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PDGFRαα Signaling Is Regulated through the Primary Cilium in Fibroblasts

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Summary

Recent findings show that cilia are sensory organelles that display specific receptors and ion channels, which transmit signals from the extracellular environment via the cilium to the cell to control tissue homeostasis and function [1–6]. Agenesis of primary cilia or mislocation of ciliary signal components affects human pathologies, such as polycystic kidney disease [7] and disorders associated with Bardet-Biedl syndrome [8]. Primary cilia are essential for hedgehog ligand-induced signaling cascade regulating growth and patterning [9,10]. Here, we show that the primary cilium in fibroblasts [11] plays a critical role in growth control via platelet-derived growth factor receptor α (PDGFRα), which localizes to the primary cilium during growth arrest in NIH3T3 cells and primary cultures of mouse embryonic fibroblasts. Ligand-dependent activation of PDGFRαα is followed by activation of Akt and the Mek1/2-Erk1/2 pathways, with Mek1/2 being phosphorylated within the cilium and at the basal body. Fibroblasts derived from *Tg737*^{orpk} mutants fail to form normal cilia and to upregulate the level of PDGFRα; PDGF-AA fails to activate PDGFRαα and the Mek1/2-Erk1/2 pathway. Signaling through PDGFRβ, which localizes to the plasma membrane, is maintained at comparable levels in wild-type and mutant cells. We propose that ciliary PDGFRαα signaling is linked to tissue homeostasis and to mitogenic signaling pathways.

Results and Discussion

Formation of Primary Cilia in NIH3T3 Fibroblasts

Primary cilia are formed in nearly all growth-arrested and differentiated cell types in vertebrates [12,13]. Typically, a single cilium projects from each cell, emerging from the mother centriole that has become a basal body. To study ciliary function, NIH3T3 fibroblasts were grown in DMEM containing 10% fetal calf serum for 2 days to ~80% confluency, followed by serum starvation for 24 hr, when the cells enter G_0 [14]. Prior to starvation, cells show a prominent cytoskeleton of acetylated microtubules but no primary cilia (Figures 1A and 1E). After 6 hr serum starvation, <5% of the cells had primary cilia, the majority of which had lengths of 1–2 μm (Figure 1B). By 12 hr about 40% had cilia, with lengths up to 6 μm (Figure 1C). By 24 hr >90% of the cells possessed cilia, all approximately 6 μm length; each axoneme grew above a ciliary basal body (Figures 1D and 1F). These cells now lacked the arrayed cytoskeleton containing acetylated microtubules (Figure 1D).

PDGFRα Is Upregulated and Localized to Primary Cilia during Growth Arrest

Using a gene trap technique to identify genes that are transcriptionally active during growth arrest of NIH3T3 fibroblasts, Lih et al. [14] identified platelet-derived growth factor receptor α (PDGFRα) as a growth arrest-specific (GAS) gene whose mRNA was preferentially expressed in serum-deprived cells. The intimate connection between cilia formation and growth arrest led us to ask if PDGFRα localized to cilia. Using rabbit polyclonal antibodies, we found that PDGFRα localized along the primary cilia of NIH3T3 cells (Figures 2A–2C, upper frames). In contrast, PDGFRβ was primarily distributed as clusters along the cell surface (Figures 2A–2C, lower frames). PDGFRβ localization is a control for how receptor localization appears when distributed in the plasma membrane; therefore, PDGFRα seems to localize predominantly in the cilium and not the plasma membrane. Preincubation of the PDGFRα antibody with an antibody-specific blocking peptide (P) reduced the ciliary PDGFRα immunofluorescence. Substitution of the primary antibody with preimmune rabbit serum gave no ciliary signal (see Figure S1 in the Supplemental Data online with this article at http://www.currentbiology.com/cgi/content/full/15/20/1861/DC1/). In Western blot analysis, anti-PDGFRα reacted uniquely with the receptor (Figure 2D). Two protein bands at ~170 kDa are seen in the immunoblot, the upper band being the mature and fully glycosylated form and the lower band the immature and only partly glycosylated form of the receptor [15]. Goat polyclonal antibody to PDGFRα also localized to the primary cilium, whereas neither anti-EGFR nor anti-IRα became associated with the cilium (Figure 2E).

