatible with a common MN-OL lineage but does not exclude other models. For example, there could be a separate pool of dedicated OLP precursors whose maturation depends on signals generated by previously formed MNs. However, our finding that OLs develop normally in explants of *Isll(-/-)* spinal cords, in the absence of MNs, tends to argue against a central role for MN-derived signals. It still leaves open the possibility that feedback from other classes of ventral neurons such as the *Lim3/Chx10* (V2) INs might be required. I think this is unlikely because normal numbers of *Chx10* INs accumulate in the *Sey/Sey* spinal cord before E12, prior to the normal onset of OLP development (Burrill et al. 1997). A lineage relationship between MNs and OLs was previously suggested by retroviral clonal analysis in the embryonic chick spinal cord (Leber et al., 1990; Leber and Sanes, 1995). Common neuron-oligodendrocyte precursors have also been identified in cell cultures established from developing cerebral cortex (Davis and Temple, 1994; Qian et al., 1997; Williams et al., 1991). Moreover,
common neuron-glial precursors are a well-established feature of invertebrate nervous systems (e.g. Udolph, et. al., 1993).

2. The lack of Pax6 causes the dorsal shift of ventral cell fates in Sey/Sey spinal cord

In Sey/Sey mice, which lack functional Pax6 protein, the position of the first PDGFRα OLPs to be specified in the VZ was shifted dorsally relative to wild-type. This is in keeping with the idea that Pax6 interprets positional information relayed by Shh (Ericson et al., 1997; Goulding et al., 1993). Shh normally represses Pax6 in the vicinity of the floor plate and allows other factors such as Nkx2.2, which are repressed by Pax6, to become established there. In the absence of Pax6, the Nkx2.2 expression domain can expand dorsally and "ventralize" more of the VZ. OLPs normally are specified in Pax6 territory just on the border of the ventral domain defined by Nkx2.2; presumably the special conditions that exist there, that are conducive to OLP fate selection, are recreated further dorsal in the Sey/Sey spinal cord. Since development generally proceeds in a temporal wave from ventral to dorsal (Altman and Bayer, 1984; Nornes and Das, 1974), this might have a bearing on why specification of OLPs is delayed in Sey/Sey animals. A general dorsal shift in cell fates can also explain why certain classes of ventral neurons are absent in Sey/Sey – e.g the En1/Pax2-expressing (V1) INs (Burrill et al., 1997; Ericson et al., 1997). Precursors in the most dorsal regions of the basal plate, which normally generate V1 INs, might be respecified to generate more ventral cells (e.g. V2 INs), while precursors more dorsal than that (i.e. within the alar plate) might be unable to generate V1 INs because of the overriding influence of dorsal gene products such as Pax7.

