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A human YAC transgene rescues craniofacial and neural tube development in *PDGFR α* knockout mice and uncovers a role for PDGFRα in prenatal lung growth

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The platelet-derived growth factor alpha-receptor (PDGFRα) plays a vital role in the development of vertebrate embryos, since mice lacking PDGFRα die in mid-gestation. PDGFRα is expressed in several types of crest cells, rescued the profound craniofacial abnormalities and spina bifida observed in *PDGFR α* knockout mice and prolonged survival until birth. The ultimate cause of death was respiratory failure due to a defect in lung growth, stemming from failure of the transgene to be expressed correctly in lung smooth muscle progenitors. However, the YAC transgene was expressed faithfully in oligodendrocyte progenitors, which was not previously observed with plasmid-based transgenes containing only upstream *PDGFRα* control sequences. Our data illustrate the complexity of *PDGFRα* genetic control, provide clues to the location of critical regulatory elements and reveal a requirement for PDGF signalling in prenatal lung growth, which is distinct from the known requirement in postnatal alveogenesis. In addition, we found that the YAC transgene did not prolong survival of *Patch* mutant mice, indicating that genetic defects outside the *PDGFRα* locus contribute to the early embryonic lethality of *Patch* mice.

INTRODUCTION

Three PDGF subunits (PDGF-A, -B and -C) have been identified to date. All three subunits form covalent homodimers. In addition, PDGF-A and PDGF-B can form heterodimers with each other, though probably not with PDGF-C (Heldin et al., 1998; Li et al., 2000). Each individual subunit binds one receptor, so PDGF dimers initiate signalling by inducing receptor dimerization and autophosphorylation. There are two known PDGF receptors with different ligand specificities: PDGFRα binds all three PDGF subunits whereas PDGFRβ mainly binds PDGF-B. Therefore, a cell's response to PDGF depends both on the receptor(s) that it expresses and on the particular dimeric PDGF isoform(s) to which it is exposed. Whether or not a given PDGF ligand-receptor interaction can occur in vivo depends critically on the spatial and temporal expression patterns of receptors and ligands. The present paper is mainly concerned with the regulation of *PDGFRα* gene expression.

PDGFRα is expressed in the embryo by several populations of progenitor cells that proliferate and migrate in response to PDGF (reviewed by Lindahl and Betsholtz, 1998). For example, *PDGFRα* is expressed in precursor cells of the cranial neural crest and is thought to be required for migration of crest cells into the branchial arches. In keeping with this idea, *PDGFRα* deficient mice [both the targeted knockout and the spontaneous deletion mutant *Patch* (*Ph*)] have a variety of defects in crest-derived tissues including gross craniofacial and skeletal abnormalities (Orr-Urtreger et al., 1992; Morrison-Graham et al., 1992; Schatteman et al., 1992; Payne et al., 1997; Soriano, 1997). They also have neural tube defects (spina bifida) and die in early- to mid-gestation. *PDGFRα* is also expressed by smooth muscle progenitors in the developing lung (Lindahl et al., 1997) and by glial progenitor cells in the

developing and adult central nervous system (Pringle and Richardson, 1993; Hall et al., 1996). In addition, *PDGFRα* is expressed widely throughout the embryonic mesenchyme (Orr-Urtreger et al., 1992).

The widespread and dynamic pattern of *PDGFRα* expression presumably depends on a complex set of *cis*-acting regulatory elements. A 6 kb upstream fragment of the mouse *PDGFRα* (Wang and Stiles, 1994) and a 2.2 kb upstream fragment of human *PDGFRα* (Afink et al., 1995) have been isolated and used to drive expression of a *lacZ* reporter gene in transgenic mice. Both promoter fragments direct expression faithfully to cells of mesenchymal and ectodermal origin and to neural crest, but not to oligodendrocyte progenitors in the CNS (Reinertsen et al., 1997; Zhang et al., 1998). The 6 kb fragment but not the 2.2 kb fragment was reported to direct expression to lung smooth muscle progenitors, though weakly.

