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

Tao Sun[1,*], Damith Jayatilake[1], Gijs B. Afink[2], Paris Ataliotis[3], Monica Nistér[2], William D. Richardson[1,‡] and Hazel K. Smith[1]

[1]Wolfson Institute for Biomedical Research and Department of Biology, The Cruciform Building, University College London, Gower Street, London WC1E 6BT, UK
[2]Department of Genetics and Pathology, Rudbeck Laboratory, University Hospital, S-751 85 Uppsala, Sweden
[3]Molecular Medicine Unit, Institute of Child Health, 30 Guilford Street, London WC1N 1EH, UK
*Present address: The Skirball Institute, Developmental Genetics Program and Department of Cell Biology, NYU School of Medicine, New York, NY 10016, USA
‡Author for correspondence (e-mail: w.richardson@ucl.ac.uk)

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SUMMARY

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 migratory progenitor cells in the embryo including cranial neural crest cells, lung smooth muscle progenitors and oligodendrocyte progenitors. To study *PDGFRα* gene regulation and function during development, we generated transgenic mice by pronuclear injection of a 380 kb yeast artificial chromosome (YAC) containing the human *PDGFRα* gene. The YAC transgene was expressed in neural crest cells, rescued the profound craniofacial abnormalities and spina bifida observed in *PDGFRα* 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.

Key words: PDGF receptor-alpha, YAC transgenesis, Craniofacial development, Neural tube defect, Lung development, Oligodendrocyte, Progenitor cell

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 found that the human *PDGFRα* transgene was correctly expressed in oligodendrocyte progenitors as well as in many mesoderm- and neural crest-derived tissues. Thus, many *cis*-acting regulatory elements necessary for tissue-specific expression of *PDGFRα* during development are contained within this 380 kb YAC. The human transgene complemented the neural crest and neural tube defects of *PDGFRα*$^{-/-}$ mice and prolonged their survival until birth. However, the transgene was not expressed in smooth muscle progenitors in the lung, resulting in a marked reduction in lung size and respiratory failure at birth. This reveals a requirement for PDGF signalling in prenatal lung growth in addition to the known requirement for PDGF-AA in postnatal alveogenesis (Lindahl et al., 1997).

MATERIALS AND METHODS

Human *PDGFRα*-containing YACs

YACs CEPH 449C2, CEPH 29E11 and SL B214G6, each of which contains the entire human *PDGFRα* structural gene (Spritz et al., 1994), were obtained from the Genome Technology Centre (Leiden, Holland) and the HGMP Resource Centre (Hinxton, UK). Southern blots of pulsed-field gels were hybridized with oligonucleotides specific for human *PDGFRα* exon 1 (H1, 5′-CTG GAC ACT GGG AGA TTC GGA G-3′) and the 3′-UTR (H3, 5′-GTG ATG TCC TTA AAA TGT GGT-3′). YAC 449C2 was transferred into the yeast ‘window’ strain YLBW2 (kindly provided by E. D. Green and C. Huxley), using the *Kar*1-transfer procedure (Spencer et al., 1994; Hugerat et al., 1994; Hamer et al., 1995). A haploid MAT-A mating type, YAC-containing strain was identified by PCR (Huxley et al., 1990) and the YAC (renamed 449-W2) was characterized by pulsed-field gel electrophoresis (PFGE) and Southern blot as above. The YAC was also characterized by PCR with primers specific for human *PDGFRα* exon1 (H1, see above; H2, 5′-CGA TGT TAT TCC GCA ATG AAT G-3′) and the 3′-UTR (H3, see above; H4, 5′-GTA ATA CAT TTT GTA TTG GTA G-3′). 449C2 and B214G6 were also mapped in more detail at the junction of YAC vector and *PDGFRα* 5′ end. Restriction enzyme digests were separated by PFGE, blotted onto nylon membrane and hybridized with probes specific for *PDGFRα* exon 1, *PDGFRα* promoter, and the right or left arms of pYAC4. The deduced maps are shown in Fig. 1A. Spritz et al. (1994) reported that 449C2 contained the centromeric (upstream) linkage marker D4S956, but this is presumably mistaken, based on our