Next we studied the time course for appearance of PDGFRα along the elongating cilium. After 12 hr serum starvation, PDGFRα was localized around the base and the lower part of the cilium in about 70% of the cilia (Figure 2F). By 24 hr serum starvation, the receptor was distributed along the entire length of the cilium in about 80% of the cilia (Figure 2G). Immunoblots with anti-PDGFRα showed that the receptor was present in growing interphase cells (0 hr serum starvation), but

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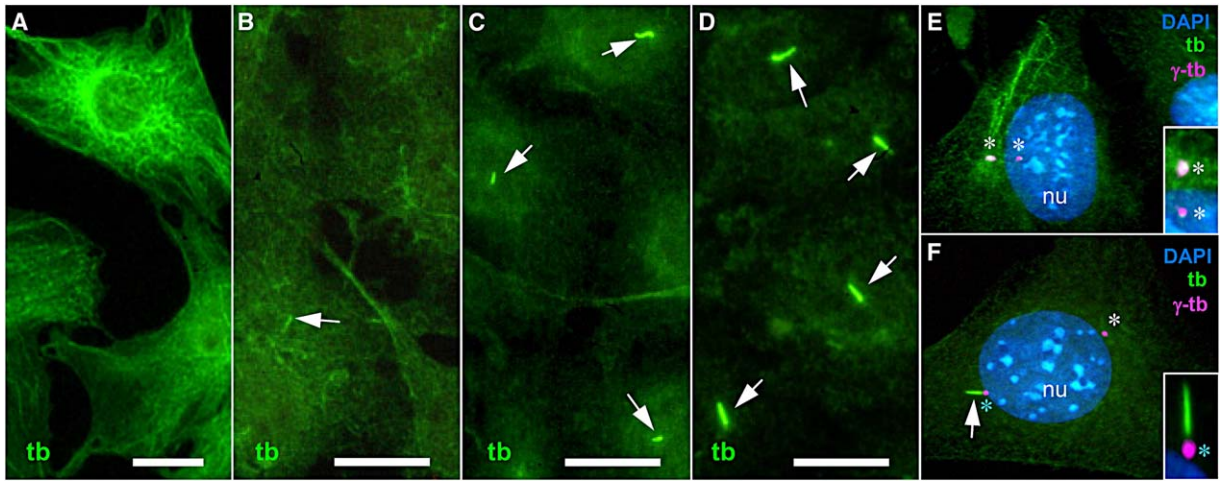

Figure 1. Time Course for Growth of the Primary Cilium in NIH3T3 Fibroblasts

Cilia and microtubules are localized with anti-acetylated α-tubulin (tb, green; arrows). Immunofluorescence microscopy analysis of nonarrested, interphase cells (A and E) and after serum starvation for 6 (B), 12 (C), and 24 hr (D and F). Centrioles are localized with anti-γ-tubulin (γ-Tb, magenta, asterisks). In (F), the mother centriole is marked with a blue asterisk and the daughter centriole with a white asterisk. Nuclei (nu) are stained with DAPI (blue). Scale bars: 10 μm.

that the level of receptor increased after serum starvation (Figure 2H). The mature receptor form was upregulated ~7-fold from 0 to 24 hr serum starvation (Figure 2I), consistent with specific placement of much of the upregulated fully glycosylated, mature PDGFRα in the ciliary membrane. In contrast, the protein level of PDGFRβ was high in interphase cells and weakly increased upon serum starvation (Figures 2H and 2I).

