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Distinct mechanisms for PDGF and FGF signaling in primitive endoderm development

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Abstract

FGF signaling is known to play a critical role in the specification of primitive endoderm (PrE) and epiblast (Epi) from the inner cell mass (ICM) during mouse preimplantation development, but how FGFs synergize with other growth factor signaling pathways is unknown. Because PDGFRα signaling has also been implicated in the PrE, we investigated the coordinate functions of PDGFRα together with FGFR1 or FGFR2 in PrE development. PrE development was abrogated in *Pdgfra; Fgfr1* compound mutants, or significantly reduced in *Pdgfra*; *Fgfr2* or $Pdgfra^{PI3K}$*; Fgfr2* compound mutants. We provide evidence that both *Fgfr2* and *Pdgfra* play roles in PrE cell survival while *Fgfr1* controls PrE cell specification. Our results suggest a model where FGFR1-engaged ERK1/2 signaling governs PrE specification while PDGFRα-and by analogy possibly FGFR2-engaged PI3K signaling regulates PrE survival and positioning in the embryo. Together, these studies indicate how multiple growth factors and signaling pathways can cooperate in preimplantation development.

Keywords

Preimplantation; cell specification; survival; ERK1/2; PI3K

Introduction

During mouse preimplantation development, the embryo undergoes two sequential cell fate decisions. At the morula stage (embryonic day (E) 2.5), outside cells differentiate to trophectoderm (TE) while inner cells constitute the inner mass cells (ICM). At the blastocyst stage, starting at E3.5, ICM cells undergo the second fate decision and become either the pluripotent epiblast (Epi), which forms the embryo proper, or the primitive endoderm (PrE) destined to produce components of the yolk sac. FGF4 signaling is critically required for the differentiation of ICM cells into PrE (Chazaud et al., 2006; Feldman et al., 1995; Goldin and Papaioannou, 2003; Kang et al., 2013; Nichols et al., 2009; Yamanaka et al., 2010). The

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FGF family includes 18 ligands and four receptors, FGFR1–4 (Brewer et al., 2016; Ornitz and Itoh, 2015). While all four FGFRs are expressed in the late mouse blastocyst (Guo et al., 2010; Ohnishi et al., 2014), only FGFR1 and FGFR2 play a critical role in PrE development (Brewer et al., 2015; Kang et al., 2017; Molotkov et al., 2017). *Fgfr1* is expressed in all cell lineages of the mouse embryo during preimplantation development (Kang et al., 2017; Molotkov et al., 2017; Ohnishi et al., 2014). Genetic ablation of *Fgfr1* on a 129S4 co-isogenic background results in decreased numbers of PrE cells and peri-implantation lethality (Brewer et al., 2015). *Fgfr2* is expressed in both the TE and PrE but is not found in Epi cells (Kang et al., 2017; Molotkov et al., 2017; Ohnishi et al., 2014). *Fgfr2*$^{-/-}$ embryos on the same 129S4 genetic background do not show any defects in PrE development, but die at E10.5 from defects in placenta development (Molotkov et al., 2017; Xu et al., 1998; Yu et al., 2003). Despite the lack of PrE defects in *Fgfr2*$^{-/-}$ embryos, targeted inactivation of both *Fgfr1* and *Fgfr2* prevents development of the PrE and phenocopies the *Fgf4*$^{-/-}$ mutant phenotype (Kang et al., 2017; Molotkov et al., 2017). Upregulation of *Fgfr2* expression in ICM cells directed them toward a PrE fate (Morris et al., 2013). This indicates that *Fgfr2* plays a role in PrE development and can compensate at some level for the lack of *Fgfr*1.

FGF4, the only FGF ligand expressed during preimplantation (Feldman et al., 1995; Ohnishi et al., 2014) is produced by Epi precursor cells in the ICM, binds to FGFRs and induces the PrE fate in some ICM cells. Culture of wild-type E2.5 embryos in the presence of FGF4 induced the PrE fate in all ICM cells and rescued PrE development in *Fgf4*$^{-/-}$ embryos (Grabarek et al., 2012; Kang et al., 2013; Krawchuk et al., 2013). Surprisingly, FGF4 treatment of *Fgfr1*$^{-/-}$ and *Fgfr2*$^{-/-}$ compound mutant embryos revealed an absolute requirement for FGF4 signaling through FGFR1 to induce PrE fate in ICM cells, since even at extremely high doses FGF4 was unable to induce PrE specification through FGFR2 (Molotkov et al., 2017). These results indicate that both FGFR1 and FGFR2 are required for PrE development, but that signaling through FGFR1 has a key role in FGF4-induced PrE fate specification.

In addition to the FGFRs, PDGFRα is expressed in the PrE (Artus et al., 2010; Plusa et al., 2008). Analysis of *Pdgfra*$^{-/-}$ blastocysts revealed an increase in PrE cell apoptosis, resulting in a decrease of the number of PrE cells (Artus et al., 2013), although loss of PDGFRα results in a range of abnormalities only at mid-gestation (Soriano, 1997). The decrease in the number of PrE cells in *Pdgfra*$^{-/-}$ embryos was not accompanied by a compensatory increase in the number of Epi cells, which is observed in *Fgf4*$^{-/-}$, *Fgfr1*$^{-/-}$ and *Fgfr1*$^{-/-}$; *Fgfr2*$^{-/-}$ embryos (Brewer et al., 2015; Kang et al., 2017; Kang et al., 2013; Molotkov et al., 2017). This suggests that PDGFRαsignaling by PDGFA, the cognate ligand expressed at this stage (Ohnishi et al., 2014), plays a role in the survival of developing PrE cells rather than in PrE specification, which is controlled mainly by FGF4 signaling through FGFR1.

FGF ligands bind to FGFRs to engage multiple downstream signaling pathways, including ERK1/2 and PI3K / AKT, which mediate cellular responses (for a review, see (Brewer et al., 2016). Culturing wild-type E2.5 embryos in the presence of a MEK inhibitor (PD0325901, 1 μM) leads to a dramatic reduction in the number of PrE cells and a compensatory increase in the number of Epi cells (Nichols et al., 2009). This closely resembles the phenotype observed in *Fgf4*$^{-/-}$ and *Fgfr1*$^{-/-}$; *Fgfr2*$^{-/-}$ embryos. This suggests that FGF activation of

MEK / ERK signaling is critical for control of PrE cell fate specification. In contrast, studies using an allelic series of mouse knock-ins designed to disable binding of Src, SHP2, PLCγ and PI3K to PDGFRs indicate that PI3K is a major signaling pathway downstream of PDGFRα (Klinghoffer et al., 2002). It is thus possible that signaling through FGFR2 and/or PDGFRα engaged PI3K signaling plays a role in PrE cell survival.