Fig. 1. Characterization of YACs and YAC transgenic mice. (A) Map of the junction between the genomic DNA insert and right arm of the YAC vector in CEPH 449C2 (drawn to scale). The pYAC4 right arm (thick line) measures approximately 3.7 kb from the telomeric (TEL) *Bam*HI site to the *Eco*RI cloning site. The 449C2 insert starts at an *Eco*RI site positioned at −3566 or −3598 relative to the *PDGFRα* transcription start site (Afink et al., 1995; our mapping data could not distinguish these sites); the B214G6 insert starts at the *Eco*RI site at position −3163. (B) Analysis of YAC 449C2 by Southern blot. The chromosomes of the wild-type yeast strain (control lane, con) and the strain carrying 449C2 were separated by PFGE, blotted onto nylon membrane and hybridized with [γ-^{32}P]dATP-labelled oligonucleotides specific for either human *PDGFRα* exon 1 or the 3′-UTR (see Materials and Methods). A 380 kb band in 449C2 was detected with both probes. Because 449C2 comigrates with endogenous yeast chromosomes and very pure DNA is required for microinjection, it was transferred into yeast ‘window’ strain YLBW2. The resulting YAC, re-named 449-W2, was again analyzed by PFGE and Southern blot using a probe to the human 3′-UTR; this indicated that the transferred YAC was still full-length. (C) DNA from transgenic mice made by injection of 449C2 was subjected to semiquantitative PCR analysis (see Materials and Methods). 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.

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′-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 and H2, see above); 3′-UTR (H3 and H4, see above); exon 20 (E20F, 5′-TGG CAC CCC TTA CCC CGG CA-3′ and E20R, 5′-ACT TCA CTG GTA GCG TGG T-3′). The integrity of the insert was confirmed by Southern blots, using ^{32}P-labelled nick-translated probes against human *PDGFRα* extracellular domain and 3′-UTR. 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 (C57BL/6J×CBA F1 hybrids).

PDGFRα knockout mice were obtained from P. Soriano (Fred Hutchinson Cancer Research Center, Seattle, USA). Heterozygous males were mated with (C57BL/6J×CBA) F_1 hybrid females and the embryos transferred to surrogate mothers. The line was maintained on the same genetic background (for more than six generations). The animals 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′. These primers generate 451 bp and 242 bp fragments for the wild-type and mutant alleles, respectively (Soriano, 1997). *Patch* mutant mice were obtained from the MRC Mammalian Genetics Unit Mouse Embryo Bank (Harwell, UK). They were received on the Harwell (H4) inbred mouse background and rederived as above. They have been maintained for more than six generations on the (C57BL/6J×CBA) background.

Determining transgene copy number

To determine transgene copy number, part of the 3′-UTRs of the human and mouse *PDGFRα* genes were co-amplified by PCR using the following primers: 5′-CCC TTG TGG TCA TGC CAA AC-3′ and 5′-GCT TTT GCC TCC ATT ACA CTG G-3′. PCR conditions were 4 minutes at 94°C followed by 30 cycles of 30 seconds at 94°C, 45 seconds at 53°C and 1 minute at 72°C, concluding with 10 minutes at 72°C. PCR products were removed during the logarithmic phase of amplification (after 20, 22 and 24 cycles), digested with *Cla*I to cleave the mouse (but not the human) PCR product once, and electrophoresed on a 2% (w/v) agarose gel. Southern blots were hybridized with a ^{32}P-end-labelled oligonucleotide (5′-ACG AAG TTA TTA GGT CCC TCG AC-3′). Autoradiographic signals were quantified on a phosphoimager (Bio-Rad) by volume integration of individual gel bands.

In situ hybridization

Fixation, embedding and cryosectioning of embryos followed by in situ hybridization with digoxygenin (DIG)-labelled antisense RNA probes has been described previously (Fruttiger et al., 1999). The mouse *PDGFRα* probe was made from an approx. 1.6 kb *Eco*RI cDNA fragment encompassing most of the extracellular domain of mouse *PDGFRα* cloned into Bluescript KS (from Chiayeng Wang, University of Illinois, Chicago), cut with *Hin*dIII and transcribed with T7 RNA polymerase (T7pol). The human *PDGFRα* probe was made from an approx. 1.7 kb *Eco*RI-*Hin*dIII expressed sequence tag (EST) corresponding to part of the 3′-UTR of human *PDGFRα* mRNA (clone #1157-i18 from the UK HGMP Resource Centre). It was cut with *Eco*RI and transcribed with T3pol to generate an approx. 1.5 kb antisense transcript. Whole-mount in situ hybridization was performed as described by Xu and Wilkinson (1998).

