

Disruption of PDGFR α -initiated PI3K activation and migration of somite derivatives leads to spina bifida

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Spina bifida, or failure of the vertebrae to close at the midline, is a common congenital malformation in humans that is often synonymous with neural tube defects (NTDs). However, it is likely that other etiologies exist. Genetic disruption of platelet-derived growth factor receptor (PDGFR) α results in spina bifida, but the underlying mechanism has not been identified. To elucidate the cause of this birth defect in PDGFR α mutant embryos, we examined the developmental processes involved in vertebrae formation. Exposure of chick embryos to the PDGFR inhibitor imatinib mesylate resulted in spina bifida in the absence of NTDs. We next examined embryos with a tissue-specific deletion of the receptor. We found that loss of the receptor from chondrocytes did not recapitulate the spina bifida phenotype. By contrast, loss of the receptor from all sclerotome and dermatome derivatives or disruption of PDGFR α -driven phosphatidylinositol 3' kinase (PI3K) activity resulted in spina bifida. Furthermore, we identified a migration defect in the sclerotome as the cause of the abnormal vertebral development. We found that primary cells from these mice exhibited defects in PAK1 activation and paxillin localization. Taken together, these results indicate that PDGFR α downstream effectors, especially PI3K, are essential for cell migration of a somite-derived dorsal mesenchyme and disruption of receptor signaling in these cells leads to spina bifida.

KEY WORDS: Spina bifida, PDGF, PI3 kinase, Cell migration, S6K1, PAK1, Mouse, Chick

INTRODUCTION

Spina bifida is a congenital birth defect where the vertebrae fail to protect the spinal cord (Stedman, 2000). Currently, there are several proposed etiologies for spina bifida. One category is attributed to failure of neural tube closure, also known as NTD (reviewed by Juriloff and Harris, 2000). Prenatal folic acid supplementation has been highly effective in reducing the occurrence of NTD, but analysis of several mouse models of spina bifida suggest that additional causes may exist for this birth defect (Helwig et al., 1995; Payne et al., 1997; Takahashi et al., 1992). These analyses point to defects in the mesoderm surrounding the neural tube as a potential cause of spina bifida. Previous analyses of PDGFR α and PDGF ligand null and mutant alleles have clearly demonstrated that loss of PDGFR α signaling results in spina bifida, but the cellular basis responsible for these defects has remained elusive (Ding et al., 2004; Helwig et al., 1995; Payne et al., 1997; Soriano, 1997). One reason is that PDGFR α is broadly expressed in mesenchymal populations during embryonic development, and complete loss of the receptor affects cells in many tissues and causes early embryonic lethality. Therefore, the reduced viability of later gestation embryos prohibits the study of spina bifida in these mutants.

More recently, analysis of a series of signaling point mutant alleles in the PDGFR α demonstrated that loss of PI3K signaling downstream of the receptor also resulted in spina bifida (Klinghoffer et al., 2002). These mice live until birth, providing a means to investigate the cause of spina bifida in a PDGFR α mutant background. In addition to vertebral defects, mislocalization of oligodendrocytes and melanocytes hinted that loss of PI3K activation downstream of PDGFR α may result in aberrant cell

migration. Although stimulation of PDGF receptors can induce cytoskeletal rearrangements and cell migration *in vitro*, few studies have demonstrated a clear requirement for PDGF receptor-driven cell migration *in vivo*. In *Xenopus* embryos, PDGFR α directs mesoderm towards the blastocoel roof and disruption of this signaling pathway leads to randomized movement of cells (Nagel et al., 2004). In *Drosophila*, PVR, a receptor tyrosine kinase related to the mammalian PDGF and VEGF receptors, directs border cell migration to the oocyte (Duchek et al., 2001) and hemocyte migration (Wood et al., 2006).

In this report, we demonstrate that normal vertebral arch formation requires PDGFR α signal transduction. Surprisingly, the cells dependent on this receptor are not chondrocytes. Using mice defective in PDGFR α -initiated PI3K (PDGFR α ^{PI3K/PI3K}) signaling and lacking PDGFR α from a broad range of somite cells, we demonstrate that proliferation and survival of this somite-derived population are unaffected. Instead, cell migration is defective. We further show that cells derived from mutant embryos fail to activate pathways implicated in actin reorganization and migration. PDGFR α ^{PI3K/PI3K} mutant cells fail to activate Pak1, Rac1 and S6K1 in response to PDGF stimulation. These signaling disruptions result in loss of paxillin localization to focal contacts. These findings demonstrate that a somite-derived cell population requires PDGFR α -induced PI3K activity for migration *in vivo* and that the presence of other PDGFR α signaling pathways cannot bypass the requirement for PI3K activation.

MATERIALS AND METHODS

In ovo chick studies

Fertilized White Leghorn (*Gallus gallus*) eggs were obtained from the Texas A&M Poultry Science Department (College Station, TX), incubated at 37°C, then opened and staged at 2-3 days of development. Imatinib mesylate (a kind gift from R. Ilaria) or vehicle alone was applied by bath application of 500 μ l to HH stage 14-17 embryos. Openings were sealed with tape and allowed to develop to HH stage 36. Embryos were frozen-embedded for histological sectioning. We analyzed two vehicle-treated embryos, and four each of 25 μ M and 50 μ M imatinib-treated embryos.

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Mice

The mutant and Cre alleles used in these experiments were: *PDGFR α ^{PI3K/PI3K}* (Klinghoffer et al., 2002), *PDGFR α ^{GFP}* (Hamilton et al., 2003), *PDGFR α ^{fl}* (Tallquist and Soriano, 2003), *Twist2^{Cre}* (Yu et al., 2003), and *Col2a1-Cre^{Tg}* (Ovchinnikov et al., 2000). The *Twist2^{Cre}* line was kindly provided by E. Olson. The *Col2a1-Cre^{Tg}* mice were kindly provided by G. Karsenty. Embryos possessing a tissue-specific deletion of the *PDGFR α* were generated by crossing males (*PDGFR α ^{fl/+}; Cre⁺*) to females homozygous for the *PDGFR α ^{fl/fl}*.

Cell culture

Mesenchymal cells were isolated from the thoracic and lumbar regions of E13.5 embryos by removing surface ectoderm, neural tube, dorsal root ganglia and vertebral column components. Remaining tissue was rinsed extensively with PBS and dispase (Roche, Basel, Switzerland) treated (0.5 mg/ml for 10 minutes at 37°C) to form a single cell suspension. Single cell suspensions were rinsed with PBS then plated. Cultures containing *PDGFR α ^{GFP}* alleles were scored for GFP-expression as a marker of *PDGFR α* -expression. Passage 1 cultures were 90% (\pm 3%) GFP⁺ ($n=4$). Passage 4 cultures were 89% (\pm 4%) GFP⁺ ($n=5$). Marker analysis (*Twist2* for dermatome and dermis; *Col2a1* and scleraxis for sclerotome and chondrocytes) was performed by RT-PCR to confirm cell culture identity and purity. Cultures were positive for *Twist2*, negative for *Col2a1* and scleraxis. RNA was extracted by Trizol (Invitrogen, Carlsbad, CA) and cDNA was generated using PowerScript Reverse Transcriptase (Clontech, Mountain View, CA).