In this manuscript, we demonstrate that both FGFR2 and PDGFRα play a role in PrE cell survival while only FGFR1 is involved in ICM cell fate specification. We also hypothesize that ERK and PI3K play sequential roles in PrE development: activation of ERK1/2 by FGF4 / FGFR1 is required for ICM cell fate decision towards the PrE, while subsequent activation of PI3K by PDGFA / PDGFRα and possibly FGF4 / FGFR2 is needed for PrE cells to survive and adopt their proper position in the embryo.

Materials and methods

Mice

Fgfr1$^{+/-}$ mice (Hoch and Soriano, 2006), *Fgfr2*$^{+/-}$ mice (Molotkov et al., 2017), *Fgfr2*$^{T2A\text{-}H2B\text{-}mCherry/T2A\text{-}H2B\text{-}mCherry}$ mice referred to as *Fgfr2*^{mCherry} (Molotkov et al., 2017), *Pdgfra*$^{+/H2B\text{-}GFP}$ mice referred to as *Pdgfra*$^{+/-}$ (Hamilton et al., 2003) and *Pdgfra*$^{+/tm5Sor}$ mice referred to as *Pdgfra*$^{+/PI3K}$ (Klinghoffer et al., 2002) were maintained on a 129S4/SvJaeSor (MGI:3044540) co-isogenic background, further referred to as 129S4. All animal experiments were conducted according to protocols approved by the Institutional Animal Care and Use Committee of Icahn School of Medicine at Mount Sinai.

Embryo dissection and embryonic culture

Female mice were super-ovulated by *i.p.* injection of 5 IU of PMSG (Pregnant Mare Serum Gonadotropin) followed 48 h later by *i.p.* injection of 5 IU of hCG (human Chorionic Gonadotropin) prior to mating with stud males. Vaginal plugs were checked the following day (E0.5). E2.5 embryos (8-cell stage) were flushed from oviducts and cultured for 72h in DMEM (11965–118, ThermoFisher Scientific, USA) with 1/100 of 100 mM sodium pyruvate solution (11360–070, ThermoFisher Scientific, USA), 1/100 of MEM non-essential amino acid solution (11140–050, ThermoFisher Scientific, USA) and 1/200 of penicillin / streptomycin mix (15140–122, ThermoFisher Scientific, USA) at 37°C and 5% CO_2; E3.5 embryos were flushed from uteri and cultured for 48h. All embryos were fixed for 30 min. in 4% PFA and stored in 30% Ethanol in PBST at 4°C until analysis.

Embryos immunostaining

Blastocyst immunostaining was performed as described previously (Nichols et al., 2009). Briefly, embryos were washed once in PBST and once in PBS/PVP (P0930, Sigma), permeabilized for 45 min. in 0.5% Triton X100 (T9284, Sigma) in PBS/PVP, blocked in 0.1% BSA with 2% donkey serum in PBST for 2h and incubated overnight with primary antibodies used at 1/100 dilution. Primary antibodies used were: NANOG (RCAB0002P-F, Cosmo Bio Co., Ltd.), CDX2 (MU392A-UC, BioGenex), GATA4 (sc-1237, Santa Cruz), mCHERRY (ab167453, Abcam) and GFP (A6455, Invitrogen). On the following day, embryos were washed in PBST and incubated for 2hours with secondary Alexa Fluor

(Invitrogen) conjugated antibodies diluted 1/400 in 1% donkey serum in PBST. Blastocysts were counterstained with DAPI and mounted individually in 20 μl PBS droplets on glass-bottom Petri dishes for imaging.

Image analysis

Optical section images were taken on a Zeiss Axio-Observer Z1 with an Apotome 2 attachment, using a Hamamatsu Orca Flash 4.0 LT camera and ZenPro 2015 software. For cell counting, we imported Zeiss z-stack images into Metamorph software (version 7.8.13.0) and manually counted individual cells by scrolling through the stack.

Quantification and statistical analysis

Statistical analysis was performed using Prism 6.0. All data are represented as mean ± standard error. Statistical p-values were calculated using two-tailed Student's t-test for unpaired samples.

RESULTS

PDGFRα and FGFR2 are expressed in PrE

We analyzed expression of *Fgfr2* and *Pdgfra* during preimplantation development in *Fgfr2*$^{+/mCherry}$; *Pdgfra*$^{+/H2B\text{-}GFP}$ double heterozygous embryos between E2.5 and E3.5. We also cultured a subset of E3.5 blastocysts for 48h to obtain expanded blastocysts (E3.5+48h). In *Fgfr2*^{mCherry} embryos, a T2A self-cleavage peptide links *H2B-mCherry* to *Fgfr2*, which puts it under the control of *Fgfr2* regulatory elements to allow reporting of *Fgfr2* expression without affecting FGFR2 levels (Molotkov et al., 2017). In *Pdgfra*$^{+/H2B\text{-}GFP}$ knock-in embryos, *H2B-GFP* is expressed under control of the *Pdgfra* promoter. *Pdgfra*$^{+/H2B\text{-}GFP}$ blastocysts do not display any PrE phenotype (Artus et al., 2010).

We found that at E3.25, *Fgfr2*^{mCherry} is expressed at various levels in all ICM cells (red in Fig. 1), whereas *Pdgfra*$^{H2B\text{-}GFP}$ expression (green in Fig. 1) is observed in some but not all ICM cells (compare cells labeled with arrow and arrowhead in Fig.1). We also noted a lack of correlation between the levels of *Fgfr2*^{mCherry} and *Pdgfra*$^{H2B\text{-}GFP}$ expression at this stage, suggesting that these genes may be independently regulated. While *Pdgfra* is expressed exclusively in the PrE, *Fgfr2* expression is also observed in TE cells in addition to ICM cells (TE cells are specifically labeled with Cdx2 antibody, cyan, in Fig. 1). By E3.5, *Fgfr2*^{mCherry} and *Pdgfra*$^{H2B\text{-}GFP}$ co-localize and are uniformly expressed in all developing PrE cells. In expanded blastocysts, as expected, *Fgfr2* and *Pdgfra* are both co-expressed in the same PrE cells (E3.5+48h, Fig.1), and can be co-labeled with antibodies to GATA4, a cell-type specific marker of mature PrE cells.