Phenotypic rescue of *PDGFRα* mutant mice

Heterozygous *PDGFRα*$^{+/-}$ mutant mice were crossed with hemizygous *PDGFRα* YAC transgenic mice from line 3-3 and *PDGFRα*$^{+/-}$ offspring carrying the human YAC transgene were identified by PCR using primers listed above. These offspring were sibling-mated to generate homozygous *PDGFRα*$^{-/-}$/YAC offspring (called KO-YAC mice). Alternatively, *PDGFRα*$^{+/-}$/YAC mice were crossed with *PDGFRα*$^{+/-}$ 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, although we only recovered singles. Pregnant females were killed and embryos removed and staged by morphology (Theiler, 1972) before fixation for in situ hybridization. Genotypes were determined retrospectively by PCR on tail-tip DNA (see above). YAC complementation of *Ph* mutant mice was also attempted by either *Ph*-YAC×*Ph*-YAC or *Ph*-YAC×*Ph* matings.

Skeletal preparations and lung histochemistry

Embryos were fixed and stained in 20% acetic acid, 80% ethanol (v/v) containing 0.015% (w/v) Alcian Blue for 24 hours. Following dehydration in absolute ethanol for 72 hours, embryos were stained in 0.05% (w/v) Alizarin Red in 1% (w/v) KOH for 4 hours, then cleared in a 4:1 mixture of 1% KOH/ glycerol for several hours to several days, depending on the age and size of the embryos. The skeletal preparations were then transferred into a 1:1 mixture of 1% KOH/glycerol then into a 1:4 mixture of the same reagents and finally into undiluted glycerol for long-term storage, allowing several days for equilibration between each solution change.

Lungs were dissected and fixed in 4% (w/v) paraformaldehyde (PF) overnight at 4°C. Following dehydration through increasing concentrations of ethanol, tissue was equilibrated in xylene, impregnated with paraffin wax, embedded and sectioned at 6 μm. Sections were stained with Hematoxylin and Eosin.

Spinal cord cell culture and immunohistochemical analysis

Spinal cords were dissected free of surrounding tissue and meningeal membranes, in Hepes-buffered minimal medium (obtained from ICN) using tungsten needles. The tissue was transferred to EBSS containing 0.0125% (w/v) trypsin (Boehringer) and incubated at 37°C in 5% CO_2 for 30 minutes. Tissue was washed in DMEM containing 10% fetal calf serum (FCS), transferred to fresh DMEM containing 10% FCS and 0.005% (w/v) DNase-I (Sigma) and immediately dissociated by gentle trituration with a Paster pipette. The resulting cell suspension was filtered through a 20 μm pore-diameter mesh and washed by

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.

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.

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

Fig. 3. 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 widely throughout the embryo, including in branchial arch mesenchyme (white arrowheads), and somites (black arrows). The transgene was also expressed in the limb buds (white arrows), though at a low level compared to the endogeneous gene. At E12.5, both the endogenous gene (C) and the transgene (D) are expressed in sclerotome and dermatome, but expression of the transgene was generally weaker and less extensive. The human transgene was ectopically expressed in the dorsal and ventral spinal cord (D). This mimics elements of the expression pattern of the adjacent *c-kit* locus. The endogenous *PDGFRα* gene but not the transgene was expressed in the aortic valve and aortico-pulmonary spiral septum of the heart (E,F). At E14.5, the endogenous gene (G,H), and the transgene (J,K), were expressed in the periosteum/perichondrium of developing vertebrae (G,J) and ribs (H,K). However, the transgene was also expressed in the chondrocytic core of the cartilage (arrows in J,K) where endogenous *PDGFRα* is not expressed. In the E12.5 eye, endogenous *PDGFRα* was expressed in the anterior lens epithelium (I), whereas the transgene was aberrantly expressed in posterior lens fibres (L).

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* (Reinertson et al., 1997; Zhang et al., 1998).

PDGFRα is expressed widely in mesodermal tissues 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 (Table 1), most noticeably in the spinal cord and associated neural crest (Fig. 3B).