Migration

Primary cells in single suspension were plated in triplicate at a density of 4×10^4 cells per well in a 96-well chemotaxis chamber (NeuroProbe), on a filter (8 μ m pore size, PVP-free) pre-treated for 1 hour with 10 μ g/ml rat tail collagen (Sigma, St Louis, MO). Growth factors were added to the bottom wells at the indicated concentrations. PDGFAA, PDGFBB and pTGF β 1 ligands were obtained from R&D Systems; bFGF was from Sigma. Long-term rapamycin treatment (22 μ M, Sigma) was 48 hours before plating cells in the chemotaxis chamber at 1×10^4 cells per well. For short-term rapamycin treatment, cells were plated in the chemotaxis chamber at 2×10^4 cells per well in rapamycin (44 μ M, Sigma) or vehicle. After addition of cells and growth factors, the migration chamber was incubated at 37°C for 6 hours. Each experiment was performed at least twice with independent cell lines, and each condition was assayed in triplicate.

Histological procedures and skeletal staining

For H&E and Safranin-O staining, embryos were fixed in 4% paraformaldehyde at 4°C overnight, embedded in paraffin, sectioned at a thickness of 7 μ m, and stained according to standard procedures. Skeletal preparations were performed according to (Hogan, 1994). For green fluorescent protein (GFP) expression, embryos were fixed in 4% PFA overnight, saturated in 10% sucrose at 4°C overnight, embedded in OCT and sectioned at 10 μ m. For BrdU analysis, pregnant females were injected with 10 μ g BrdU (Sigma) per gram of body weight 1 hour prior to sacrifice. Embryos were fixed and sectioned as described above. BrdU was detected by anti-BrdU (Becton Dickinson, Franklin Lakes, NJ) primary antibody (1:50 dilution in block).

Cell number, proliferation and TUNEL index

Images of high-quality sections through the lumbar region were taken at 40 \times . For all quantification, the region that was quantified was demarcated by a box of consistent width that extended below the surface ectoderm to the perineural plexus in three sections for each embryo. Cells were identified by the nuclear hematoxylin-QS counterstain (Vectastain). One embryo for each genotype at each time point was examined. Cells were counted in the region dorsal to the neural tube. Proliferation index was calculated by dividing the number of BrdU-positive cells by the total number of cells within the boxed area and multiplying by 100. TUNEL index was performed by standard procedures using biotinylated-14-dATP and detected using the streptavidin-HRP and DAB (Vectastain) kits. The TUNEL index was calculated by dividing the number of TUNEL-positive cells by the total number of cells in the demarcated region. No TUNEL-positive cells were

detected in this region although TUNEL positive cells could be found in other tissues of the embryo. We also performed the assay on a positive control (DNase I treated section) to verify the detection of fragmented DNA.

Micromass culture analysis

Limb buds isolated from E11.5 embryos were rinsed extensively with PBS, dispase treated (1 U/ml for 30 minutes at 37°C), rinsed and dispersed to make a single cell suspension. Cells were plated in 25 μ l droplets at a density of 1×10^7 cells/ml of basal media (DMEM with 2% serum) and incubated at 37°C for 1 hour. Wells were flooded with basal media. After 24 hours, conditioned media (1:1) and growth factors (concentrations as indicated) were added. Growth factor and conditioned media treatments were performed in triplicate. Conditioned media was harvested from cells cultured in 10% serum at density of 5×10^5 cells per well after 24 hours. Micromass cultures were fixed in ethanol after 5 or 7 days of culture, and chondrocyte nodules were stained with 1% Alcian Blue. Stained cultures were rinsed with 3% acetic acid to remove excess stain. Alcian Blue was extracted with 300 μ l 4 M guanidine-HCl. Extracted Alcian Blue was measured by OD₆₀₀ (Amersham Biosciences GeneQuant pro spectrophotometer). The 4- to 5-day culture was repeated three times, and the 7-day culture was repeated twice.

Western blot

For western blot analysis, cells were starved (in DMEM with 0.1% serum) for 48 hours, then stimulated with 10 ng/ml PDGF-AA or 10% serum for 5 minutes. Whole cell lysates were run on 7.5% SDS-PAGE and transferred to PVD membranes. Rac1 pull downs were performed as previously described (del Pozo et al., 2000). Cells were plated at 1×10^6 cells per 10 cm dish and then starved for 24 hours in 0.1% serum. After starvation, cells were stimulated with either 10% serum or 25 ng/ml PDGFAA for 2-20 minutes. Following stimulation, cells were washed with ice cold PBS prior to lysis. Lysis buffer (400 μ l) plus aprotinin (Sigma, A6279-5ML) and PMSF were added. GTP-bound Rac1 was then isolated using the Rac1 activation kit (Upstate, Billerica, MA, 17-283).

Immunocytochemistry

GFP-expressing cells were plated for 24 hours in 1% FBS in DMEM. Cells were stimulated for 10 minutes with either 10% serum containing media or 10 ng/ml PDGFAA. *PDGFR α ^{PI3K/PI3K}* and control cells were plated overnight and then starved in 0.1% serum for 6 hours. After starvation cells were stimulated for 10 minutes with either 10% serum-containing media or 25 ng/ml PDGFAA. Cells were then fixed in 3% paraformaldehyde, permeabilized, blocked and stained for paxillin (BD Transduction Laboratories, Franklin Lakes, NJ, 51-9002034) at a dilution of 1:400. Secondary antibody was donkey anti-mouse 594 (Molecular Bioprobes, Carlsbad, CA). Cells were imaged on a Zeiss Axiovert 200 microscope.

Antibodies

Cytoskeletal actin loading control (Novus, Littleton, CO 1:5000), AKT-p(Ser473) (Cell Signaling, Danvers, MA 1:1000), AKT (Cell Signaling, 1:1,000), FAK (Cell Signaling, 1:1000), FAK-p(Tyr576/577) (Cell Signaling, 1:1000), MAPK-p(P44,42) (Cell Signaling, 1:5000), MAPK (Cell Signaling, 1:2500), PAK1 (Cell Signaling, 1:1000), PAK1-p(Ser199/204) (Cell Signaling, 1:1000), PAK1-p(Thr423) (Cell Signaling, 1:1000), PDGFR α (Santa Cruz, Santa Cruz, CA 1:333), p70S6K-p(Thr389) (Cell Signaling, 1:1000) and p70S6K (Cell Signaling, 1:1000).

In situ hybridization

Embryos were fixed in 4% PFA overnight, saturated in 10% sucrose at 4°C overnight, embedded in OCT, and sectioned at 14 μ m. In situ analyses were performed as previously described (Wilkinson, 1992).

RT-PCR primers

Twist2: forwards, CAGAGCGACGAGATGGACAATAAG; reverse GGTTGGTCTTGTTTCCTCAGG. *Col2a1*: forwards, TATGGAAGC-CCTCATCTTGCCG; reverse, TCTTTTCTCCCTTGTCACCAGC. *Scleraxis*: forwards, TTCTCACCTGGGCAATGTGC; reverse AGT-GTTCGGCTGCTTAGAGTCAAG.