Activation of PDGFRα in the Primary Cilium of NIH3T3 Cells

The ligand PDGF-AA binds and activates the homodimer of PDGFRα (PDGFRαα) but not homodimers of PDGFRβ (PDGFRββ) or heterodimers of PDGFRαβ, whereras PDGF-BB binds and activates all three receptor dimers [15]. As a control for ligand specificity, activation of PDGFRα and PDGFRβ was analyzed by immunoprecipitation of the receptors in cells stimulated with either PDGF-AA or PDGF-BB for 3 min in nonarrested interphase cells (growth) and in 24 hr serum-starved, growth-arrested cells (arrest) followed by Western blotting with anti-phosphotyrosine (Figures 3A and 3B). PDGFRβ was not activated by phosphorylation upon stimulation with PDGF-AA, whereas both receptor forms were phosphorylated by PDGF-BB. Further, PDGFRβ was activated in both growing and growth-arrested cells, whereas PDGFRα was heavily phosphorylated only during growth arrest because the level of immunoprecipitated PDGFRα was high only during growth arrest (Figure 2H). We examined tyrosine phosphorylation of the immunoprecipitated receptor after 0, 12, and 24 hr of serum starvation and subsequent PDGF-AA stimulation. Immunoprecipitated PDGFRα was loaded on SDS-PAGE at equal concentrations and corresponding Western blots monitored. After 3 min of PDGF-AA stimulation, tyrosine phosphorylation of PDGFRα increased about 17-fold after 24 hr serum starvation compared to cells in interphase growth (Figure 3C). These results support the conclusion that the activation response to PDGF-AA increased in correspondence with development of the primary cilium.

Prior to ligand addition, there was little phosphotyrosine labeling along the cilium. When PDGF-AA was added to the cells after 24 hr serum starvation, a marked increase in tyrosine phosphorylation within cilia was detected about 3 min after ligand addition, whereas little new immunofluorescence was detected in the cytoplasm (Figure 3D). Among the proteins that became tyrosine phosphorylated after PDGF-AA addition was the glycosylated PDGFRα itself (Figure 3E), which we surmise to be primarily PDGFRαα homodimer activation in the ciliary membrane. Substantial PDGFRα phosphorylation seen by 3 min, analyzed by immunoprecipitation of receptor followed by Western blotting with anti-phosphotyrosine, persisted for at least 10 min; the immature receptor was unaffected. Addition of PDGF-BB led to increased tyrosine phosphorylation of proteins both in the cilia and around the plasma membrane (Figure 3D), suggesting that PDGF-BB may activate PDGFRββ, residual PDGFRαα homodimers, and PDGFRαβ heterodimers in the plasma membrane as well as PDGFRαα homodimers in the ciliary membrane.

Two sites of tyrosine phosphorylation of PDGFRα (Y^{720} and Y^{742}) can be monitored by phosphospecific antibodies. Y^{720} acts as a docking site for adaptor proteins with SH2 domains, including Shp [16]. In response to PDGF in 3T3 fibroblasts, Shp becomes tyrosine phosphorylated and contributes to Ras and normal Erk activation [17]. Y^{742} is a docking site for p85, which is required for the phosphoinositide 3-kinase (PI3K)/Akt signaling axis [15]. After PDGFRαα activation, both Mek1/2-Erk1/2 and Akt pathways were activated by phosphorylation beginning at 3 min of PDGF-AA stimulation (Figure 3F). The increased level of Mek1/2 (by