Both PDGFRα and FGFR2 play roles in survival of PrE cells

The number of PrE cells has been shown previously to not change in *Pdgfra*$^{+/-}$ embryos, but it is reduced by ~40% in *Pdgfra*$^{-/-}$ embryos by E4.5 due to apoptosis (Artus et al.,2010). To determine if *Fgfr2* contributes to the survival of these remaining PrE cells and can partially rescue *Pdgfra*$^{-/-}$ embryos, we bred *Fgfr2*$^{+/-}$ and *Pdgfra*$^{+/-}$ mice to generate double

heterozygous mice. We next intercrossed these mice to obtain E3.5 blastocysts deficient for both *Fgfr2* and *Pdgfra* (Fig. 2A).

We and others have previously shown that PrE development is normal in $Fgfr2^{-/-}$ embryos (Kang et al., 2017; Molotkov et al., 2017). We noted a reduction of the number of PrE cells (red $GATA4^{+}$ cells in Fig. 2A) in $Fgfr2^{+/+}$; $Pdgfra^{-/-}$ embryos, consistent with a previous report (Artus et al., 2010). This deficiency in the number of PrE cells was additionally enhanced in $Fgfr2^{+/-}$; $Pdgfra^{-/-}$ and was particularly severe in $Fgfr2^{-/-}$; $Pdgfra^{-/-}$ double homozygous embryos, which had only a few remaining PrE cells (Fig. 2A). To further analyze the defect in PrE development in these mutant embryos, we counted the total number of Epi and PrE cells (Fig. 2B–G and Table 1). We found that the average number of Epi, PrE and total cells in the embryos were similar between $Fgfr2^{+/+}$; $Pdgfra^{+/+}$ (wild-type control), $Fgfr2^{+/-}$; $Pdgfra^{+/-}$ and $Fgfr2^{-/-}$; $Pdgfra^{+/-}$ embryos (Fig. 2B, C, D and Table 1). The number of PrE cells was slightly decreased in the $Fgfr2^{+/+}$; $Pdgfra^{-/-}$ embryos, it was significantly lower in $Fgfr2^{+/-}$; $Pdgfra^{-/-}$ ($p=0.02$), and most reduced in $Fgfr2^{-/-}$; *Pdgfra*–/– ($p=0.006$) embryos. Interestingly, the number of Epi cells trended higher but did not change significantly in any of the mutant embryos we analyzed. This is different from the phenotype observed earlier in *Fgfr1* and *Fgfr2* compound mutants (Kang et al., 2017; Molotkov et al., 2017) or in $Fgf4^{-/-}$ embryos (Kang et al., 2013; Krawchuk et al., 2013). We observed in $Fgfr2^{-/-}$; $Pdgfra^{+/-}$, $Fgfr2^{+/-}$; $Pdgfra^{-/-}$ and $Fgfr2^{-/-}$; $Pdgfra^{-/-}$ embryos an increased incidence of pyknotic nuclei, consistent with apoptosis (Table 2 and supplementary Fig. S1). To exclude an effect of embryo size on the relative size of the Epi, we calculated the percentage of Epi and PrE cells in the embryos and the size of the ICM (Fig. 2 E–G). We found that the relative size of the Epi was similar in all mutant classes except for $Fgfr2^{-/-}$; $Pdgfra^{-/-}$ embryos, where it was significantly reduced (Fig. 2F). Similar to the relative size of the PrE, the combined number of $Nanog^{+}$ Epi cells and $Gata4^{+}$ PrE cells was considerably decreased in $Fgfr2^{-/-}$; $Pdgfra^{-/-}$ embryos, while it remained unchanged in the rest of the mutants we analyzed (Fig. 2G).

PrE development is abrogated in $Fgfr1^{-/-}$; $Pdgfra^{-/-}$ embryos

Previously we (Molotkov et al., 2017) and others (Kang et al., 2017) demonstrated that both FGFR1 and FGFR2 play roles in PrE development, and that genetic inactivation of both receptors leads to the total absence of PrE development. Our results also suggested that FGFR1 plays a major role in PrE fate specification, while FGFR2 is involved in PrE cell survival / proliferation. Our data on *Fgfr2* and *Pdgfra* compound mutants suggest that while both FGFR2 and PDGFRα play roles in PrE cell survival, only $Pdgfra^{-/-}$ embryos exhibit deficient PrE development, whereas the PrE is normal in $Fgfr2^{-/-}$ embryos (Fig. 2). This raises the intriguing possibility that $Fgfr1^{-/-}$; $Pdgfra^{-/-}$ mutants might exhibit a phenotype similar to the one we described earlier for $Fgfr1^{-/-}$; $Fgfr2^{-/-}$ embryos (Molotkov et al., 2017). To test this hypothesis, we crossed $Fgfr1^{+/-}$ and $Pdgfra^{+/-}$ mice to obtain double heterozygous $Fgfr1^{+/-}$; $Pdgfra^{+/-}$ mice. We were unable to recover any $Fgfr1^{+/-}$; $Pdgfra^{+/-}$ double heterozygous mice at weaning on the 129S4 genetic background (0 out of 55 pups), similar to the background dependency problem we encountered previously with $Fgfr1^{+/-}$; $Fgfr2^{+/-}$ double heterozygous mice (Molotkov et al., 2017). To overcome this difficulty, we intercrossed $Pdgfra^{+/-}$ mice on a 100% 129S4 background with $Fgfr1^{+/-}$ mice on a 75% B6 /

25% 129S4 background, allowing us to obtain *Fgfr1*$^{+/-}$; *Pdgfra*$^{+/-}$ mice on a mixed 129S4 / B6 genetic background.