I could not directly test whether neuronal precursors are shunted dorsally in Sey/Sey spinal cords because the available markers of these cells are not fully expressed until after the cells leave the VZ. However, our data on OLPs suggested that a dorsal shift might also be accompanied by a developmental delay. To test this, I repeated and extended previous studies of neuronogenesis in Sey/Sey spinal cord by following the
expression of \( Isl1, Isl2, \) and \( Lim3 \) from earlier to later ages than before. I found a delay of up a day in the onset of expression of \( Isl2 \) and \( Lim3 \). \( Isl2 \) is a specific marker of MMC-MNs in the cervical spinal cord (Tsuchida et al., 1994), so I can definitely conclude that development of this class of somatic MNs is delayed in \( Sey/Sey \). The fact that \( Lim3 \) is also delayed is consistent with this conclusion and also suggests that \( Lim3/Chx10 \) (V2) INs might be delayed as well. A delay in production of V2 INs was already suggested by previous studies; Ericson et al. (1997) found reduced numbers of \( Chx10 \) INs in \( Sey \) homozygotes at E11 yet Burrill et al. (1997) found essentially normal numbers of these cells at E12. Therefore, it appears that development of ventral neurons, like OLPs, is retarded but not blocked by the loss of Pax6.
Fig. 5.1. Site of origin of PDGFRα⁺ OLPs relative to the Pax6- and Nkx2.2-positive domains of the wild type spinal cord. Neighboring sections from an E12.5 wild type spinal cord were hybridized in situ with DIG-labeled probes for Pax6 (A, E), PDGFRα (B, F) or Nkx2.2 (C, G) and hybrids visualized by alkaline phosphatase. Pax6 is expressed throughout the VZ except for a small region abutting the floor plate; and also in a population of cells at the edge of the VZ in the ventral part of the cord (A). Nkx2.2 is expressed in a complementary pattern, restricted to the region adjacent to the floor plate (C). PDGFRα⁺ is first expressed in a few cells at the ventricular surface (arrow in B). We mapped the positions of 28 PDGFRα⁺ OLPs onto the dorsal-ventral axis (see 2.3.12) (panel D). The median position was between 10% and 11% (panel D) and the mean position 11% ± 2% (mean ± s.d., n=28). This corresponds to about 10-15 cell diameters from the edge of the floor plate. When higher-magnification images of adjacent sections were aligned carefully (E to H), it was clear that the PDGFRα⁺ cells (signified by red crosses in H) lay close to the boundary of the Pax6- and Nkx2.2 domains. Most PDGFRα⁺ cells mapped to the ventral-most part of the Pax6-positive domain (panel H). Some cells whose sites of origin can be defined accurately because they retain visible contact with the ventricular surface are clearly within Pax6-positive, Nkx2.2-negative territory (e.g. the upper two cells visible in panel F). Others fall in the small area of overlap between Pax6 and Nkx2.2, but whether these cells express both Pax6 and Nkx2.2 cannot be determined at this level of analysis. Panels E to G illustrate consecutive sections from a single embryo. Arrowheads mark the ventral limit of the spinal cord lumen. H is a juxtaposition of Pax6 and Nkx2.2 expression patterns. Red crosses mark the positions of PDGFRα⁺ cell bodies in four consecutive sections including those shown in panel F. Scale bars: 100 μm (A-C), 20 μm (E-H).
Position % of V-D axis