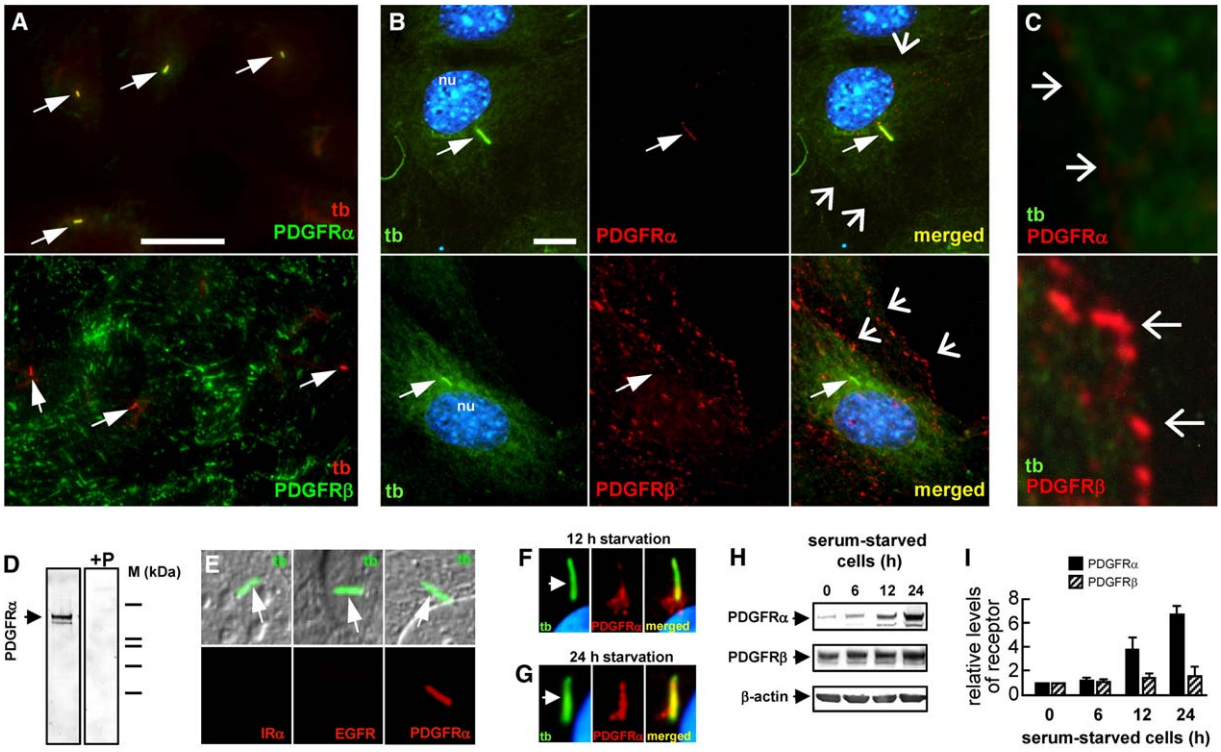

Figure 2. PDGFRα Is Upregulated and Localized to Primary Cilia in Growth-Arrested NIH3T3 Fibroblasts

(A) Localization of rabbit anti-PDGFRα (green) and rabbit anti-PDGFRβ (green) in 24 hr serum-starved cells. Arrows indicate the primary cilia detected by anti-acetylated α-tubulin (tb, red). Scale bar: 50 μm.

(B and C) High-magnification images of rabbit anti-PDGFRα (red, top) and rabbit anti-PDGFRβ (red, bottom) localization to 24 hr serum-starved cells. Bold arrows indicate the cilium (tb, green) and open arrows the cell surface. Scale bar: 10 μm.

(D) Rabbit anti-PDGFRα crossreactivity to the receptor in whole-cell lysate of 24 hr serum-starved cells in the absence and in the presence of antibody blocking peptide, P. Molecular mass marker (kDa) from top to bottom: 200, 116.3, 97.4, 66.3, 55.4, 36.5, and 31.

(E) Ciliary localization (Tb, green, arrows) of rabbit anti-IRα, rabbit anti-EGFR and goat anti-PDGFRα in 24 hr serum-starved cells (red). PDGFRα targeting (rabbit anti-PDGFRα, red) to the elongating cilium (Tb, green, arrows) in cells serum starved for 12 (F) and 24 hr (G). Nuclei (nu) are stained with DAPI (blue) or visualized with DIC images.

(H) Level of PDGFRα, PDGFRβ and β-actin (control) upon serum starvation for 0, 6, 12, and 24 hr.

(I) Quantification of PDGFRα and PDGFRβ protein levels upon serum starvation for 0, 6, 12, and 24 hr relative to β-actin and receptor levels at 0 hr of serum starvation.

Error bars indicate standard errors from three separate experiments.

c-Raf-dependent Mek1/2 phosphorylation on serines 217 and 221 in the activation loop to activate Erk1/2) preferentially localized along and at the base of the cilium; i.e., the mother centriole (Figures 3G and 3I). No phospho-Mek1/2 was detected at the cell surface (Figure 3H and Figure S2). In contrast, PDGF-BB increased the level of phospho-Mek1/2 in cilia, in the basal body and partly in the cell cytosol (Figure 3G), supporting the conclusion that although PDGF-BB acts on receptors at the cell surface outside the cilium, receptors for PDGF-AA respond predominently in the cilium. We also observed ciliary localization of Mek1 phosphorylated on serine 298 (Figure 3J), produced by p21-activated protein kinase, PAK, which is a convergence point for integrating growth factor signaling via the MAPK pathway [18]. These results support the conclusion that Raf-dependent activation of Mek1/2 is assisted by PAK in the cilium and that the primary cilium contains the signaling machinery from PDGFRααα activation to Mek1/2 through the Ras-Raf pathway, controlling cell growth and proliferation.