We intercrossed *Fgfr1*$^{+/-}$; *Pdgfra*$^{+/-}$ compound mutants, and isolated E3.5 blastocysts that we cultured for 48h prior to analysis. We found that simultaneous deletion of both copies of *Fgfr1* and *Pdgfra* disrupts PrE development in double homozygous embryos (n=3; Fig. 3 and Table 3). Interestingly, in 2 out of 3 *Fgfr1*$^{-/-}$; *Pdgfra*$^{-/-}$ embryos we observed a group of 2 (embryo 1) or 6 (embryo 2, shown with asterisks on Fig. 3A) cells that expressed GATA4 faintly, and that were also located at a considerable distance from the Epi. We did not score these cells as PrE, due to their weak GATA4 expression level and ectopic location. To assess the individual contributions of *Fgfr1* and *Pdgfra* to this phenotype, we counted the number of Epi (NANOG^{+} cells), PrE (GATA4^{+} cells) and the total number of cells in embryos of different genotypes (Fig. 3B–D). We found that in all embryos deficient for one or two *Fgfr1* alleles, the number of Epi cells was increased compared to wild-type control, while *Pdgfra*$^{-/-}$ embryos showed no change in Epi number (Fig. 3B and Table 3). This further reinforces the concept that FGFR1 plays a major role in ICM cell fate decision towards the PrE lineage. *Fgfr1* deficiency causes fewer ICM cells to become PrE, with a compensatory increase in the number of Epi cells. Whereas PDGFRα plays a more important role than FGFR1 in the survival of formed PrE cells, *Pdgfra* deficiency does not directly affect the number of Epi cells. The number of PrE cells (Fig. 3C) trended lower in *Fgfr1*$^{-/-}$; *Pdgfra*$^{+/+}$, *Fgfr1*$^{+/+}$; *Pdgfra*$^{-/-}$, *Fgfr1*$^{+/-}$; *Pdgfra*$^{-/-}$ embryos and was significantly lower in *Fgfr1*$^{-/-}$; *Pdgfra*$^{+/-}$ (n=5, p=0.004) embryos. *Fgfr1*$^{-/-}$; *Pdgfra*$^{-/-}$ embryos were also considerably smaller (p=0.002) than the other embryos (Fig. 3D). Preliminary results suggest that TE development is compromised in these mutants (data not shown).

To exclude an effect of embryo size on our results, we calculated the percentage of Epi and PrE relative to the total number of cells (Fig. 3E–G). The proportion of Epi cells significantly increased in *Fgfr1*$^{+/-}$; *Pdgfra*$^{-/-}$ (p=0.04), *Fgfr1*$^{-/-}$; *Pdgfra*$^{+/-}$ (p=0.05) and *Fgfr1*$^{-/-}$; *Fgfr2*$^{-/-}$ (p=0.02) embryos (Fig. 3E), with a corresponding decrease in the number of PrE cells (Fig. 3G). The combined number of Epi and PrE cells trended lower in *Fgfr1*$^{+/+}$; *Pdgfra*$^{-/-}$ and *Fgfr1*$^{+/-}$; *Pdgfra*$^{-/-}$ embryos and was significantly lower in *Fgfr1*$^{-/-}$; *Pdgfra*$^{-/-}$ embryos.

PI3K is downstream of PDGFR signaling in PrE development

Previous genetic studies in our lab have demonstrated that PI3K is the main signaling pathway downstream of PDGFRα (Fantauzzo and Soriano, 2014, 2016; Klinghoffer et al., 2001; Vasudevan et al., 2015). The PI3K / AKT pathway is active in preimplantation embryos (Riley et al., 2005). To test if PDGFRα-mediated PI3K signaling plays a critical role in PrE development, we intercrossed *Pdgfra*$^{+/PI3K}$ mice (Klinghoffer et al., 2001), in which PI3K cannot bind to the PDGFRα and become activated upon PDGFRα engagement, with *Fgfr2*$^{+/-}$ mice to obtain *Fgfr2*$^{+/-}$; *Pdgfra*$^{+/PI3K}$ double heterozygous animals. We crossed *Fgfr2*$^{+/-}$; *Pdgfra*$^{+/PI3K}$ mice to obtain E3.5 embryos, which we further cultured for 48h (Fig. 4A). We found that double homozygous *Fgfr2*$^{-/-}$; *Pdgfra*$^{PI3K/PI3K}$ embryos (n=2) have severely reduced number of PrE cells, similar to *Fgfr2*$^{-/-}$; *Pdgfra*$^{-/-}$ embryos (Fig. 4B).

This result strongly supports the concept that PDGF-engaged PI3K signaling plays an important role in PrE development.

DISCUSSION

Both *Pdgfra* and *Fgfr2* are expressed in the PrE of the implanting blastocyst. *Pdgfra* is expressed exclusively in the PrE at this stage (Plusa et al., 2008), while *Fgfr2* starts to be expressed in the future TE cells at the 16-cell stage and is later expressed in both the PrE and TE (Kang et al., 2017; Molotkov et al., 2017; Ohnishi et al., 2014). Interestingly, we observed that while both *Pdgfra* and *Fgfr2* are expressed in the same PrE cells in the late blastocyst, *Fgfr2* and *Pdgfra* expression does not entirely overlap at the morula and midblastocyst stages. This suggests that induction of *Fgfr2* and *Pdgfra* expression in ICM cells is controlled by independent mechanisms.

Pdgfra$^{-/-}$ blastocysts have reduced numbers of PrE cells due to increased apoptosis of the developing PrE cells (Artus et al., 2013). PrE development in *Fgfr2*$^{-/-}$ blastocysts is normal (Kang et al., 2017; Molotkov et al., 2017). Culturing blastocysts in the presence of high doses of external FGF4 failed to convert ICM cells into PrE in embryos deficient for both copies of *Fgfr1*, while external FGF4 induces the PrE fate in all wild-type and *Fgfr1*$^{+/-}$ ICM cells (Molotkov et al., 2017). This demonstrated a primary role for FGFR1 but not for FGFR2 in the determination of the PrE fate in ICM cells during development (Kang et al., 2017; Molotkov et al., 2017). However, while around 30% of *Fgfr1*$^{-/-}$ embryos did not contain any PrE cells, PrE development was still maintained in 70% of *Fgfr1*$^{-/-}$ blastocysts, with some mutant embryos having normal number of the PrE cells (Brewer et al., 2015). We and others recently demonstrated that simultaneous deletion of both *Fgfr1* and *Fgfr2* is required to completely block PrE development in all embryos (Kang et al., 2017; Molotkov et al., 2017). This strongly suggests that while FGFR2 may not play a critical role in ICM cell fate decision toward PrE, it may play an auxiliary role. At that time we speculated that, similar to PDGFRα (Artus et al., 2013), FGFR2 might be involved in the control of PrE cell survival and proliferation (Molotkov et al., 2017). The results presented here demonstrate that deletion of both FGFR2 and PDGFRα receptors further reduced the number of PrE cells and was associated with increased apoptosis. Interestingly, this reduction in the number of PrE cells was not accompanied by a compensatory increase in the number of Epi cells, observed in *Fgfr1*$^{-/-}$; *Fgfr2*$^{-/-}$ or *Fgf4*$^{-/-}$ embryos (Kang et al., 2017; Kang et al., 2013; Krawchuk et al., 2013; Molotkov et al., 2017). This suggests that the reduction in PrE cell number in *Fgfr2*$^{-/-}$; *Pdgfra*$^{-/-}$ embryos is due to decreased survival of these cells rather than a switch in the fate of the developing ICM toward Epi, which is controlled by FGF4 signaling to FGFR1.