PDGFRα Pax6 Nkx2.1t

PDGFRα cells per interval

Position, % of V-D axis

Pax6  PDGFRα  Nkx2.2  Pax6  Nkx2.2
Fig. 5.2. Development of PDGFRα+ OLPs in wild type and Sey/Sey spinal cords. (A) Transverse sections through cervical spinal cords at the indicated ages were hybridized in situ with DIG-labeled PDGFRα probes. PDGFRα+ cells first appeared in the wild-type cord at E12.5 (see Fig. 5.1). By E13 PDGFRα+ cells had already started to spread through the ventral part of the wild-type cord (panel A) but had not yet appeared in their age-matched Sey/Sey littermates (panel B). PDGFRα+ cells first appeared at the ventricular surface of the Sey/Sey cord at E13.5 (see Fig. 5.4). Numbers of PDGFRα+ cells in Sey/Sey lagged behind wild type until after E15.5 (panels C-F) but caught up by E18.5 (panels G, H). Scale bar: 200 μm. (B) PDGFRα+ OLPs were counted in transverse sections of wild type, Sey/+ and Sey/Sey spinal cords. Data (means ± s.d.) were obtained from at least six sections from two embryos at each age, either from one (E13 and E13.5) or two litters. The time of first appearance and subsequent proliferation of PDGFRα+ cells was retarded by about a day in Sey homozygotes compared to both wild type and heterozygous Sey mice.
Fig. 5.3. Cell cycle dynamics of PDGFRα+ OLPs analyzed by BrdU incorporation *in vivo*. Pregnant females were given a single intraperitoneal injection of BrdU (50 μg per gram body weight) and their embryos were removed 2 hours later and prepared for histochemistry. Transverse sections through cervical spinal cords of Sey/Sey embryos and their wild type littermates were hybridized *in situ* with a DIG-labeled PDGFRα probe, followed by antibody labeling for BrdU. (A) An immunofluorescence micrograph of an E15.5 Sey/Sey spinal cord showing BrdU-labeled cells distributed throughout the cross section of the cord, superimposed on the corresponding PDGFRα in situ hybridization micrograph (scale bar: 200 μm). (B) Part of the same image at higher magnification; in this field there are three PDGFRα+ cells, two of which have BrdU-positive nuclei (arrows). Numbers of (PDGFRα, BrdU) double-positive cells were plotted as proportions of the total numbers of PDGFRα+ cells (BrdU labeling index) for embryos of different ages (C). Data (mean ± s.e.m.) were obtained from at least five sections from two embryos of each age, either from one litter (E12.5 and E13.5) or two litters. In both wild type and Sey/Sey cords, the BrdU labeling index dropped from around 60% at early ages to around 15% at E18.5 and later; however, the curve for Sey/Sey was displaced to the right by about a day, consistent with the developmental delay in Sey homozygotes.
Fig. 5.4. Site of origin of PDGFRα^ OLPs in the ventral VZ of Sey/Sey spinal cord shifts dorsally relative to wild-type. Sections through the cervical cord of E13.5 Sey/Sey embryos were subjected to in situ hybridization with DIG-labeled probes for Pax6 (A, D) or PDGFRα. (B, E). An E12.5 wild type cord is shown at the same magnification for comparison (F). The positions of 11 OLPs in the VZ (six sections from one animal) were measured relative to the ventral and dorsal midlines (C) as described in 2.3.12. All mapped between 20% and 32% on the ventral-dorsal axis (25% ± 3%, mean ± s.d.). This is shifted dorsally compared to wild type OLPs (11% ± 2%; see Fig. 5.1). Arrowheads mark the ventral limit of the spinal cord lumen. The small arrow in B indicated the position of the PDGFRα^ OLPs. Red crosses in D mark the positions of PDGFRα^ OLPs in four adjacent sections including that shown in E. Magnifications are as in Fig. 5.1.
Fig. 5.5. Expression of LIM-domain transcription factors in E9.5 wild-type and Sey/Sey spinal cords. Transverse sections through the cervical region of E9.5 cords (25 somite stage) were hybridized in situ with DIG-labeled probes forIsl1, Isl2, and Lim3. Isl1 expression is relatively normal in Sey/Sey embryos at this age, whereas expression of Isl2 and Lim3 is not detected in the mutant. Scale bar: 100 μm.
Fig. 5.6. Expression of LIM domain transcription factors in E10.5 wild type and Sey/Sey spinal cords. Transverse sections through the cervical regions of E10.5 cords were hybridized in situ with probes for Isl1, Isl2, and Lim3. Expression of all three genes seems to be depressed in the Sey/Sey cord compared to wild type, but most markedly for Isl2. In addition, there is a ventro-medial population of Isl1-positive cells in the wild type that is missing from the mutant at this age. Scale bar: 100 μm.
Fig. 5.7. Expression of LIM domain transcription factors in E11.5 wild type and Sey/Sey spinal cords. Transverse sections through the cervical regions of E11.5 cords were hybridized in situ with probes for Isl1, Isl2, and Lim3. Fewer cells were labelled with Isl2 and Lim3 in the Sey/Sey spinal cord than in wild-type. In addition, there is a reduction of Isl1-positive cells in the dorsal and ventral Sey/Sey spinal cord. Scale bar: 100 μm.
Fig. 5.8. Expression of LIM domain transcription factors in E13.5 wild type and Sey/Sey spinal cords. Transverse sections through the cervical regions of E13.5 cords were hybridized in situ with probes for Isl1, Isl2, and Lim3. At this age, there appear to be no significant differences between wild type and Sey/Sey. Scale bar: 200 μm.
Fig. 5.9. Oligodendrocytes develop normally in explant cultures of *Isl1(-/-)* spinal cords. Embryonic (E9.5) offspring of heterozygous *Isl1(-/+)* parents were genotyped by PCR (A). Segments of spinal cord from *Isl1(-/-), Isl1(-/+)* and wild type littermates were cultured in collagen gels on a rocking platform. Some explants were fixed after 2 days and immunolabeled with anti-Isl1/2 monoclonal antibodies. Isl1/2-positive neurons developed in wild type and *Isl1(-/+)* explants (B) but not in *Isl1(-/-)* explants (C). The majority of explants were cultured for 11 days (until the equivalent of the day of birth) and immunolabeled with monoclonal anti-GC. GC-positive OLs developed in all explants, whether from wild type (not shown), *Isl1(-/+)* or *Isl1(-/-)* cords (D and E, respectively). The inset in E shows a control immunolabeling in which the primary anti-GC antibody was omitted.
Chapter 6