Ciliary Assembly and Upregulation of PDGFRα Expression Is Blocked in *Tg737*^{orpk} Mutant Cells

Tg737 encodes the IFT particle protein IFT88/polaris required for ciliary assembly [7]. No other function of *Tg737* is known, and IFT protein-encoding genes are found only in organisms that possess cilia. Homozygous Tg737 mutant mice normally die within 2 weeks of birth with multiple tissue pathologies including cystic lesions in the pancreas and kidney [19].

To test the hypothesis that the ciliary-localized PDGFRα receptor is responsible for the PDGF-AA responses we observed in NIH3T3 cells more directly, we compared primary cultures of wt mouse embryonic fibroblasts (MEFs) and MEFs derived from *Tg737*^{orpk} mutant mice. Wild-type MEFs serum starved for 48 hr developed primary cilia with lengths of 5–10 μm; PDGFRα localized along these cilia (Figure 4A). Mutant MEFs grown similarly presented no or very short, <1 μm long, cilia. PDGFRα localized at the base of these short stubs (Figure 4B). Importantly, PDGFRα was present at a low level at the cell surface as in wt cells (Figures 4A and

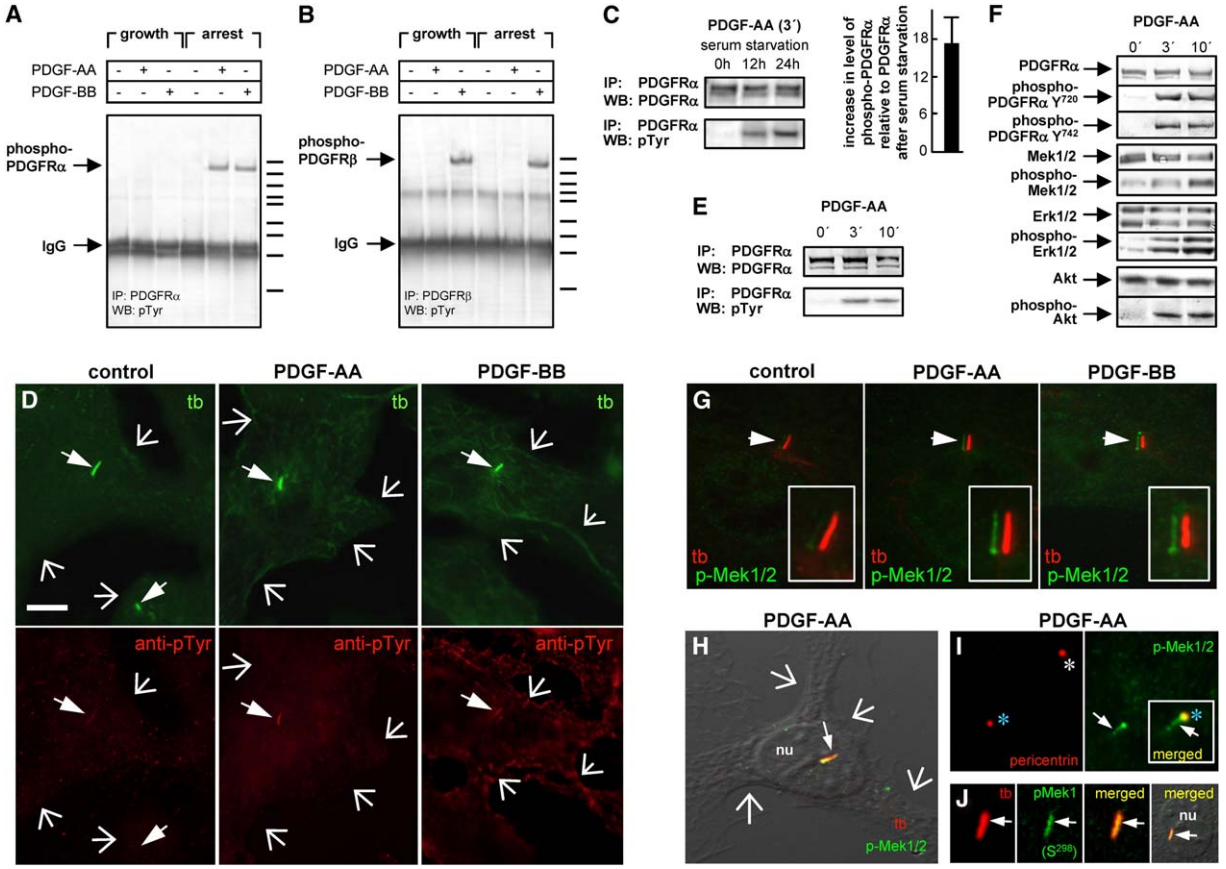

Figure 3. PDGF-AA Activates PDGFRα in the Primary Cilium of NIH 3T3 Fibroblasts Followed by Mitogenic Signaling through Akt and the Mek1/2-Erk1/2 Pathway

(A and B) Western blot with anti-phosphotyrosine on immunoprecipitated PDGFRα and PDGFRβ in nonarrested, interphase cells (growth) versus 24 hr serum-starved, growth-arrested cells (arrest) after stimulation with either PDGF-AA or PDGF-BB for 3 min. Molecular mass markers (kDa) from top to bottom: 200, 150, 120, 100, 85, 70, 60, 50, and 40.

(C) Time course of tyrosine phosphorylation of immunoprecipitated PDGFRα in cells serum starved for 0, 12, and 24 hr and stimulated with PDGF-AA for 3 min (left) and quantification of the level of tyrosine phosphorylated PDGFRα in cells starved for 24 hr relative to immunoprecipitated PDGFRα and cells starved 0 hr (right). Immunoprecipitated PDGFRα in cells starved for 0 and 12 hr was increased to match the amount of receptor in cells starved for 24 hr.