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). To analyze *PDGFRα* expression and function during development, we produced transgenic mice by pronuclear injection of a 380 kb YAC (CEPH 449C2; Spritz et al., 1994) containing human *PDGFRα* genomic DNA. We fou001 5at the

mapping data and the fact that D4S956 hybridized only weakly to 449C2 in our hands. We were also unable to demonstrate hybridization of a *c-kit* exon 1 probe to 449C2 on Southern blots, contrary to Spritz et al. (1994).

STS content mapping of the 3′ end of the YAC was performed using the markers SHGC-103244 (Accession #G57545), CHLC.GATA61B02 (#G16733) and SHGC-4228 (#G16733), which are located 92,244 bp, 87,139 bp and 23,318 bp upstream of *c-kit* exon 1, respectively (for the complete sequence of the region see #NT_000121). Primer sequences and reaction conditions are available from GenBank. YAC 449C2 included the two upstream STS markers but the third (SHGC-4228) was absent. Therefore, the 3′ end of the YAC falls between approx. 87 kb and approx. 23 kb upstream of the *c-kit* transcription start site.

Generation and genotyping of transgenic and mutant mice

Transgenic mice were produced by standard pronuclear microinjection of purified YAC DNA. DNA was released from yeast embedded in high-density agarose plugs as described (Schedl et al., 1996). YAC DNA was purified for microinjection by preparative PFGE (1% agarose in 0.5 TBE; Schedl et al., 1996). The gel was run for 30 hours at 4°C at 6 V/cm, using a switching time of 30 seconds (Bio-Rad CHEF-DRII system). Purified YAC DNA was again checked by PFGE before injection into eggs from (C57BL/6J×CBA) F_1 hybrid donors.

Transgenic founders were identified by PCR and Southern blot analysis of tail-tip DNA. The following primers were used to verify the presence of the YAC ends and the *PDGFRα* insert: YAC vector left arm (L1, 5′-CAC CCG TTC TCG GAG CAC TGT CCG ACC GC-3′ and L2, 5′

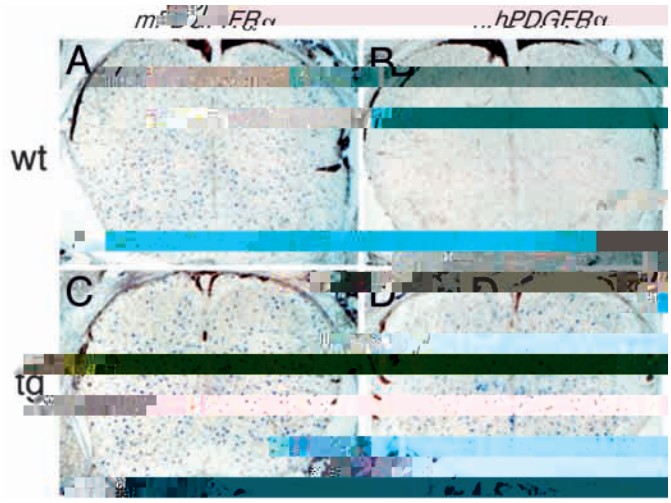

Fig. 2. Comparison of endogeneous and transgenic *PDGFRα* expression in newborn spinal cords. Transverse sections through spinal cords of wild-type (A,B) and YAC transgenic mice (C,D) were hybridized with DIG-labelled mouse (A,C) and human (B,D) *PDGFRα* probes. At this age, expression of the transgene, like the endogenous *PDGFRα* gene, is restricted to oligodendrocyte progenitors scattered throughout the cord (D,C). Note that the human probe does not cross-hybridize to endogeneous *PDGFRα* transcripts (A,B). Scale bar, 250 μm.

centrifugation and resuspension in DMEM containing 10% FCS. Cells were plated on poly-D-lysine-coated 13 mm diameter glass coverslips in a 50 μl droplet, and allowed to attach for 30 minutes at 37°C. 350 μl of Bottenstein and Sato (1979) medium with 10 ng/ml of PDGF-AA (Peprotech) was added and incubation continued at 37°C in 5% CO_2 with medium being refreshed every 2 days. Cells were cultured until the equivalent of the day of birth before staining with antibodies against the oligodendrocyte marker galactocerebroside (GC) (a gift from M. C. Raff).