Future directions
Specific regulatory elements for PDGFRα transcript in oligodendrocyte progenitors: summary and future work

In this thesis, I have described the expression and function of PDGFRα gene in mouse embryonic development, particularly in oligodendrocyte progenitor (OLP) development. During development, PDGFRα displays a complex and dynamic expression pattern in the central nervous system (CNS) and outside the CNS, and participates in the development of diverse tissues and organs. Among its many functions, PDGFRα is crucial for the development of OLPs, which originate from the ventral spinal cord.

A main purpose of my Ph.D. work is to study PDGFRα regulation, particularly in OLPs. In this study, I have obtained evidence that PDGFRα transcribes from the same start site in the CNS and outside the CNS. This implies that the reason that previously characterized promoter fragments do not appear to function in OLPs is not because OLPs use a special promoter but that they require distant cis-acting enhancer elements. For the long-term goal of identifying distant cis-acting regulatory elements for PDGFRα expression in OLPs, I have generated transgenic mice by pronuclear injection of a 380 kb YAC containing the entire human PDGFRα gene. The faithful expression of the YAC transgene in OLPs indicates that the OLP-specific elements driving PDGFRα expression are within this YAC. Moreover, the YAC transgene can rescue the craniofacial abnormalities and spina bifida observed in the PDGFRα knockout (KO) mouse and prolong their survival until birth.

This work forms the foundation for future studies aimed at identifying the OLP-specific control elements. This will require production of new transgenic mice using partially deleted YACs by homologous recombination. The analysis of transgenic mice generated with deleted YACs will provide information about where the OLP-specific regulatory elements are located.
Identifying the OLP-specific regulatory elements of the PDGFRα will eventually provide clues to the direct DNA targets of Shh signaling. The Gli genes have been suggested to act downstream of Shh (see 1.3.2.4) and play a general role in glial generation (Ruiz i Altaba, 1998). It will be interesting to test whether Gli genes regulate PDGFRα expression. The binding sites for Gli genes have been found in 5' flanking region of the PDGFRα gene (personal communication from Gijs Afink, Uppsala University, Sweden). Biochemical evidence of the interaction between Gli and PDGFRα will enrich our understanding of Shh signalling in specifying ventral cell fates. It will also be instructive to study oligodendrocyte development in the Gli mutant mice. Furthermore, isolation of OLP-specific regulatory elements of the PDGFRα will provide us a means to express conventional transgene directly in OLPs. Then the potential for investigation into glial fate determination and subsequent development of the glial lineage will be great.

In addition, study of PDGFRα expression will help us to understand the molecular mechanisms that cause human glioma. Since PDGF-A and PDGFRα are frequently overexpressed in glioma cells, an autocrine activation of the PDGFRα has been suggested to drive the proliferation of glioma cells (reviewed by Westermark et al., 1995). Our understanding of PDGFRα expression in normal glia will help us to investigate the autocrine activation of the PDGFRα in human glioma.

PDGFRα YAC transgenic mice can also provide a unique opportunity to study structure-function relationships of the PDGFRα in vivo. For example an interesting observation is that PDGF-A/PDGFB double null mice display a less severe phenotype compare to PDGFRα mutant mice, for instance the craniofacial defects and spina bifida. One of the possible explanations is that there might be some independant activation of PDGFRα in vivo. A candidate molecule could be the transmembrane chondroitin sulfate proteoglycan NG2 (see Discussion in chapter 4). To test this
prediction, a significant experiment would be to generate mice with YACs that contain targeted mutations to the extracellular PDGF-A binding domain of human $PDGFR\alpha$ gene. If this mutated YAC could still rescue the defects in neural crest-derived tissues in $PDGFR\alpha$ null mutant mice (see Chapter 4), this would suggest PDGF-A-independent activation of PDGFR$\alpha$. 
References


demonstration that mesoderm induction establishes the lineage-specific pattern of ligand and receptor gene expression. *Dev Genet, 14*:185-93.