(D) Localization of tyrosine phosphorylation (anti-pTyr, red) in the primary cilium (bold arrow; anti-acetylated α-tubulin, tb, green) and at the plasma membrane (open arrows) before (control) or upon stimulation with PDGF-AA or PDGF-BB for 3 min. Scale bar: 10 μm.

(E) Time course for tyrosine phosphorylation of immunoprecipitated PDGFRα in cells serum starved for 24 hr and stimulated with PDGF-AA for 0, 3, and 10 min.

(F) Level of phosphorylation of PDGFRα, Mek1/2, Erk1/2 and Akt upon stimulation with PDGF-AA for 3 and 10 min in cells serum starved for 24 hr.

(G) Shifted overlays of ciliary localization of anti-phospho-Mek1/2 (green) and anti-tb (red, arrows) in cells stimulated with either PDGF-AA or PDGF-BB for 10 min.

(H) Merged DIC image of a single cell with anti-phospho-Mek1/2 (green) and anti-tb (red, bold arrow) stimulated with PDGF-AA for 10 min. Open arrows indicate the edge of the cell.

(I) Localization of anti-phospho-Mek1/2 (green) and anti-pericentrin (red) that marks the mother and daughter centrioles (blue and white asterisks, respecetively).

(J) Colocalization of anti-phospho-Mek1 S^{298} (green) and the primary cilium (red, tb, arrows). Abbreviation: nu, nucleus detected by DIC.

Error bars indicate standard errors from three separate experiments.

4B), supporting the conclusion that the majority of PDGFRα in quiescent wt MEFs is ciliary. In wt MEFs, PDGFRα was upregulated essentially as in NIH3T3 cells, whereas in serum-starved mutant cells PDGFRα expression remained similar to that of nonarrested, interphase cells (Figures 4C and 4D). In order to validate that mutant cells had entered growth arrest, we analyzed the level of cdk4-mediated phosphorylation of tumor suppressor protein retinoblastoma (Rb) at serines 807 and 811, which controls progression through the late G_1 restriction point, and is a major regulator of the G1/S transition that marks cycling cells [20]. Western blot analysis confirmed that the phospho-Rb in mutant cells decreased during serum starvation and was at a low level similar to wt cells after 48 hr starvation. Further, mutant cells had neither mitotic spindles nor the