RESULTS

Treatment with imatinib mesylate recapitulates PDGFR α spina bifida phenotype

One of the most widely accepted causes of spina bifida is failure of neural tube closure (Juriloff and Harris, 2000). Several PDGF receptor mutant alleles exhibit spina bifida and wavy neural tubes, but no neural tube defects have been reported in these embryos. To determine whether disruption of PDGF receptor activity can cause spina bifida after neural tube closure has occurred, we used a pharmacological inhibitor of PDGF receptor tyrosine kinase activity (imatinib mesylate) in developing embryos (Buchdunger et al., 2002). Using the avian system, we treated HH stage 14-17 embryos with imatinib mesylate. This stage is just after neural tube closure and compartmentalization of the rostral somites occurs (equivalent to an E11.5-12.5 mouse embryo). We then examined vertebral formation at stage 36 after chondrogenesis of the lumbar vertebrae is completed (reviewed by Romanoff, 1960). Comparing imatinib mesylate-treated and untreated embryos, we observed no differences in embryo size. In the majority of drug-treated embryos, their outward appearance was normal, including the craniofacial region and limbs. However, in two embryos treated with 50 μ M of imatinib mesylate, we observed failure of ventral closure, which has also been reported in PDGFR α -null embryos (Soriano, 1997). Serial sections of the embryos revealed that all drug-treated embryos exhibited spina bifida. In each of these embryos, we observed failure of the spinal lamina (plates of bone that form the dorsal wall of the vertebrae) to extend to the dorsal midline throughout the lumbar and lumbar-sacral regions. All vehicle-treated embryos possessed normal vertebral development (Fig. 1). In addition, the neural tubes of drug-treated embryos were comparable with vehicle-treated embryos in that they were closed and exhibited no obvious defects. In the slightly older drug-treated embryos, we also observed feather primordia. These data suggest that inhibition of PDGF receptor signaling after neural tube closure leads to spina bifida.

Loss of PDGFR α from cartilage does not result in spina bifida

The lumbar vertebrae form by extension of the somite-derived sclerotome around the neural tube. PDGFR α is expressed in the epithelial somite but becomes restricted to a subset of cells, including the perichondrium, the condensing mesenchyme surrounding the vertebral arch and the dermis (Hamilton et al., 2003; Orr-Urtreger and Lonai, 1992; Payne et al., 1997; Takakura et al., 1997). To gain a better understanding of the cellular disruption leading to spina bifida, we examined three independent mouse lines with defective PDGFR α signaling. Using a PDGFR α allele that is flanked by loxP sites (Tallquist and Soriano, 2003) and tissue-specific Cre-expressing mouse lines, we generated mice that lacked PDGFR α in distinct somitic cell populations. Embryos deficient in PDGFR α in vertebral cartilage populations were generated using *Col2a1-Cre^{Tg}* mice (Ovchinnikov et al., 2000) (referred to as PDGFR α^{CKO}). In these embryos, Cre is expressed in the sclerotome as early as E9.5 and results in recombination of loxP-flanked alleles in all cartilage and perichondrium of the axial skeleton (Day et al., 2005; Yoon et al., 2005). A broader deletion in condensed mesenchyme was generated using mice that express Cre from the *Twist2* locus (Yu et al., 2003) (referred to as PDGFR α^{TKO}). In this mouse line, Cre is expressed in the sclerotome condensing mesenchyme, the dermatome and osteoblasts. Finally, we also examined vertebral development in embryos expressing a PDGFR α that was incapable of signaling through PI3K (PDGFR $\alpha^{PI3K/PI3K}$) and had been previously reported to exhibit spina bifida (Klinghoffer

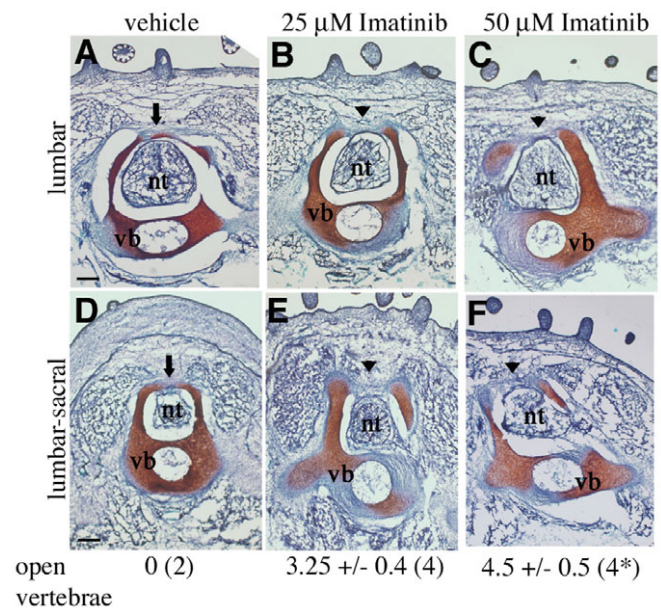


Fig. 1. Induction of spina bifida by imatinib-mesylate.

(A-F) Representative Safranin-O stained transverse sections through the lumbar and lumbar-sacral regions of chick embryos (HH stage 36). (A,D) Vehicle treated, and (B,E) 25 μ M and (C,F) 50 μ M imatinib-mesylate treated. The lamina (indicated by an arrow in A,D) failed to form across the dorsal midline in imatinib-treated embryos (indicated by arrowheads in B,C,E,F), resulting in spina bifida. Images show the most advanced arch formation found in serial sections of each treatment group. Scale bars: 200 μ m. nt, neural tube; vb, vertebral body. Values listed below each column indicate the number of open vertebrae in all serial sections through the lower thoracic, lumbar and sacral region in each embryo. The number of embryos examined is indicated in parentheses. *Two out of four embryos treated with 50 μ M imatinib-mesylate also exhibited failure of ventral closure.

et al., 2002). This allele contains engineered point mutations in the domain of the PDGFR α that normally binds PI3K, resulting in a loss of this signaling pathway downstream of the receptor.

Examination of skeletal preparations at E18.5 demonstrated that loss of PDGFR α in chondrocytes resulted in normal formation and closure of the vertebrae (Fig. 2B) when compared with controls (Fig. 2A), although 100% of these mice died at birth from a clefting of the secondary palate similar to mice with neural crest cell deletion of the receptor (Tallquist and Soriano, 2003). This suggests that deletion of PDGFR α occurred in chondrocyte populations, but that loss of the receptor in vertebral chondrocytes and perichondrium did not affect its development. By contrast, defects were observed in the dorsal-most sclerotome derivatives in PDGFR α^{TKO} and PDGFR $\alpha^{PI3K/PI3K}$ embryos. In the lumbar region, the progression of the lamina was significantly impeded, and the spinous process was absent (Fig. 2C,D). This phenotype was specific to the lumbar vertebrae (L1-L6) and sometimes included the immediate surrounding thoracic (T10-13) and sacral (S1-2) vertebrae. In all embryos examined, the vertebral bodies, vertebral arches, pedicles, ribs and appendicular skeleton developed normally (Fig. 2 and data not shown). When postnatal day 1 (P1) PDGFR α^{TKO} mice were recovered, we observed protrusion of the spinal cord through the opening in the vertebrae (data not shown). This suggested that during the process of birth, constriction of the embryo caused the spinal cord to emerge between the open vertebrae, resulting in subcutaneous myelomeningocele (or spina bifida aperta). Because