The critical role of FGFR1 in the control of PrE fate was further substantiated by our analysis of *Fgfr1*$^{-/-}$; *Pdgfra*$^{-/-}$ embryos. We found that, similar to *Fgfr1*$^{-/-}$; *Fgfr2*$^{-/-}$ embryos, deletion of *Pdgfra* together with *Fgfr1* prevented PrE development. In line with this observation, we observed a compensatory increase in the number of Epi cells in *Fgfr1*$^{-/-}$; *Pdgfra*$^{-/-}$ embryos, also seen previously in some *Fgfr1*$^{-/-}$ and in all *Fgfr1*$^{-/-}$; *Fgfr2*$^{-/-}$ embryos. This further supports the hypothesis that FGF4 signaling through FGFR1 is required for PrE fate specification. Interestingly, two out of three *Fgfr1*$^{-/-}$; *Pdgfra*$^{-/-}$

embryos contained a few cells that expressed GATA4 faintly, but these cells were located far from NANOG^{+} Epi cells, suggesting that they do not acquire a “classical” PrE cell fate and location adjacent to Epi. Such cells were never observed in *Fgfr1*$^{-/-}$; *Fgfr2*$^{-/-}$ embryos. We hypothesize that these few faintly expressing *Fgfr1*$^{-/-}$; *Pdgfra*$^{-/-}$ GATA4^{+} cells retain sufficient levels of *Fgfr2* that allowed them to survive, and that in the absence of both FGFR1 and PDGFRα they are unable to migrate to their proper position adjacent to Epi, leaving them in ectopic locations in the embryo.

PI3K is the main signaling pathway downstream of the PDGFRα (Fantauzzo and Soriano, 2014, 2016; Klinghoffer et al., 2001). In contrast, FGFRs have long been thought to be dependent on downstream activation of ERK for their biological activity, but they are also known to engage PI3K signaling (Brewer et al., 2016; Brewer et al., 2015; Vasudevan et al., 2015). Our data now suggests that both ERK and PI3K signaling might be involved in PrE development (Fig. 5). First, in the mid-blastocyst, FGF4 produced by a subpopulation of ICM cells binds to FGFR1 on neighboring ICM cells and activates ERK1/2, which induces the PrE fate. The involvement of ERK1/2 in PrE fate determination was demonstrated earlier by studies using selective inhibitors (Nichols et al., 2009). Second, selective inhibition of PI3K significantly reduced number of embryos developing to the blastocyst stage and increased the proportion of cells with a fragmented nuclei (Lu et al., 2004). In the expanded blastocyst, PDGFA (Ohnishi et al., 2014) signaling to PDGFRα, and potentially FGF4 signaling to FGFR2, activates PI3K in newly forming PrE cells, which may control their survival and possibly position (Brewer et al., 2016; Manning and Toker, 2017). Our results thus suggest a sequential role for multiple signaling pathways in establishing cell fates in the preimplantation embryo.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse monoclonal to CDX2	BioGenex	MU392A-UC
Goat polyclonal to GATA4	Santa Cruz	sc-1237
Goat polyclonal to GATA6	R&D Systems	AF1700
Rabbit polyclonal to GFP	Invitrogen	A6455
Rabbit polyclonal to mCHERRY	Abeam	ab167453
Rabbit polyclonal to NANOG	Cosmo Bio Co., Ltd.	RCAB0002P-F
Bacterial and Virus Strains		
N/A		
Biological Samples		
N/A		
Chemicals, Peptides, and Recombinant Proteins		
N/A		
Critical Commercial Assays		
N/A		
Deposited Data		

REAGENT or RESOURCE	SOURCE	IDENTIFIER
N/A		
Experimental Models: Cell Lines		
N/A		
Experimental Models: Organisms/Strains		
Fgfr1$^{+/-}$ mouse strain	This lab	N/A
Fgfr2$^{+/-}$ mouse strain	This lab	N/A
Pdgfra$^{+/H2B\text{-}GFP}$ mouse strain	This lab	N/A
Pdgfra$^{+/tm5Sor}$ mouse strain	This lab	N/A
Fgfr2$^{T2A\text{-}H2B\text{-}mCherry/T2A\text{-}H2B\text{-}mCherry}$ mouse strain	This lab	N/A
Oligonucleotides		
N/A		
Recombinant DNA		
N/A		
Software and Algorithms		
Excel	Microsoft	2016
FIJI (ImageJ)	NIH	ImageJ ver 2
MetaMorph Image Analysis	Molecular Devices	Ver 7.8.13.0
Prism	GraphPad	Ver 6
Other		
N/A	Ibidi	80826

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- PDGF and FGF signaling are both critical in primitive endoderm (PrE) development in the mouse.
- FGF4/FGFR1 engaged ERK1/2 signaling regulates cell allocation towards the PrE.
- PDGFA/PDGFRα engaged PI3K signaling regulates PrE survival and positioning.
- FGF4/FGFR2 also regulates cell survival, thus suggesting a role for PI3K signaling in this process.