By E13.5, derivatives of the sclerotome have normally given rise to the cartilaginous framework of the spinal column and ribs. At E14.5, endogenous *PDGFRα* is expressed in the perichondrium/periosteum at the surface of developing bones, for example the vertebrae (Fig. 3G) and ribs (Fig. 3H) (Schatteman et al., 1992). The human *PDGFRα* transgene was also expressed in the perichondrium/periosteum, but continued to be expressed in the chondrocytic core of the cartilage where *PDGFRα* is normally downregulated (arrows in Fig. 3J,K). A similar failure to downregulate the transgene was seen in the developing eye lens. Both endogenous and transgenic *PDGFRα* were expressed in the anterior lens epithelium (Schatteman et al., 1992; Orr-Urtreger et al., 1992; Mudhar et al., 1993) (Fig. 3I,L). However, the human transgene was also

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 human transgene (e.g. the heart) and vice versa (e.g. dorsal spinal cord). We did not notice any differences between transgenic lines 3-3 and 5A1, suggesting that the disparities between transgene and endogenous gene expression are not a function of the site of integration. In both lines transgenic embryos were phenotypically indistinguishable from non-transgenic littermates, indicating that any increases in overall PDGFRα level due to the YAC transgenes had no overt developmental consequences. Presumably, ligand availability is the limiting factor for PDGF function in vivo (e.g. Calver et al., 1998).

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

PDGFRα null mice have severe developmental defects and most die before E16 (Soriano, 1997). To test whether the human *PDGFRα* YAC transgene can rescue these defects, we set up appropriate crosses to introduce the YAC transgene onto the *PDGFRα* null background (see Materials and Methods). We 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). The frontonasal and mandibular processes had failed to fuse at the midline. Many *PDGFRα* null embryos died before E14.5; those that survived later than this were smaller than their wild-type littermates, had a cleft face (Fig. 4A-C) and showed signs of internal bleeding at various sites (not shown). Neural tube abnormalities were also observed: the neural tube was not completely closed in the cervical region (spina bifida) and it had an abnormal shape.

Skeletal preparations of an E16.5 *PDGFRα* null embryo clearly showed that the anterior skull plates were severely reduced in size and failed to fuse at the midline (Fig. 4D,E). The path of the spinal column was distorted (Fig. 5H) and the cervical and upper thoracic vertebrae had an abnormal shape (compare Fig. 5J,K), having failed to close on the dorsal side as described for 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. 4G,H and J,K). We did not recover any live *PDGFRα* null embryos older than E16.5.

KO-YAC mice were alive and apparently normal with respect to external and skeletal morphology up to birth (e.g. Fig. 4C,F,I,L, which shows an E17.5 embryo). There were no signs of bleeding and the heart was beating. We recovered four KO-YAC embryos just before birth (E18.5-19) and four KO-YAC pups that had died shortly after birth. All of these animals were normal in appearance but slightly smaller than their wild-type littermates (see Table 2).

The human *PDGFRα* YAC rescues oligodendrocyte development in *PDGFRα* null spinal cords

Oligodendrocyte development was severely affected in *PDGFRα* null mice. Since differentiated oligodendrocytes do not normally appear in significant numbers until after birth, we were forced to establish cultures of spinal cord cells from E14.5 *PDGFRα* null embryos and study oligodendrogenesis in vitro. After 5 days in vitro (equivalent to the first day after birth, P1) we fixed the cells and labelled with antibodies against galactocerebroside (GC) to visualise differentiated oligodendrocytes (Raff et al., 1978) (Fig. 5A,B). Many fewer GC^+ oligodendrocytes developed in cultures of *PDGFRα* null spinal cords than in wild-type cultures (Fig. 5C). The numbers of oligodendrocytes that developed in cultures of KO-YAC spinal cords were indistinguishable from wild type (Fig. 5C).

Table 2. Lung and body mass of normal and KO-YAC embryos

Genotype (*n*)	Age	Lung mass (mg)	Body mass (g)
KO-YAC (3)	E18.5	19.2±3.75	1.1±0.06
Control (5)	E18.5	46.5±4.08	1.4±0.05
KO-YAC/Control	E18.5	0.41	0.79
KO-YAC (1)	P0	14.4	1.139
Control (2)	P0	39.4±4.2	1.4±0.0
KO-YAC/Control	P0	0.37	0.82

Lungs were weighed immediately after dissection and excess fluid had been dabbed off with a tissue. Controls were wild-type littermates. Values are means ± s.d. (*n*=3) or means ± range (*n*=2). Note that care was taken to ensure that the lungs did not fill with blood before weighing (by cooling the embryos to 4°C before decapitation).