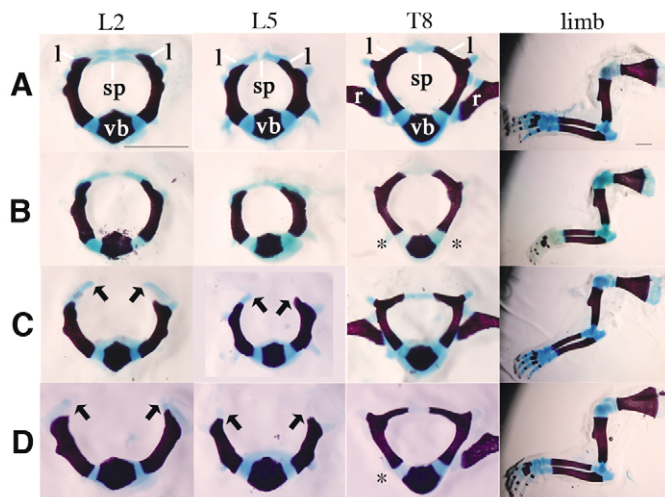


Fig. 2. Spina bifida in $PDGFR\alpha$ mutant embryos. Skeletal preparations of E18.5 embryos display defects in lumbar vertebrae formation. (A) Control, (B) $PDGFR\alpha^{CKO}$ (C) $PDGFR\alpha^{PI3K/PI3K}$ and (D) $PDGFR\alpha^{TKO}$. Headings at the top of each column indicate the bone isolated. L, lumbar; T, thoracic. The lumbar vertebral arches form normally in A and B but fail to progress in C and D. Vertebral arch development of the thoracic vertebrae and limbs was normal in all embryos. Arrows indicate the extent that Alcian Blue cartilage can be detected. Scale bars: 1 mm. Asterisks indicate location of bones lost during dissection. sp, spinous process; l, lamina; vb, vertebral body; r, rib. Data representative of skeletal preparations performed on E18.5 and P1 embryos: wild type ($n=10$); $PDGFR\alpha^{CKO}$ ($n=4$); $PDGFR\alpha^{PI3K/PI3K}$ ($n=10$); and $PDGFR\alpha^{TKO}$ ($n=9$).

of the distinct phenotypes of the $PDGFR\alpha^{CKO}$ and the $PDGFR\alpha^{TKO}$ embryos, we conclude that the defect in lamina development does not result from a primary abnormality in perichondrium or chondrocytes. Instead, the defect was probably caused by a loss of $PDGFR\alpha$ signaling in a somite cell population other than chondrocytes.

Loss of progression of the dorsal vertebrae

Because spina bifida is commonly associated with NTD, we examined the $PDGFR\alpha^{TKO}$ and $PDGFR\alpha^{PI3K/PI3K}$ embryos throughout gestation for neural tube closure and found that the neural tube had a normal morphology and closed at the expected time point (Fig. 3B and data not shown). To identify when the defect in vertebral development appeared, we examined the lumbar region by H&E and Safranin O (cartilage) staining between E14.5 and E16.5 (Fig. 3A–J and data not shown). At E14.5, control, $PDGFR\alpha^{PI3K/PI3K}$ and $PDGFR\alpha^{TKO}$ embryos appeared similar (Fig. 3A,B), but by E15.5, defects in vertebral development were observed (Fig. 3E,F,I–L). In wild-type embryos, the chondrocytes expanded along the dorsal aspect of the neural tube, but in mutant embryos small condensations of unstained mesenchyme were observed (Fig. 3E,F). By E16.5, mesenchymal condensations could be identified, but the area was dramatically reduced in size and did not stain with Safranin O (Fig. 3 I–J). Using collagen type 2a1 as an earlier marker for chondrocyte progenitors and chondrocytes, we found that the precursors of the lamina were present, but they failed to extend towards the dorsal midline (see Fig. S1A in the supplementary material). Examination of cells dorsal to the neural tube revealed an increase in cell density between E14.5 to E15.5 in the wild type but not in the mutant (Fig. 3C,D,G,H; see Table S1 in the supplementary material). When we examined the lumbar region

of E18.5 $PDGFR\alpha^{TKO}$ embryos, we found a similar result. Although the skin was similar to control sections with regards to epidermis, dermis and subcutis formation, the lamina and the adjacent dense mesenchyme were absent (Fig. 3K,L). These data demonstrate that the defect in the vertebral arch formation in the $PDGFR\alpha^{PI3K/PI3K}$ and $PDGFR\alpha^{TKO}$ embryos occurred after E14.5 and specifically affected the lamina of the lumbar vertebrae.

Mesenchymal cell-secreted factors promote chondrogenesis

Previous reports suggest that growth factor secretion from adjacent tissues may affect the development of the dorsal vertebrae (Boyd et al., 2004). To investigate the possibility that loss of $PDGFR\alpha$ signaling negatively affected expression of chondrocyte growth factors, we isolated primary cells from the lumbar region of E13.5 control, $PDGFR\alpha^{PI3K/PI3K}$ and $PDGFR\alpha^{TKO}$ embryos (see Materials and methods) and compared their abilities to promote chondrogenic growth and differentiation in a limb bud micromass culture system. In this assay, undifferentiated limb bud mesenchyme is stimulated with conditioned media from primary cells. Because the limb bud mesenchyme is capable of cartilage differentiation in vitro, one can determine whether secreted factors, such as growth factors or extracellular matrix, in conditioned media are capable of enhancing the chondrocyte differentiation (Stott and Chuong, 2000). We found that conditioned media from both wild-type and $PDGFR\alpha$ mutant cells (TKO and PI3K/PI3K) were capable of inducing the production of cartilage proteoglycans, similar to stimulation with TGF β 1, as measured by Alcian Blue binding (see Fig. S2 in the supplementary material). To determine whether chondrocyte induction was specific to primary mesenchymal cells, we also tested conditioned media from mouse embryonic fibroblasts (MEF) and HEK293T epithelial cell lines. MEF-conditioned media promoted chondrocyte differentiation, whereas HEK293T conditioned media induction was similar to control media. These data indicate that disruption of $PDGFR\alpha$ signaling does not alter the secretion of factors enhancing chondrocyte differentiation.

Disrupted sclerotome migration in $PDGFR\alpha$ mutant embryos

A major function of the $PDGFR\alpha$ during development is to promote the proliferation of progenitor cell populations (Betsholtz, 2003). To determine whether a proliferation defect was the cause of the aberrant vertebral formation, we compared proliferation of control, $PDGFR\alpha^{TKO}$ and $PDGFR\alpha^{PI3K/PI3K}$ embryos. All exhibited similar rates of proliferation in comparable regions of the neural arch, as well as the loose sclerotome cells adjacent to the developing vertebrae and the dermis (see Fig. S3 in the supplementary material). We also investigated the proliferation of primary cells from E13.5 embryos and found that whereas control and $PDGFR\alpha^{PI3K/PI3K}$ cells proliferated in response to media containing 10% serum, neither cell type proliferated in response to PDGFAA stimulation (data not shown). Thus, in contrast to many other cell populations that rely upon $PDGFR\alpha$ signal transduction for proliferation, sclerotome is unaffected by loss of the receptor.