Cells on coverslips were fixed in 2% (w/v) paraformaldehyde in PBS for 5 minutes at room temperature then washed with PBS. Coverslips were incubated for 30 minutes in monoclonal anti-GC diluted 1:1000 in PBS containing 0.1% (v/v) Triton X-100, then washed in PBS, incubated with rhodamine-conjugated goat anti-mouse Ig (Pierce) for 30 minutes at room temperature and finally postfixed in 4% (w/v) paraformaldehyde before mounting in glycerol for fluorescence microscopy.

RESULTS

Generation of human *PDGFRα* YAC transgenic mice

PDGFRα-containing YACs CEPH 449C2, CEPH 29E11 and SL B214G6 (Brownstein et al., 1989; Cohen et al., 1993) were originally characterized using primers specific for human *PDGFRα* exons 3 and 20 (Spritz et al., 1994). We obtained these YACs from the Genome Technology Centre, Leiden (449C2 and 29E11) and the UK Human Genome Mapping Project (HGMP) Resource Centre, Hinxton (B214G6). Each YAC was examined by pulsed-field gel electrophoresis (PFGE) and Southern blot, using probes specific for human *PDGFRα* exon 1 and exon 23 (the 3′-untranslated region, UTR) (e.g. Fig. 1B). This indicated that YAC B214G6 is approx. 190 kb in length and 449C2 is approx. 380 kb, as reported; however, 29E11 was approx. 470 kb, not 290 kb or 195 kb as reported by Spritz et al. (1994). Since 29E11 seemed to be unstable and, of the other two, 449C2 contains slightly more 5′ *PDGFRα* sequence than B214G6 (Fig. 1A; see Materials and Methods), we selected 449C2 for transgenesis. Because it is in the same size range as endogenous yeast chromosomes, and pure YAC DNA is required for microinjection, we transferred 449C2 into yeast strain YLBW2 which has a 'window' (yeast chromosome-free zone) in the size range 250-450 kb (Hamer et al., 1995). A haploid window yeast strain carrying 449C2 was identified by PCR; the resulting YAC was renamed 449-W2. PFGE and Southern blot using a probe to the human *PDGFRα* 3′-UTR indicated that the transferred YAC remained intact (Fig. 1B). PCR with primers specific for exon 1 and the 3′-UTR suggested that the whole *PDGFRα* structural gene is contained within the YAC, while STS content analysis showed the 3′ end of the YAC to lie 23-87 kb upstream of the adjacent *c-kit* gene (see Materials and Methods).

Transgenic mice were produced by microinjection of gel-purified YAC DNA into fertilized mouse oocytes. DNA from tail tips of the newborn mice was analyzed by PCR and Southern blot (see Materials and Methods). After injection of 449-W2 YAC DNA, four transgenic mice were detected among 43 newborns. Founders 3-3, 5A1 and 8A4 all appeared to contain full-length YAC DNA by PCR and Southern blot. They were bred with wild-type mice to establish lines. In the fourth founder we detected the 3′-UTR but not exon 1 of human *PDGFRα*, so the YAC was presumably broken.

To determine the numbers of integrated YAC transgenes, semiquantitative PCR was carried out using primers corresponding to matching sequences within the mouse and human *PDGFRα* 3′-UTRs (see Materials and Methods). 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

Table 1. Expression of endogenous mouse *PDGFRα* and the human *PDGFRα* YAC transgene

Tissues	Endogenous *PDGFRα*	Human *PDGFRα*
Dorsal mesoderm		
Sclerotome	+++	++
Dermatome (dermis)	++	+
Myotome	–	–
Perichondrium/periosteum	+++	+++
Chondrocytic core	–	+
Lateral mesoderm		
Mesenchyme (connective tissues)	+++	+
Heart	++	–
Blood vessels	–	++
Limb	+++	+
Tongue	+++	++
Notochord	–	–
Endoderm		
Trachea	+++	–
Oesophagus	+++	–
Lung	++	+
Thymus	++	++
Ectoderm		
Lens epithelium	+++	+++
Branchial arches	+++	++
Dorsal root ganglia	+	++