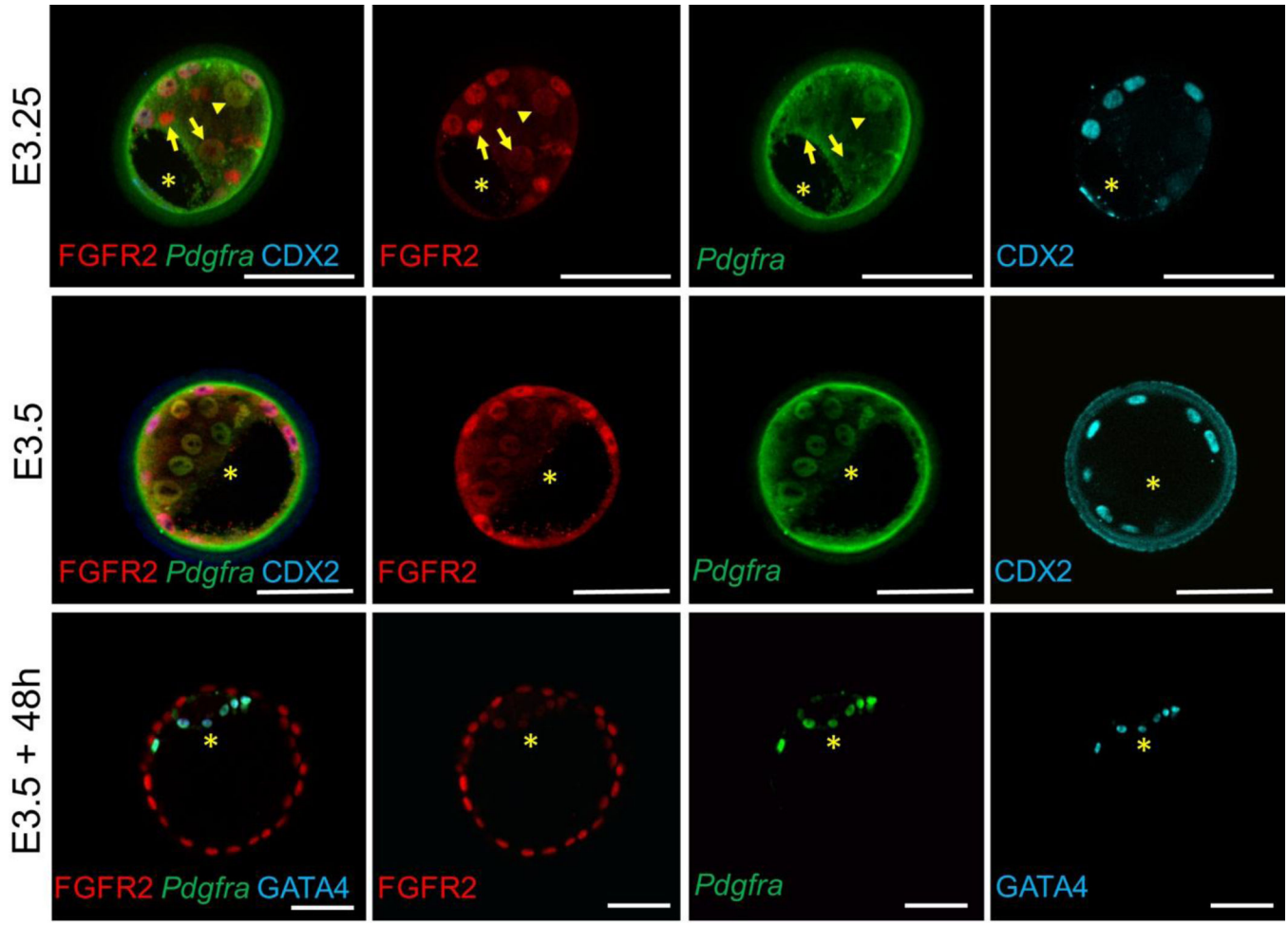

Figure 1.

Expression of *Fgfr2* and *Pdgfra* in E3.0 and E3.5 preimplantation embryos. Note, while $Fgfr2^{mCherry}$ and $Pdgfra^{H2B\text{-}GFP}$ are differentially expressed in ICM cells at E3.0, both receptors are expressed in the same cells in E3.5 expanded embryos (E3.5 + 48h). Green background fluorescence in E3.0 and E3.5 embryos is due to the presence of the Zona Pellucida that surrounds the embryos. *, blastocoel cavity. Scale bars, 50 μm.