The KO-YAC mice were slightly smaller than their normal littermates at birth (approx. 80% normal mass), while the lungs were much smaller (only 40% of normal).

PDGFRα knockouts 'rescued' with the YAC die after birth, probably from respiratory failure

On post-mortem examination of newborn KO-YAC mice, we found no obvious internal abnormalities except that the heart was slightly smaller than normal and the lungs dramatically so (Table 2, Fig. 6A). Lung histology appeared grossly normal (Fig. 6B-E), but on closer examination we discovered a defect in the development of a specific lung mesenchymal cell type.

In E15.5 wild-type embryos, two distinct types of cell in the lung express *PDGFRα*. These are interstitial mesenchymal cells, which express *PDGFRα* weakly, and putative smooth muscle progenitors (Lindahl et al., 1997), which express *PDGFRα* more strongly. The same expression pattern of the endogenous *PDGFRα* gene was observed in our wt-YAC embryos (Fig. 7A,D). Smooth muscle progenitors are flattened cells that are associated tightly with the outer surfaces of developing bronchial (epithelial) tubules (Fig. 7D, arrows). In the same wt-YAC animals, the human *PDGFRα* transgene was expressed in the interstitial mesenchymal cells but not in the smooth muscle progenitors (Fig. 7B,E). Consistent with this, in the KO-YAC lungs (which rely entirely on the human *PDGFRα* transgene), interstitial mesenchymal cells were present but smooth muscle progenitors were greatly reduced in number (Fig. 7C,F). (Due to the structure of the targeted *PDGFRα* allele (Soriano, 1997), a truncated, non-functional transcript is produced, which allows us to visualize cells that express the gene even in $PDGFR\alpha^{-/-}$ animals.) Our data therefore confirm that *PDGFRα* function is required for development of smooth muscle progenitors, as previously deduced from studies with *PDGF-A* knockout mice (Böstrom et al., 1995; Lindahl et al., 1997).

The loss of *PDGFRα*-positive smooth muscle progenitors was more obvious at E17.5 and later. In wt-YAC lungs probed for mouse *PDGFRα* these cells were abundant and individual cells were scattered throughout the lung tissue, in contact with the epithelial tubules (Fig. 7G, arrows). As at earlier ages, cells adjacent to the tubules in the same wt-YAC lungs did not express detectable human *PDGFRα* (Fig. 7H). In the KO-YAC lungs (which depend entirely on transgene-derived PDGFRα), smooth muscle progenitors were completely absent, judging by the lack of cells labelled with the mouse *PDGFRα* probe (Fig. 7I). The size of the KO-YAC lungs was markedly smaller than wild type at E18.5, as at birth (Table 2, Fig. 6). These data suggest either that smooth muscle progenitors play an important role in regulating the growth of the prenatal lung or, alternatively, that growth of lung mesenchymal cells is particularly sensitive to alterations in the level of expression of PDGFRα.

The YAC transgene does not prolong survival of *Patch* mutant mice

The *Patch* (*Ph*) mouse is a spontaneous mutant with a large deletion in the *PDGFRα* locus that includes the entire *PDGFRα* structural gene and flanking sequences. The phenotype of the *Ph* homozygote is similar but not identical to that of the targeted *PDGFRα* knockout (e.g. heart development is more severely affected in *Ph* and they also die earlier during embryogenesis), raising the possibility that the *Ph* deletion affects another gene(s) in addition to *PDGFRα*. We asked whether we could extend embryonic survival of *Ph/Ph* as with *PDGFRα* knockout mice by crossing in the YAC transgene (either *Ph*-YAC×*Ph*-YAC or *Ph*-YAC×*Ph* matings). We have so far not detected any offspring with the *Ph*/*Ph* genotype from a total of 46 embryos from nine litters aged between E12.5 and E18.5 (between 5 and 8 might have been expected if one or two copies of the YAC could complement *Ph* homozygotes). We tentatively conclude that the YAC is unable to rescue the early embryonic lethality of *Ph*.