Because we had noted a specific decrease in the cell population dorsal to the neural tube, we examined the expansion of this cell population in the lumbar region during vertebral development. Between E14.5 and E15.5 control embryos demonstrated an expansion in the total number of cells present, whereas $PDGFR\alpha^{PI3K/PI3K}$ cells showed little increase (see Table S1 in the supplementary material). To determine whether the failure in expansion of this cell population was caused by reduced

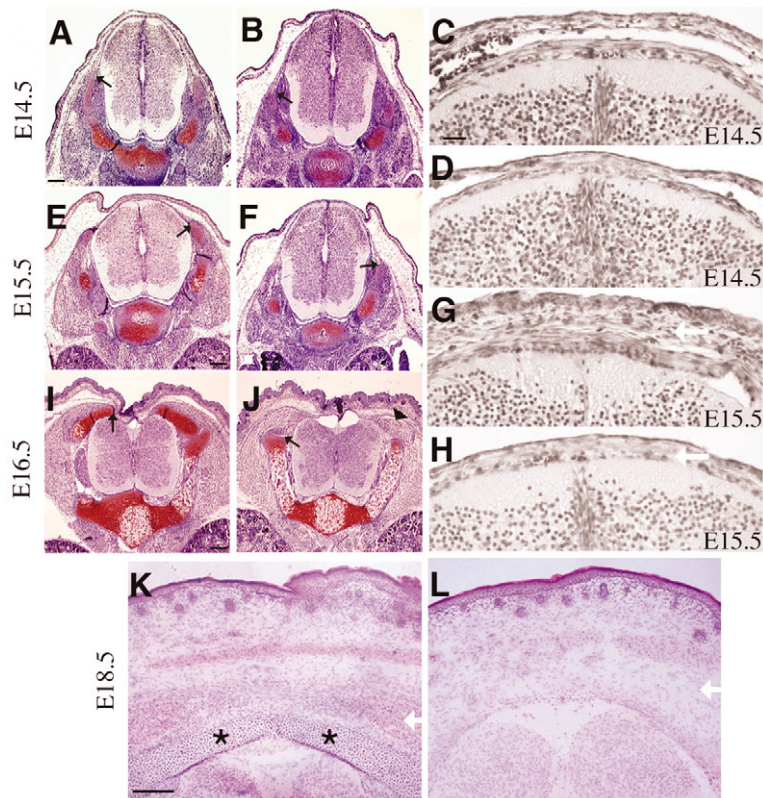


Fig. 3. Timing of vertebral arch defect.

(A,B,E,F,I,J) Safranin-O stained transverse sections through lumbar vertebrae at the indicated ages. (A,E,I) are control and (B,F,J) are $PDGFR\alpha^{PI3K/PI3K}$ embryos. Safranin-O sections are representative of the furthest progression of the vertebral arch in the lumbar region at each time-point. By E15.5 the development of the vertebral arch in the mutant lags behind that of the control littermate, and failure of the vertebral arch to fully form was observed at E16.5. Arrowhead indicates condensing cartilage that fails to stain with Safranin-O. Black arrows denote furthest progression of vertebral arch. (C,D,G,H) H&E (in black and white) of (C,G) control and (D,H) $PDGFR\alpha^{PI3K/PI3K}$ embryos at the indicated ages. (K,L) Hematoxylin and Eosin staining of E18.5 (K) control and (L) $PDGFR\alpha^{TKO}$. Asterisks indicate the lamina of the vertebral arch. White arrows indicate region of mesenchymal cells dorsal to the neural tube and their absence in the mutant embryos. Scale bars: 100 μm , except C,D,G,H (20 μm).

proliferation or increased apoptosis, we quantified the number of cells incorporating BrdU and the number staining with TUNEL labeling. We found that there was no significant difference in the number of TUNEL-positive cells or in the proliferation index of cells dorsal to the neural tube (see Table S1 in the supplementary material), suggesting that cell survival and proliferation in this region are normal in the $PDGFR\alpha^{PI3K/PI3K}$ embryos.

To determine which cell populations in these regions expressed the $PDGFR\alpha$, we examined embryos that expressed GFP from the $PDGFR\alpha$ locus ($PDGFR\alpha^{GFP}$) (Hamilton et al., 2003). Sections of E14.5 control embryos demonstrated the location of $PDGFR\alpha$ -expressing cells in the lumbar region. The most abundant receptor expression was in the mesenchyme surrounding developing vertebrae and in the dermis. Receptor expression was also present in the perichondrium, but only a low expression level was observed in the developing chondrocytes (Fig. 4A). A similar expression pattern has been reported previously for $PDGFR\alpha$ transcripts (Payne et al., 1997). At E16.5, $PDGFR\alpha$ (GFP⁺) cells had migrated to the dorsal neural tube. The outer cell population was probably dermis, whereas the cells adjacent to the neural tube were connective mesenchyme derived from somites (see Fig. S1D in the supplementary material).

These observations suggested that failure in migration of $PDGFR\alpha$ -expressing cells might lead to the observed reduction in cells in the dorsal region of the embryo. Comparison of sections through control $PDGFR\alpha^{GFP/+}$ embryos and $PDGFR\alpha$ mutant embryos further supported this possibility. In control embryos, the mesenchyme immediately adjacent to the forming arch had advanced to the extent of the tip of the vertebral arch. By contrast, in $PDGFR\alpha^{GFP/PI3K}$ embryos GFP⁺ cells were present lateral to the vertebral arch, but the majority of GFP⁺ cells had not advanced even half the distance of the vertebral arch (Fig. 4B). This effect was even more pronounced when we examined GFP⁺ cells in $PDGFR\alpha^{TKO/GFP}$ embryos (Fig. 4C). At E16.5, a stage when

vertebral arch development is halted in the mutant animals, we find a cluster of GFP⁺ cells dorsal to the developing vertebral arches in the lumbar region of $PDGFR\alpha^{TKO/GFP}$ embryos (see Fig. S1D,E in the supplementary material). These results suggest that $PDGFR\alpha$ signaling is likely to be required for migration of this somite cell population. Furthermore, because these cells are capable of secreting chondrogenic factors (as demonstrated in the micromass assay), failure of these cells to migrate could result in disrupted vertebral development.

To examine the ability of PDGF receptor stimulation to induce migration, we tested primary cells from embryos whose genotypes were either $PDGFR\alpha^{PI3K/GFP}$ or $PDGFR\alpha^{GFP/+}$ in a modified Boyden chamber assay. Cells isolated from wild-type embryos migrated towards PDGF ligands and serum. By contrast, mutant cells had a reduced ability to migrate towards PDGFAA and PDGFBB (Fig. 4D) but were still capable of migration induced by other stimuli. These results indicate that loss of PI3K signals downstream of the $PDGFR\alpha$ lead to a defect in cell motility.

Normal Ras/MAPK and FAK activation but aberrant Akt and S6K1 phosphorylation in $PDGFR\alpha^{PI3K/PI3K}$ cells

Generation of PtdIns(3,4,5) P_3 by $PDGFR\alpha$ -initiated PI3K activity leads to activation of multiple downstream pathways. We examined how efficiently these pathways were disrupted in $PDGFR\alpha^{PI3K/PI3K}$ cells by western blot. Both mutant and wild-type cells expressed similar levels of $PDGFR\alpha$ (Fig. 5A). However, we found that phosphorylation of Akt on S473 and p70S6 kinase (S6K1) on T389 was drastically reduced in the $PDGFR\alpha^{PI3K/PI3K}$ cells in response to PDGFAA stimulation (Fig. 5B). Loss of phosphorylation of both of these effectors confirmed that the PI3K pathway was inactive in PDGF stimulated $PDGFR\alpha^{PI3K/PI3K}$ cells. By contrast, MAPK phosphorylation of S6K1 (T421/S424) was similar in control and