+, Expression detected; –, no expression.

product, suggesting that around 22 copies of human *PDGFRα* had integrated per diploid mouse genome (Fig. 1C). There were around ten copies of the YAC in line 5A1 and two copies in line 8A4

Expression of the human *PDGFRα* transgene

We looked at the transgene expression patterns in our three transgenic lines by in situ hybridization with a probe to the human *PDGFRα* 3′-UTR. Under our experimental conditions, this probe did not cross-hybridize with the endogenous mouse gene (compare Fig. 2A and B). Transgene expression was similar and wide-ranging in lines 3-3 and 5A1 (see below and Table 1). Expression of the transgene in line 8A4 was only detectable in the anterior lens epithelium, so this line was not studied further.

The human *PDGFRα* transgene was expressed correctly in oligodendrocyte progenitors in the CNS (Fig. 2). *PDGFRα*$^+$ oligodendrocyte progenitors first appear at the midline of the spinal cord on E12.5 in the mouse (Pringle et al., 1996; Calver et al., 1998). Subsequently, these progenitors proliferate and migrate through the spinal cord in response to PDGF-AA (Fruttiger et al., 1999), becoming evenly distributed throughout the cord before birth (Fig. 2A). In the spinal cords of transgenic mice the *PDGFRα* transgene was specifically expressed in oligodendrocyte progenitors at a level comparable to that of the endogeneous gene (Fig. 2C,D). Expression in oligodendrocyte progenitors was not previously observed with conventional transgenes containing up to 6 kb of sequences upstream of *PDGFRα* (Benningston et al., 1997; Zhang et al., 1998).

PDGFRα is expressed widely in the developing mesoderm during early development (Orr-Urtreger and Lonai, 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 the somites and, very weakly, in the limb buds though this was difficult to see in whole mounts (Fig. 3B). Both endogenous and transgenic *PDGFRα* were strongly expressed in the mesenchyme of the branchial arches, which give rise to the jaws and other structures (Fig. 3A,B). By E12.5, expression of the human *PDGFRα* transgene outside the nervous system had become much weaker in comparison to the endogeneous gene (e.g. compare the sclerotome expression in Fig. 3C,D). Transgene expression was undetectable in some tissues that express the endogenous gene, such as the aortic valve and aortico-pulmonary spiral septum of the heart (Fig. 3E,F). Ectopic expression of the transgene was observed in a number of tissues

expressed in the posterior lens fibres, where endogenous *PDGFRα* expression is normally extinguished (Fig. 3L).

To summarize, the human *PDGFRα* transgene was expressed at many of the normal sites of *PDGFRα* expression, although outside the nervous system transgene expression was generally at a lower level than that of the normal gene (Table 1, Figs 2, 3). However, there were also significant differences; there were places where the endogenous gene was expressed but not the

oligodendrocyte progenitors. *PDGFRα*$^+$ oligodendrocyte progenitors are first specified in the ventral neural tube under the influence of Sonic hedgehog (SHH) from the ventral midline (Pringle et al., 1996; Poncet et al., 1996; Orentas et al., 1999). Subsequently, they proliferate and migrate throughout the developing spinal cord in response to PDGF-AA from neurons and astrocytes (Calver et al., 1998; Fruttiger et al., 1999). Human *PDGFRα* was expressed appropriately in oligodendrocyte progenitors 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* (i.e. in KO-YAC mice). Therefore, the progenitor-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 oligodendrocyte progenitors (Reinertsen et al., 1997; Zhang et al., 1998). Since YAC 449C2 contains less than 6 kb of upstream sequence, this strongly suggests that the progenitor-specific element(s) is located within the gene (e.g. in an intron) or downstream of it.