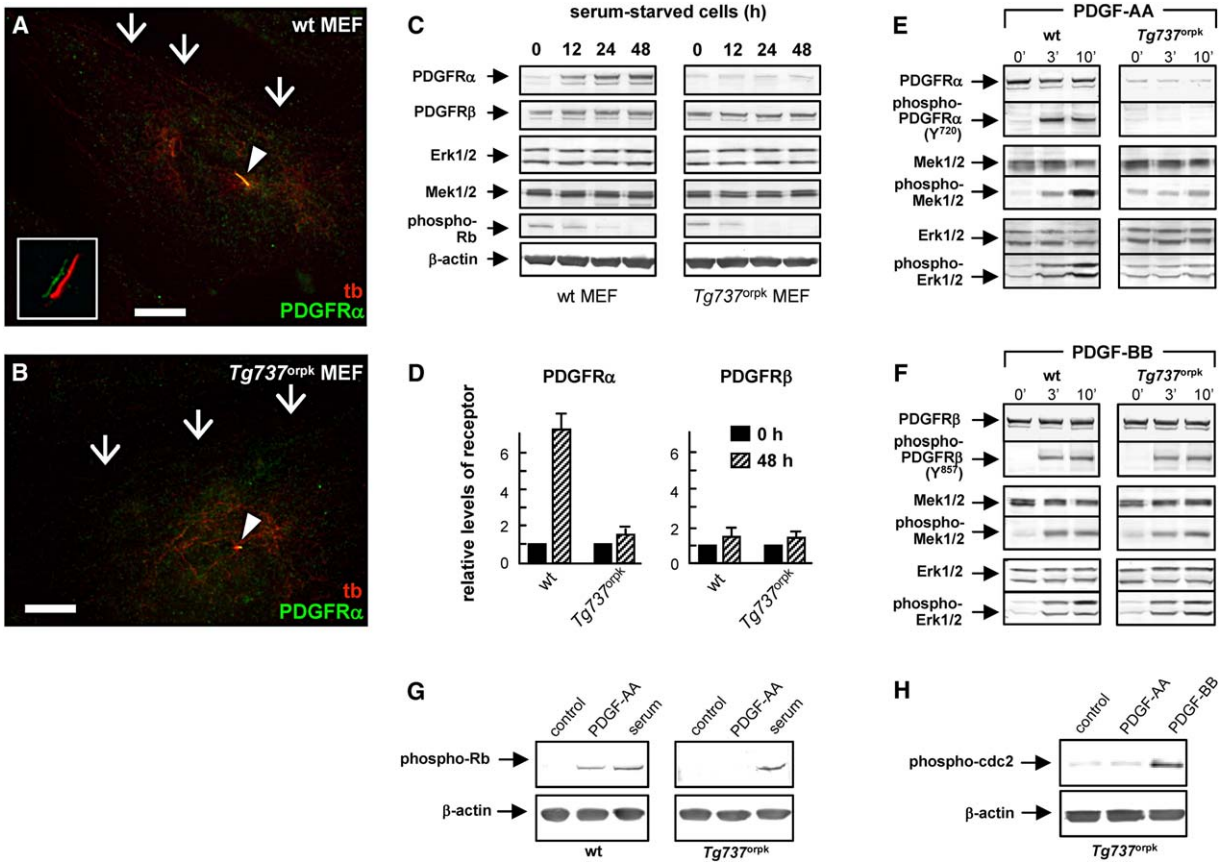

Figure 4. Ciliary Formation, Upregulation of PDGFRα- and PDGFRα-Mediated Signal Transduction, and Cell Cycle Entrance Is Impaired in *Tg737* Mutant Mouse Embryonic Fibroblasts

Ciliary formation (tb, red, bold arrows) and localization of rabbit anti-PDGFRα (green) in wild-type (A) and *Tg737*^{orpk} (B) mutant embryonic mouse fibroblasts, serum starved for 48 hr. Open arrows indicate the edge of cells. Scale bars: 10 μm.