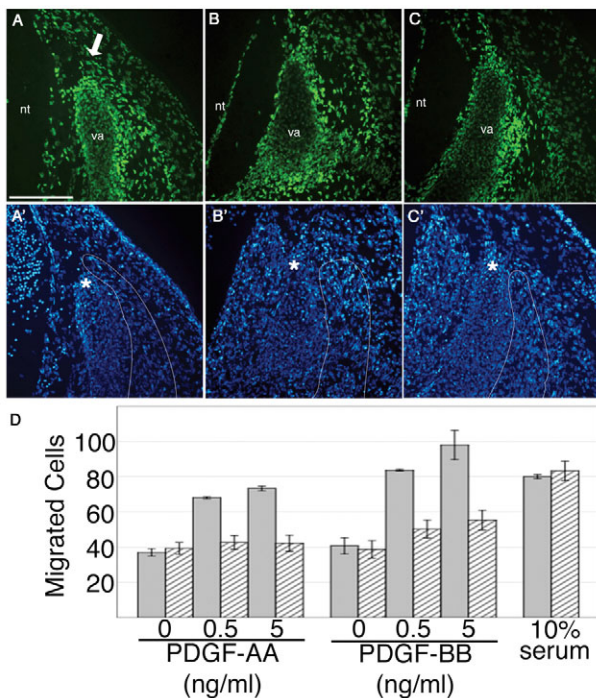


Fig. 4. Loss of PDGFR α -PI3kinase signaling results in defective migration. (A,A') PDGFR $\alpha^{GFP/+}$, (B,B') PDGFR $\alpha^{PI3K/GFP}$ and (C,C') PDGFR $\alpha^{TKO/GFP}$. PDGFR α -expressing cells (as detected by PDGFR α^{GFP}) are present in the perichondrium and in the mesenchyme surrounding the vertebral arch. (A,A') Heterozygote control section is at the level of the perichondrium and therefore displays more PDGFR α -positive chondrocytes. The PDGFR α -positive mesenchyme adjacent to the vertebral arch extends just beyond the tip of the arch in the heterozygote control, but has failed to advance in the (B,C) mutants. Sections are representative of furthest advancement of the vertebral arch and adjacent mesenchyme population in two embryos of each genotype. (A'-C') DAPI fluorescence of the sections in A-C. Adjacent mesenchyme is outlined. Asterisks denote the tip of the vertebral arch. Arrow indicates mesenchyme. va, vertebral arch; nt, neural tube. Scale bar: 100 μ m. (D) In vitro migration assay. Heterozygous cells (grey bars) migrated in a dose-dependent manner in response to PDGF-AA and PDGF-BB ligands, whereas mutant cells (striped bars) were unable to migrate in response to PDGF-AA and showed a decreased response to PDGF-BB. Both genotypes were able to migrate in response to 10% serum. This experiment is representative of three independent experiments.

PDGFR $\alpha^{PI3K/PI3K}$ cells in response to PDGFAA and 10% serum (data not shown). We then examined the activation status of other key motility pathways downstream of the PDGFR α . The Ras effectors ERK1/2 have been associated with establishment of pseudopodia and focal adhesions during chemotaxis (Brahmbhatt and Klemke, 2003; Fincham et al., 2000). Therefore, we determined whether ERK1/2 activation was disrupted in the PDGFR $\alpha^{PI3K/PI3K}$ cells. We found that phosphorylation of these proteins was comparable in mutant and control samples (Fig. 5A). These data are in agreement with previous analysis of MEF cells isolated from the PDGFR $\alpha^{PI3K/PI3K}$ embryos that suggest the PI3K mutation does not disrupt other signaling pathways downstream of the PDGFR α (Klinghoffer et al., 2002). Activation of focal adhesion kinase (FAK) in response to Src phosphorylation has also been demonstrated to be important for migration (reviewed by Hanks et al., 2003; Parsons, 2003). Similar to ERK1/2 activation, we observed no differences in

FAK phosphorylation between wild-type and PDGFR $\alpha^{PI3K/PI3K}$ cells (Fig. 5A). These data support the idea that disruption of pathways directly downstream of PI3K activity is the cause of the motility defect and that other downstream pathways, such as Src and MAPK, remain intact.

Recent data have suggested that rapamycin affects both mTOR complexes (mTORC1 and mTORC2) and that this effect varies depending on the cell type and the duration of rapamycin treatment (Sabatini, 2006; Sarbassov et al., 2006; Zeng et al., 2007). Because we observed a failure in S6K1 phosphorylation on the mTOR site in PDGFR $\alpha^{PI3K/PI3K}$ cells, we next examined the consequence of disrupting the mTOR pathway during PDGFR α -stimulated migration. To accomplish this we treated wild-type primary, somite-derived mesoderm cells with rapamycin and determined their ability to migrate towards PDGFAA. We observed a loss of motility in response to PDGFAA, bFGF and TGF β 1 stimulation (see Fig. S4A in the supplementary material). By contrast, rapamycin had no effect on the migration of these cells towards serum. Interestingly, migration was inhibited regardless of the treatment time, either 24 hours prior to migration or during the migration assay (see Fig. S4B in the supplementary material).

PI3K activity at the cell membrane leads to translocation and activation of a variety of proteins at the cell surface, including phosphoinositide-dependent kinase 1 (PDK1), Akt and mTOR. One of the downstream effectors of these signals is Rac1, which is important for cytoskeletal organization. We therefore tested the ability of PDGFR $\alpha^{PI3K/PI3K}$ cells to activate Rac1. We found that Rac1 activation occurred as early as 1 minute after stimulation and persisted up to 20 minutes in PDGFAA stimulated wild-type cells (Fig. 6A). By contrast, the amount of active Rac1 did not increase above background when we stimulated PDGFR $\alpha^{PI3K/PI3K}$ cells with PDGFAA (Fig. 6A), but we were able to recover activated Rac1 in response to stimulation by serum (Fig. 6A). We next investigated a downstream effector of activated Rac1: p21-activated kinase (Pak1). Pak proteins are serine/threonine kinase effectors of Rho family GTPases (Dharmawardhane et al., 1997; Sells et al., 1997). Upon Rac1 binding, Pak1 becomes autophosphorylated on S199 and T204. Using a phosphospecific antibody to these sites, we found that Pak1 autophosphorylation did not occur in PDGFR $\alpha^{PI3K/PI3K}$ cells stimulated with PDGFAA (Fig. 5C). Because Pak1 can also be directly phosphorylated on T423 by PDK1 downstream of PI3K activity, we investigated this event via western blot and observed a lack of phosphorylation on T423 in response to PDGF stimulation in PDGFR $\alpha^{PI3K/PI3K}$ cells (Fig. 5C). By contrast, when these same cells are stimulated with serum containing media, we observe phosphorylation of Pak1 at both the autophosphorylation site and the PDK1 site, demonstrating that other signaling pathways could still activate these pathways. These data demonstrate that PDGFR α association with PI3K leads to Pak1 phosphorylation, and suggest that failure of this activation may result in defective migration.