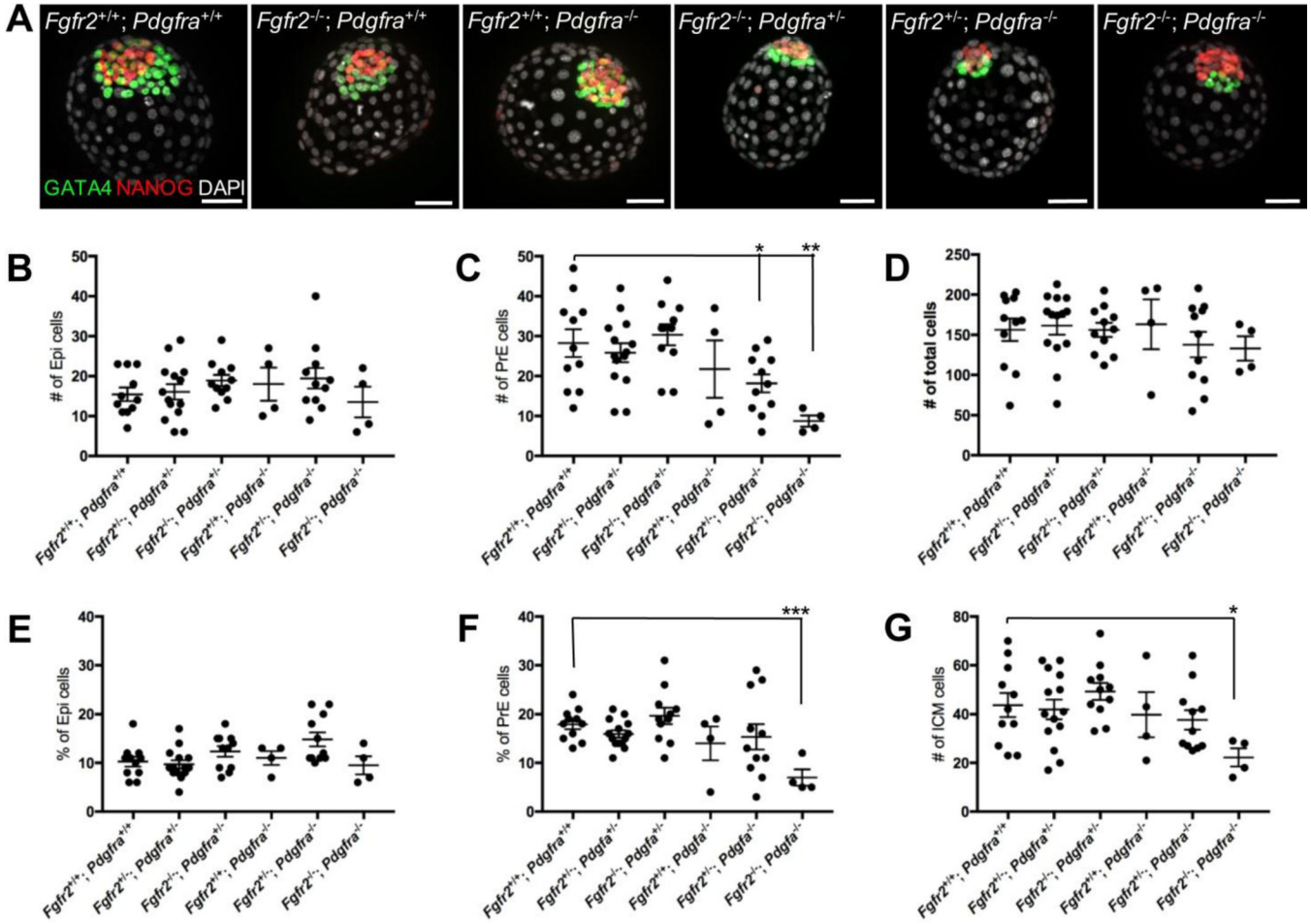

Figure 2.

PrE development is deficient in *Fgfr2*$^{-/-}$; *Pdgfra*$^{-/-}$ embryos. (**A**) E3.5 embryos were cultured for 48h in DMEM and stained with antibodies to NANOG (red) and GATA4 (green). DAPI (white) was used to counter-stain nuclei. Number of Epi (**B**), PrE (**C**) and total cells (**D**) were counted in E3.5 embryos cultured for 48h in DMEM. Percentages of Epi (**E**) and PrE (**F**) cells relative to the total number of cells are shown. (**G**) Number of ICM cells is shown. Data represented as mean + SEM. *, p=0.02; **, p=0.006. Scale bars, 50 μm.

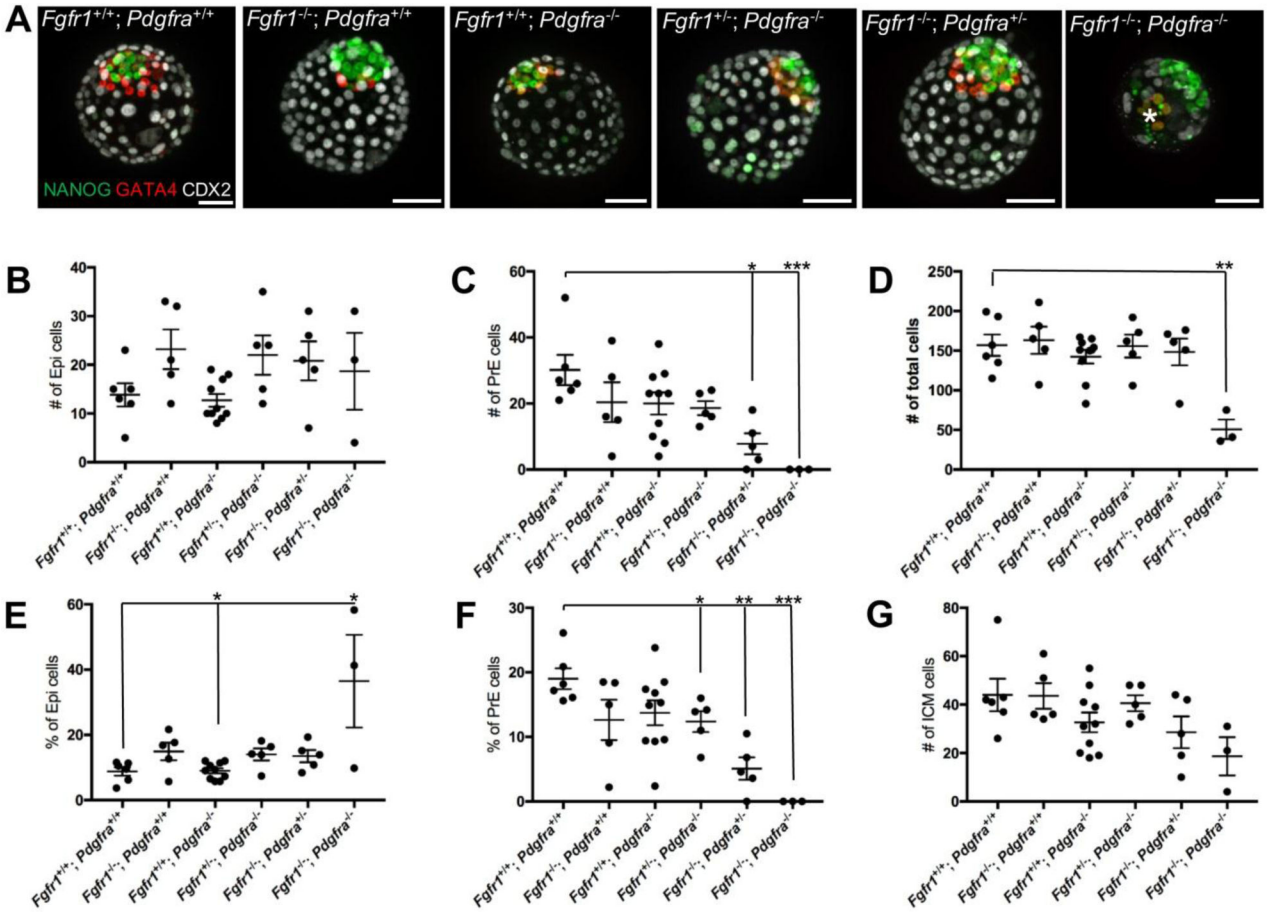

Figure 3.

PrE development is disrupted in *Fgfr1*$^{-/-}$; *Pdgfra*$^{-/-}$ embryos. (**A**) Embryos were dissected at E3.5, cultured for 48h in DMEM and stained with antibodies to NANOG (green), GATA4 (red) and CDX2 (white). Number of Epi (**B**), PrE (**C**) and total number of cells (**D**) were counted and are shown for the individual embryos. The proportion of Epi (**E**) and PrE (**F**) cells in the embryos are shown. (**G**) Number of ICM cells. Data represented as mean + SEM. *, p=0.002; **, p=0.003. Scale bars, 50 μm.