(C) Level of PDGFRα, PDGFRβ, Erk1/2, Mek1/2, phospho-Rb and β-actin in wild-type and mutant cells after serum starvation for 0, 12, 24, and 48 hr.

(D) Quantification of PDGFRα and PDGFRβ upon serum starvation for 48 hr relative to β-actin and receptor at 0 hr of serum starvation.

(E) Time course for phosphorylation of PDGFRα, Mek1/2 and Erk1/2 upon stimulation with PDGF-AA for 3 and 10 min in wild-type and mutant cells serum starved for 48 hr.

(F) Time course for phosphorylation of PDGFRβ, Mek1/2 and Erk1/2 upon stimulation with PDGF-BB for 3 and 10 min in wild-type and mutant cells serum starved for 48 hr.

(G) Phospho-Rb and β-actin (control) in wild-type and mutant cells after serum starvation for 48 hr unstimulated or stimulated with PDGF-AA or serum for 26 hr.

(H) Phospho-cdc2 and β-actin (control) in mutant cells after serum starvation for 48 hr unstimulated or stimulated with PDGF-AA or PDGF-BB. Error bars indicate standard errors from three separate experiments.

characteristic interphase cell cytoskeleton (Figure 4B). No major differences were observed between wt and mutant in the levels of PDGFRβ, Mek1/2 and Erk1/2 in growth-arrested versus nonarrested, interphase cells (Figure 4C).

PDGFRαα-Mediated Signaling and Cell Cycle Entrance in Wt and *Tg737*^{orpk} Mutant Cells

To investigate the role of the primary cilium in mitogenic signaling through PDGFRαα in quiescent wt and mutant MEFs, cells were stimulated with PDGF-AA and subjected to Western blot analysis. Receptor and Mek1/2-Erk1/2 activations in wt MEFs occurred as in NIH3T3 fibroblasts, whereas in mutant cells PDGF-AA failed to activate PDGFRα and the Mek1/2-Erk1/2 pathway (Figure 4E). Importantly, receptor activation by tyrosine phosphorylation in wt MEFs increased about 8-fold compared to mutant cells, in which the relative level of receptor activation was nearly unchanged during the first 10 min of PDGF-AA stimulation, supporting the conclusion that activation of PDGFRαα in cellular proliferation and growth signaling depends on its ciliary localization. In contrast, PDGF-BB-mediated activation of PDGFRβ and signaling through the Mek1/2-Erk1/2 pathway were largely unaffected by the inability to form a primary cilium (Figure 4F). To investigate whether PDGF-AA and PDGF-BB stimulated wt and mutant cells to reenter the cell cycle, we monitored phosphorylation of Rb on $S^{807/811}$ in cells serum starved for 48 hr followed by addition of either PDGF-AA or serum as a

positive control for 16 and 26 hr. In wt cells, both additions induced the phosphorylation of Rb, whereas in mutant cells the PDGF-AA-mediated response was blocked (Figure 4G). We also followed phosphorylation of cdc2 on tyrosine 15, which distinguishes G_2 phase entrance [21]. In Tg737 cells only PDGF-BB, but not PDGF-AA, increased cdc2 phosphorylation (Figure 4H), whereas wt cells responded to both ligands, showing that signaling through the cilium plays a major role in PDGF-AA cell cycle regulation.

Our study shows that primary cilium formation correlates with an elevation in PDGFRα expression and that PDGFRα localizes with the cilium. It seems likely that in quiescent fibroblasts PDGFRα-mediated signaling is dependent upon its ciliary localization and that much of the physiologically important PDGFRα signaling occurs via the cilium. PDGFRα is widely expressed in human tissues, controlling migration, proliferation, and apoptosis [15], and mutations in the receptor play a role in the generation of human malignancies, notably in the pathogenesis of gastrointestinal stromal tumors [22], lung tumors [23], and ovarian carcinoma [24]. PDGFRαα signaling through the fibroblast primary cilium or comparable pathways in the primary cilium in other tissues may be important in tissue homeostasis whereas perturbations in this pathway could lead to oncogenesis.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures and two figures and are available with this article online at http://www.currentbiology.com/cgi/content/full/15/20/1861/DC1/.

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