Finally, because we observed profound motility defects in the mutant cells, we examined the ability of the PDGFR α mutant cells to form focal contacts and reorganize actin in response to PDGFAA stimulation. Paxillin is a multidomain protein that localizes to focal contacts and is a docking site for many regulators of actin dynamics (Brown and Turner, 2004). Using cells derived from PDGFR α^{TKO} that were heterozygous for the PDGFR α^{GFP} allele, we found that control and mutant cells localized paxillin to the cellular periphery when stimulated with media containing 10% serum (Fig. 6B). In cells stimulated with PDGFAA, we found control cells possessed localized regions of intense paxillin staining, but mutant cells appeared similar to unstimulated cells. We next examined paxillin

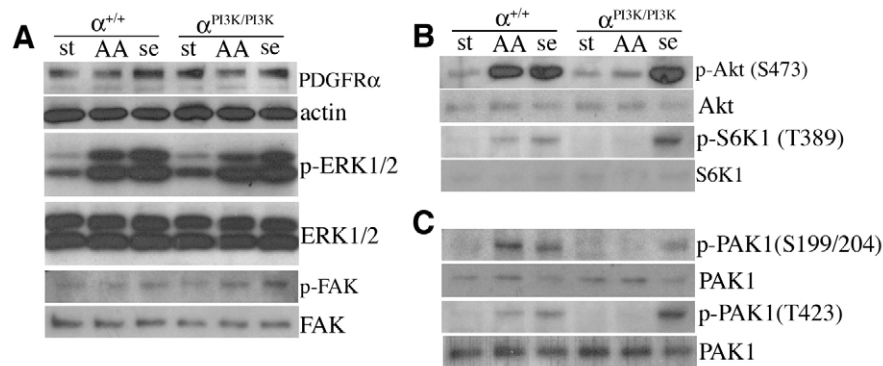


Fig. 5. Disruption of PI3K and PAK pathway activation in PDGFR $\alpha^{PI3K/PI3K}$ cells. (A-C) Western blot analysis of whole cell lysates from primary mesenchymal cells. (A) PDGFR α is expressed and ERK1/2 is phosphorylated in PDGFAA and serum-stimulated control and PDGFR $\alpha^{PI3K/PI3K}$ cells. (B) Akt and S6K1 are not phosphorylated in PDGFAA-stimulated PDGFR $\alpha^{PI3K/PI3K}$ cells. (C) PAK1 is not phosphorylated in PDGFAA stimulated PDGFR $\alpha^{PI3K/PI3K}$ cells. Loading controls (immediately beneath the sample) were either actin (for the PDGFR α) or stripped blots that were reblotted for the unphosphorylated form of the protein. Stimulation conditions: st, starved; AA, 10 ng/ml PDGF-AA; se, 10% serum. Blots are representative of three independent experiments.

localization in PDGFR $\alpha^{PI3K/PI3K}$ cells. Analogous to what we observed in the PDGFR α -deficient cells, paxillin localized to cellular protrusions in control cells upon PDGF stimulation, but

failed to localize in the PDGFR $\alpha^{PI3K/PI3K}$ cells (Fig. 6B). Taken together, these data indicate the PDGFR α -mediated migration and cytoskeletal rearrangement is dependent on PI3K signal transduction.

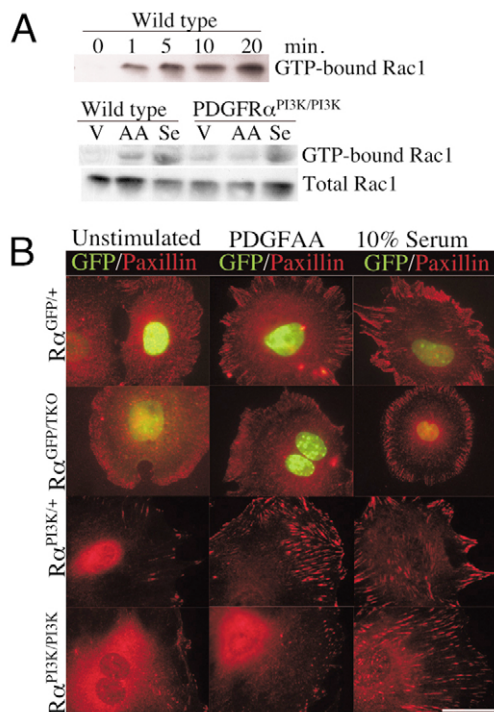


Fig. 6. Effects of PDGFR $\alpha^{PI3K/PI3K}$ mutation on Rac1 activation and paxillin localization. (A) Western blot for GTP-bound Rac1 and total Rac1 in control and PDGFR $\alpha^{PI3K/PI3K}$ cells. Upper blot demonstrates the time course of Rac1 activation in control cells. Lower blot illustrates the failure of PDGFR $\alpha^{PI3K/PI3K}$ cells to stimulate Rac1 activation when stimulated with PDGFAA. Cells were stimulated for 10 minutes. Results are representative of three independent experiments. (B) Immunocytochemistry for paxillin localization in control and PDGFR α mutant cells. Genotypes are indicated on the left. Cells were plated for two hours, starved for 24 hours, and then stimulated for 30 minutes with the indicated stimulation. Images are representative of five independent experiments and represent the majority of cells within the culture for each stimulation. Scale bar: 50 μ m.

DISCUSSION

Failure of neural tube closure is commonly believed to be the major cause of spina bifida. In this report, we demonstrated that a likely cause of spina bifida in PDGFR α mutant mice was a defect in a non-sclerotome somite cell population and that neural tube defects did not precede the spina bifida. The failure of this cell population to migrate towards the dorsal neural tube halted further development of vertebral elements in the lumbar region. We also showed that migration of these cells was directed by PI3K signaling downstream of the PDGFR α . Similar to a requirement for PI3K signaling downstream of the PDGFR β (Aoki et al., 2001; Franke et al., 1995), stimulation of PDGFR $\alpha^{PI3K/PI3K}$ cells resulted in failure in activation of Akt (this report) (Klinghoffer et al., 2002) and S6K1. These signaling disruptions resulted in loss of Rac1 and Pak1 activation, and a failure in generation of focal contacts. Thus, PI3K is an essential signaling pathway for cytoskeletal reorganization downstream of the PDGFR α , and signaling through Src and MAPK pathways does not compensate for loss of PI3K.

Until now, the cause of spina bifida in PDGFR α mutant mice has been unclear. The difficulty in identifying the etiology of this defect previously has been the requirement of PDGFR α in many tissues in the developing embryo. Using chick embryos, as well as conditional and hypomorphic PDGFR α mouse lines, we have been able to define the cell population responsible for spina bifida, as well as identifying a potential cellular function mediated by the receptor. The imatinib-treated chick embryos most closely resembled spina bifida occulta, whereas defects in the mutant mouse embryos were similar to spina bifida aperta. We attribute this difference to the fact that, in the mouse, a genetic lesion exists, whereas, in the chick, a single treatment of the PDGF receptor inhibitor was the inducing factor. One surprising outcome of our data is the limited region of vertebrae affected by disruption of PDGFR α signaling. In both the imatinib-treated embryos and the genetic disruption of the receptor, defects were limited to the lumbar region and the immediately adjacent thoracic and sacral vertebrae. This is in striking contrast to the vertebral defects reported for PDGFR α -null alleles (Morrison-

Graham et al., 1992; Payne et al., 1997; Soriano, 1997). In these mouse lines, defects occur along the entire length of the vertebral column. One explanation for the disparity in the phenotypes is that the *PDGFR α* -deficient mutants may have mesodermal hypoplasia and exhibit surface ectoderm detachment (Morrison-Graham et al., 1992; Orr-Urtreger et al., 1992; Soriano, 1997). These disruptions may cause a more global defect in cell organization, survival and growth factor production. Therefore, complete loss of *PDGFR α* signaling during embryogenesis may affect many cell populations leading to more severe disruption of somite morphogenesis, vasculature or even neural tube closure.