DISCUSSION

We produced transgenic mice carrying the complete human *PDGFRα* structural gene with 5′- and 3′- flanking sequences by pronuclear injection of a YAC, CEPH 449C2. We chose 449C2 for transgenesis because (1) it is of manageable size (approx. 380 kb) and is not chimeric, (2) it was reported to contain long flanking sequences both 5′ and 3′ of the gene and (3) it contains human *PDGFRα*, allowing discrimination between the transgene and endogenous gene (Spritz et al., 1994). Detailed mapping of the 5′ *PDGFRα* flank showed that YAC 449C2 contained much less upstream sequence than originally reported, only approx. 3.6 kb. Another YAC, SL B214G6, which was isolated from a different library, turned out to terminate within 500 bp of 449C2 at the 5′ end. This might be coincidence, or alternatively might reflect some property of the sequence further upstream that renders it difficult to propagate in yeast.

Nevertheless, the profound craniofacial defects and spina bifida observed in mice with a targeted *PDGFRα* null mutation were completely rescued by the YAC. The craniofacial defects – severe shortening of the jaw bones, for example – presumably reflect reduced proliferation and/or migration of cranial neural crest in the *PDGFRα* knockout, so complementation of these defects is consistent with the observation that the YAC appeared to be expressed correctly in branchial arch mesenchyme. More caudal skeletal elements including vertebrae and ribs are generated from somitic mesoderm, not crest. The YAC transgene corrected malformations of these structures despite the fact that YAC expression was not completely normal there. For example, the transgene was expressed in cells in the chondrocytic core of developing vertebrae and ribs where *PDGFRα* expression is normally absent. Note that neither *PDGF-A* null mice nor *PDGF-A/PDGF-B* double-mutant mice develop any craniofacial defects or spina bifida like the *PDGFRα* nulls (personal communication from C. Betsholtz, University of Göteborg, Sweden). This raises the possibility that the recently described PDGF-C, also a ligand for PDGFRα, might contribute to craniofacial/skeletal growth (Li et al., 2000).

Despite the striking rescue of skeletal defects, the KO-YAC mice still died at birth. This was probably from respiratory failure due to defective lung development. Early lung branching and growth is reduced by antisense inhibition of PDGF-A in culture (Souza et al., 1995), but no comparable effects on in vivo lung development have been described in *PDGF-A* knockout mice (Boström et al., 1996; Lindahl et al., 1997). *PDGF-A* null mice die after birth from alveogenesis failure, apparently because *PDGFRα*-positive smooth muscle progenitors do not proliferate and migrate properly in the mutant lungs (Lindahl et al., 1997). It is to be expected that a similar defect in proliferation and migration of these cells should occur in *PDGFRα* null lungs but it has not been possible to address this because *Patch* (*Ph*) mutant mice and targeted *PDGFRα* knockout mice die as embryos, mostly before E16. The fact that the KO-YAC mice survived until birth, together with our finding that lung smooth muscle progenitors were essentially missing from the KO-YAC lungs allowed us to follow lung development up to birth in the absence of smooth muscle progenitors. We found that lung mass in KO-YAC mice was reduced by almost two-thirds compared to their wild-type siblings, although lung histology appeared normal. Since smooth muscle progenitors are tightly associated with growing epithelial tubules in wild-type lungs, it seems plausible that these cells play a role in regulating the extension and/or branching of the tubules and that this is the underlying cause of the reduced growth of KO-YAC lungs. This is in keeping with the generally held view that reciprocal epithelial-mesenchyme interactions are crucial for the development of the lung and other organs (Lindahl et al., 1997; Hogan, 1999). An alternative explanation might be that the absolute level of PDGFRα expression in interstitial mesenchymal cells of the KO-YAC lungs is abnormal and this, rather than the loss of smooth muscle progenitors, is responsible for reducing lung growth. However, this would imply that growth of lung tissue must be more sensitive than other tissues to alterations to cellular levels of PDGFRα.

One of our longer-term aims in generating the YAC transgenics is to identify regulatory elements that are responsible for driving *PDGFRα* transcription in various progenitor cell populations in the embryo, especially

Fig. 5. 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-GC and photographed under fluorescence (A) and phase contrast optics (B). Many fewer GC^+ oligodendrocytes developed in cultures of *PDGFRα* null spinal cord than in wild-type and KO-YAC cultures. (C) 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 and only 57±6 GC^+ cells per coverslip in homozygous *PDGFRα* null cultures (means ±range, duplicate coverslips, one embryo of each genotype).