In contrast, the YAC was not expressed in smooth muscle progenitors in the lung. The *PDGFRα* structural gene spans about 65 kb (Kawagishi et al., 1995) so, taking into account 15 kb of vector arms plus upstream sequence, YAC 449C2 (380 kb) must contain around 300 kb of 3′- flanking sequence. Therefore, in all likelihood lung-specific elements will be found further upstream of the gene. Reinertsen et al. (1997) report that their 6 kb mouse *PDGFRα* promoter fragment drives *lacZ* expression weakly in lung smooth muscle progenitors (‘alveolar myofibroblasts’) after birth. If so, and if the mouse and human promoters are closely conserved, this

Fig. 6. Growth and morphology of the lungs in KO-YAC mice. (A) Lungs of *PDGFRα*$^{+/-}$, wild-type and KO-YAC newborn mice. The KO-YAC lungs are much smaller than wild type, i.e. one-third to one-half normal mass (Table 2), although gross morphology is normal. (B-E) Hematoxylin/Eosin staining of paraffin sections of wild-type (B,D) and KO-YAC (C,E) lungs at E18.5. B,C and D,E show the same specimens at low and high magnification, respectively. No obvious differences between wild type and mutant lungs are evident. Both have clearly reached the saccular stage of development. Similar histology was found in lungs from two KO-YAC embryos from different litters and two wild-type littermates. Scale bar: 150 μm for B-C; 50 μm for D-E.

chromosome 5 and human chromosome 4 (Bernex et al., 1996; Kluppel et al., 1997). This suggests that there might be regulatory elements (enhancers) in the YAC that normally communicate with the *c-kit* promoter, but are misdirected towards the *PDGFRα* promoter in our transgenic mice. Our YAC transgene extends downstream of *PDGFRα* to within 87 kb of the *c-kit* promoter (both genes are transcribed in the same direction). There appears to be a spinal cord-specific enhancer(s) between 38 kb and 146 kb upstream of the *c-kit* promoter because *c-kit* expression in the cord seems to be abolished in the W^{57} mouse mutant, which lacks these sequences (Berrozpe et al., 1999; also see Fig. 4 in Klüppel et al., 1997). This *c-kit* enhancer might be present in our YAC transgene where it might compete with genuine *PDGFRα* elements for the *PDGFRα* promoter. Such competition would be facilitated if any of the multiple copies of the YAC in our transgenic lines (3-3 and 5A1) are arranged head-to tail, as this could bring *c-kit* elements close to the *PDGFRα* promoter. A similar promoter affinity switch happens in reverse in the *Patch* (*Ph*) mouse, in which a large deletion in the *PDGFRα* locus brings 5′ regulatory sequences of *PDGFRα* close to the *c-kit* promoter (Stephenson et al., 1991; Wehrle-Haller et al., 1996) resulting in ectopic expression of *c-kit* in places that normally express *PDGFRα* (Duttlinger et al., 1995; Wehrle-Haller et al., 1996). Interference by misplaced regulatory elements could

Fig. 7. Lung development in wt-YAC (A,B,D,E,G,H) and KO-YAC (C,F,I) lungs, visualized by *PDGFRα* in situ hybridization. (A-C) and (D-F) show the same specimens at low and high magnification, respectively. (A,D) E15.5 wt-YAC lung probed with mouse *PDGFRα*. Endogenous *PDGFRα* is expressed in many cells in the interstitial mesenchyme of the wt-YAC lung and, more strongly, in smooth muscle progenitors closely associated with the epithelial tubules (D, arrows), giving the impression of crescents of strongly-labelled cells. (B,E) E15.5 wt-YAC lung probed with human *PDGFRα*. The human transgene is expressed in the interstitial mesenchymal cells but not in smooth muscle progenitors (E, arrows). (C,F) E15.5 KO-YAC probed with mouse *PDGFRα*. Smooth muscle progenitors were greatly reduced in number (F, arrows) although the interstitial mesenchymal cells remained. Note that *PDGFRα* null mice still transcribe a non-functional mouse *PDGFy.48 (.7(O-Y)119.8(A)39.8(C)0((C,F)79.8(,I) lungsabt Tc(C)C)0(probed)]Tend, more str8(,I) lukee19.8(2.)Tj T* 0.0i32114.8(xpres*
PDGFR

explain not only the ectopic *PDGFRα*