Our observations are in agreement with studies in the avian system that investigated the formation of the dorsal vertebrae. These experiments suggested that signaling of sonic hedgehog and BMP4 from the dorsal ectoderm and roof plate of the neural tube directed a superficial somite cell population to form the dorsal vertebrae (Monsoro-Burq et al., 1994; Monsoro-Burq et al., 1996; Monsoro-Burq and Le Douarin, 2000; Watanabe et al., 1998). Although our data support the idea that a somite-derived dorsal mesenchyme is required for chondrocyte development, the lack of phenotype in the *PDGFR α ^{CKO}* suggests an indirect role for *PDGFR α* signaling in vertebrae development. Chick/quail chimeras have indicated that both the dorsal chondrocyte and mesenchymal cell population arise from the ventral half of the somite (Christ et al., 2004). Therefore, the dorsal mesenchyme population is derived from the same area of the somite as the vertebral derivatives, and is also required in this signaling paradigm. The ability of soluble factors from non-chondrocyte dorsal mesenchyme to stimulate chondrogenesis supports the possibility that coordinated migration of mesenchymal cells alongside the developing vertebral arches plays a role in the development of the vertebral arch. There are multiple growth factors that could be involved in regulating the chondrocyte growth and differentiation. It is clear from a number of studies that normal bone development often requires a balance of growth factor signals (Naski and Ornitz, 1998). In many circumstances, either an excess or a deficiency in growth factor signaling can lead to impaired development. The factor(s) secreted by the mesenchymal cell population could be either a growth factor or a growth factor inhibitor (Day et al., 2005; Hung et al., 2007). Although it is possible that the secreted molecule is a growth factor, we cannot rule out the possibility that these cells may also be depositing extracellular matrix components such as collagens, matrix proteoglycans and matrix metalloproteases that also have a demonstrated role in bone growth and morphogenesis (Blair et al., 2002; Lee, 2006; Malesud, 2006).

In vitro, PDGF receptors direct multiple cellular activities including proliferation, survival, actin reorganization and motility (reviewed by Heldin and Westermark, 1999), and in *Xenopus* and *Drosophila*, PDGF receptor homologs are required for migration of several cell populations (Cho et al., 2002; Duchek et al., 2001; McDonald et al., 2003; Nagel et al., 2004). Although migration signals downstream of PDGF receptors have been implicated in the mouse (Abramsson et al., 2007; Klinghoffer et al., 2002; Richarte et al., 2007), it is often difficult to separate proliferation from migration defects (reviewed by Hoch and Soriano, 2003). Although the *PDGFR β* seems to consistently promote cell motility in multiple cell types, the ability of *PDGFR α* to regulate chemotaxis has proven to be a cell type-dependent phenomenon (reviewed by Ronnstrand and Heldin, 2001). In some cells, such as NIH and Swiss 3T3 cells, *PDGFR α* stimulation induces cell motility (Hosang et al., 1989; Rosenkranz et al., 1999), whereas in endothelial and vascular smooth muscle cells, *PDGFR α* activation represses migratory

signals from other receptors (Koyama et al., 1992; Yokote et al., 1996). Our results suggest a primary role for the *PDGFR α* in promoting migration of this somite-derived population. By contrast, cells that are destined to become dermis that also express *PDGFR α* migrated appropriately in the *PDGFR α* mutants investigated. Therefore, during development PDGF receptors are likely to play distinct roles in promoting cellular functions, and each cell type must be examined individually to define the role of the receptor.

In mammals, PI3K signaling has been linked to many cellular outcomes, including growth, metabolism, survival and migration (Fruman et al., 1998). Loss of all PI3K p85 regulatory subunits results in embryonic lethality and a phenotype strikingly similar to complete loss of *PDGFR α* , and cells from these p85 mutant embryos failed to form membrane ruffles in response to PDGF (Brachmann et al., 2005). Now, we have shown that the loss of this signaling pathway renders a specific population of cells incapable of migration during embryogenesis, even though *PDGFR α ^{PI3K/PI3K}* mutant cells retained the ability to signal through Src and MAPK. The cellular outcome of PI3K signaling not only depends on the receptor promoting it, but also depends on the cell type responding to it. For example, disruption of PI3K activation downstream of the Kit receptor resulted in a block in differentiation of gametes, and mast cell and melanocyte migration were unperturbed (Blume-Jensen et al., 2000; Kissel et al., 2000). In *Drosophila* the PDGF-related receptor PVR guides border cell migration in a PI3K-independent manner, suggesting that PDGF receptors may use different signals depending on the developmental context (Bianco et al., 2007). Mice deficient in PI3K signaling downstream of the *PDGFR β* displayed reduced interstitial fluid homeostasis but no apparent defect in pericyte migration (Heuchel et al., 1999; Tallquist et al., 2003). Our data clearly demonstrate a requirement for PI3K signaling downstream of the *PDGFR α* to initiate migration, whereas cell survival and proliferation are not dependent on this signaling.

The promotion of cell motility requires the coordination of many intracellular signaling molecules. Although PI3K activation is important for both Akt and Rac1 activation (Nobes and Hall, 1995; Wennstrom et al., 1994a), it is not the only pathway that has been implicated in cytoskeletal reorganization driven by PDGF stimulation. For example, PLC γ and Src signaling have been implicated in actin ruffle formation and cell motility driven by *PDGFR β* stimulation in vitro (Boyle et al., 2007; Kundra et al., 1994; Wennstrom et al., 1994b). Nonetheless, in our mesenchymal cells, these pathways were not sufficient to stimulate motility in vivo or in vitro.

The hierarchy of signaling is yet to be established for many of these interactions. It has been shown that paxillin phosphorylation by PAK1 leads to increased cell motility (Nayal et al., 2006), and paxillin can also modulate Rac1 activity (Chen et al., 2005). Finally, it has also been suggested that S6K1 could be involved in cell motility via interactions with Rac1 (Berven et al., 2004; Liu et al., 2006). Thus, loss of *PDGFR α* -PI3K activation results in a failure in multiple components associated with focal adhesions and cell motility. The complex interactions of these proteins suggest multiple levels of regulation, and it will be interesting to use these cells to further determine the essential actions of each of these components.

Although Rac1 activation is an important component of cell motility, and this pathway is clearly disrupted in our cells, it is unlikely that loss of Rac1 is the only disruption leading to a failure in migration. Recent evidence in *Rac1*-null mouse embryonic fibroblasts has shown that these cells are capable of migrating towards PDGF (Vidali et al., 2006). This suggests that other signaling pathways downstream of PDGF receptors can also induce

migration. Our data suggest that the mTORC1 or potentially mTORC2 complexes are also important in PDGF stimulated chemotaxis. We not only see a loss of phosphorylation of Akt on the mTORC2 site (S473), but rapamycin also inhibits migration of cells towards PDGF. This result is in contrast to the lack of inhibition of migration to serum, suggesting that the rapamycin does not render cells incapable of migration because of a generalized mechanism. Studies of neuronal migration in response to HGF stimulation have demonstrated that rapamycin does not affect migration (Segarra et al., 2006). These results emphasize that different cell types rely on distinct signaling pathways for induction of cell motility.

The role of cell polarity and cytoskeletal reorganization has been appreciated in the morphogenesis of the neural tube. Multiple lines of evidence indicate that defects in these processes can result in neural tube defects (Harris and Juriloff, 2007; Wallingford, 2006). Now, we have shown that spina bifida can be caused by defects unrelated to neural tube closure. In addition, disrupted cytoskeletal rearrangement and cell movement are involved in this second etiology. In these studies, the importance of PDGFR α signaling, especially through PI3K, in somite-derived tissues suggests that cases of human spina bifida resistant to folate supplementation may not be directly related to failure in neural tube closure. Second, because disruption of PDGF receptor signaling has been proposed for treatment of several human pathologies, concern should be placed on effects that PDGF receptor inhibition could have on fetuses.

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Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/135/3/589/DC1>

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