Fig. 4. Craniofacial and skeletal defects in homozygous *PDGFRα* null (KO) mice were rescued by the human *PDGFRα* YAC. E16.5 wild-type (A,D,G,J) and KO (B,E,H,K) mice; (C,F,I,L) E17.5 KO-YAC mice. At E16.5 KO embryos (B) displayed a cleft face and internal bleeding compared with wild type (A). Skeletal preparations of KO embryos of the same age (E) indicated that the mandibular bone (mb), nasal bone (nb) and frontal bone (fb) had failed to fuse in the midline (compare with D). E16.5 KO embryos are clearly smaller than wild type and have severe arching of the spinal column and abnormal vertebrae (H,K) (compare with G,J). The human *PDGFRα* YAC rescued all these craniofacial and skeletal 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 instead of E16.5) hence Alcian Blue cartilage staining is weaker.

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 would place these elements between 3.6 kb and 6 kb upstream of the transcription start site.

Ectopic expression of the *PDGFRα* transgene in the dorsal spinal cord and blood vessels matches some aspects of the normal expression patterns of *c-kit*, a closely related receptor tyrosine kinase that maps downstream of *PDGFRα* on mouse

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 *PDGFRα* transcript that allows us to visualize the fate of the *PDGFRα*-expressing cells in the knockout. (G) E17.5 wt-YAC lung probed for mouse *PDGFRα*. (H) E17.5 wt-YAC lung probed for human *PDGFRα*. (I) E17.5 KO-YAC lung probed for mouse *PDGFRα*. Many *PDGFRα*-positive smooth muscle progenitors are evident in the wt-YAC lung (G, arrows), but these cells do not express the human *PDGFRα* transgene (H) and, as a result, do not develop in the KO-YAC lung (I). Scale bar: 300 μm for A-C; 75 μm for D-I.

explain not only the ectopic *PDGFRα* expression that we observe in our transgenics but also the generally low level of expression. Therefore, removing sequences downstream of *PDGFRα* in the YAC might improve both the fidelity and strength of transgene expression.

We were unable to detect transgene expression in some normal sites of *PDGFRα* expression, notably in smooth muscle progenitors in the lung (see above) and in the heart. Normally, *PDGFRα* is expressed from E9 in the pericardium and the endocardial cushions of the heart, and later in the newly forming atrial and ventricular valves and in the immediately abutting myocardium (Orr-Urtreger and Lonai, 1992; Orr-Urtreger et al., 1992; Morrison-Graham et al., 1992; Schatteman et al., 1992). In homozygous *Ph* mutants the heart fails to septate, suggesting that *PDGFRα* is crucial for heart development. However no heart defect was described in the targeted *PDGFRα* null mutant (Soriano, 1997). In addition, *Ph* mice generally die earlier in gestation than the targeted *PDGFRα* null mice. These discrepancies could result from insufficiency of other genes in addition to *PDGFRα* in the *Ph* mice. We have attempted to complement the genetic defect in *Ph* by crossing in the YAC transgene but have failed to detect any rescue. Our mouse breeding colonies were maintained so as to minimize differences in genetic background among the different strains used in this study (see Materials and Methods). Our data therefore add support to the idea that early embryonic lethality of *Ph* mice (100% of *Ph/Ph* embryos die before E12 in our colony) might be due to defects outside the *PDGFRα* structural gene. For example, it is known that expression of *c-kit* is perturbed in *Ph* (Duttlinger et al., 1995; Wehrle-Haller et al., 1996; see above) and in addition the *Ph* deletion extends 50-100 kb upstream of *PDGFRα* (Brunkow et al., 1995; Berrozpe et al., 1999), possibly affecting a neighbouring upstream gene(s). The failure of YAC complementation does not allow us to distinguish between these possibilities.

Our demonstration that the targeted *PDGFRα* null mouse can be partially rescued by YAC 449C2 encourages us to think that a different YAC with more 5′-sequence might allow complete rescue. This would open the way to a mutational analysis of *PDGFRα* structure-function relationships in vivo. In addition, information gained from the present study will help us locate and isolate transcriptional control elements that function specifically in various types of progenitor cells in the embryo. Targeted deletion of individual elements might then lead to loss of *PDGFRα* expression in specific types of progenitor cells, allowing us to assess the role of *PDGFRα* in individual cell lineages or organ systems. The present study has fortuitously allowed us to do that for lung smooth muscle progenitors up to birth.

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