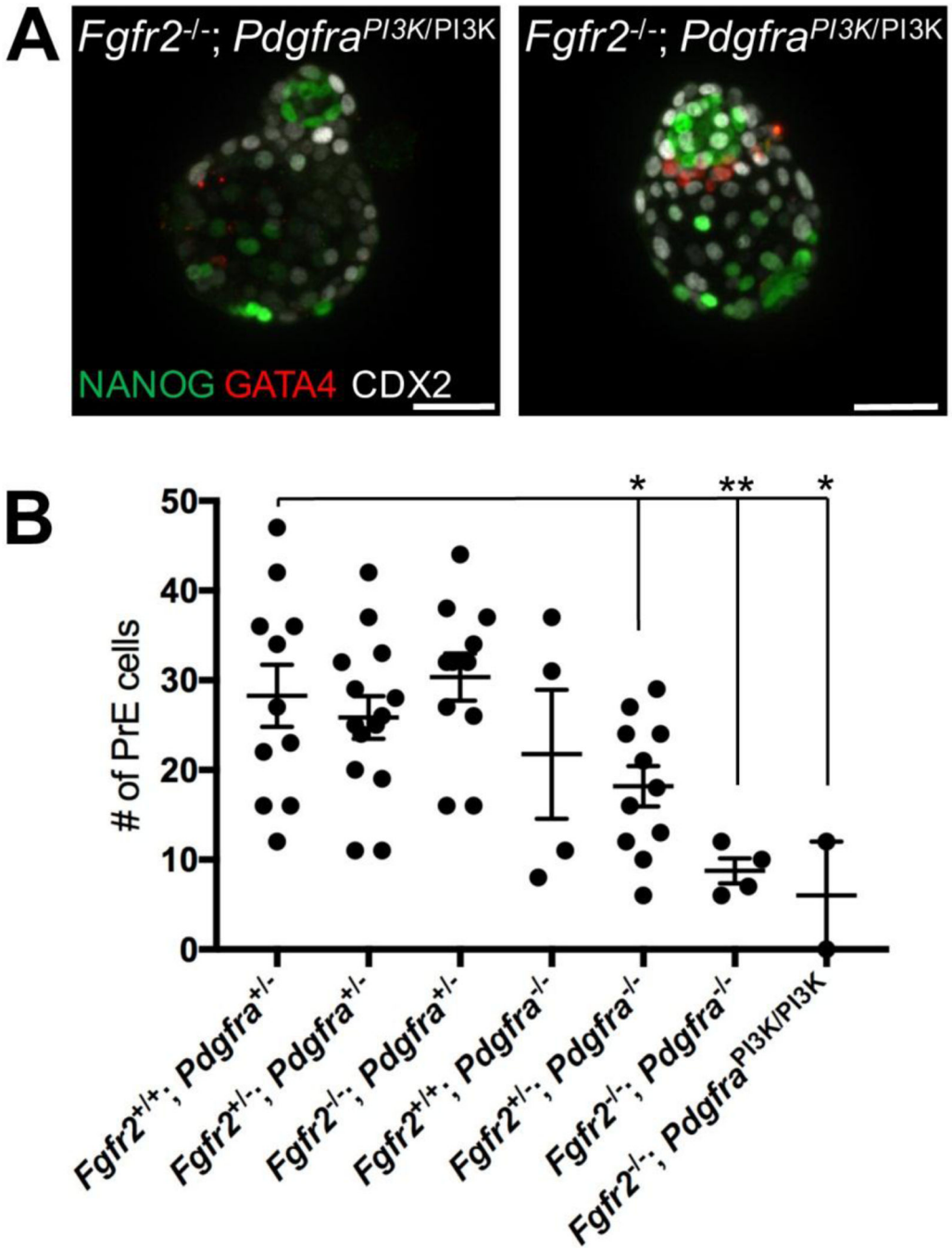

Figure 4.

PI3K is the main signaling pathway downstream PDGFRα in PrE development. (**A**) Two E3.5 *Fgfr1*$^{-/-}$; *Pdgfra*$^{PI3K/PI3K}$ embryos are shown. NANOG (green) labels Epi cells, GATA4 (red) labels PrE. DAPI (blue) used to counter label cell nuclei. (B) Number of PrE cells in *Fgfr1*$^{-/-}$; *Pdgfra*$^{PI3K/PI3K}$ embryos (red box) are shown compared to *Fgfr1* & *Pdgfra* compound mutants. Scale bars, 50 μm.

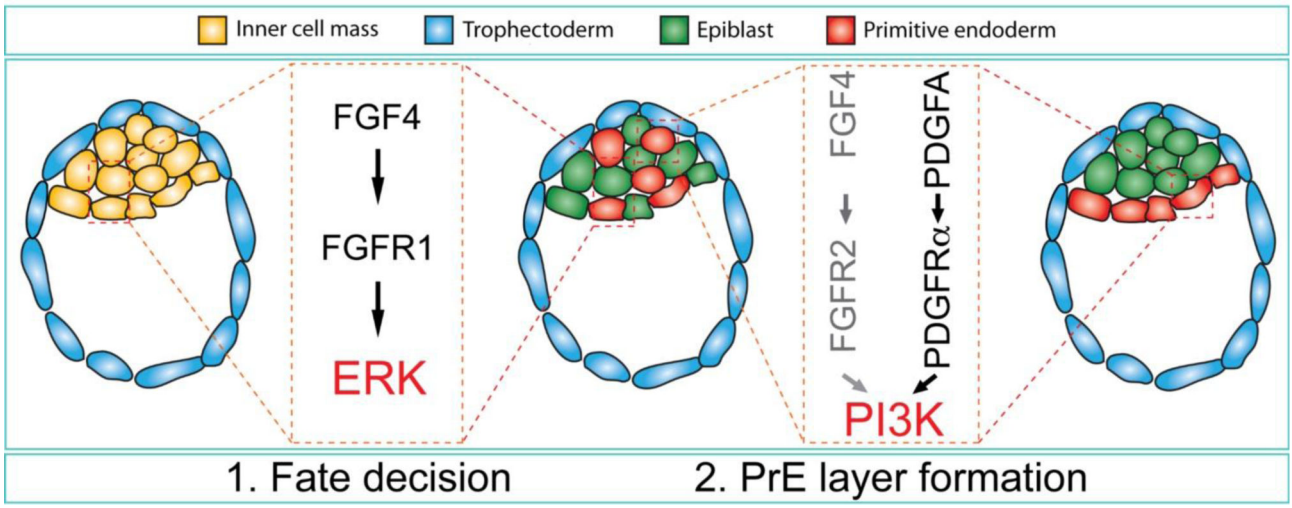

Figure 5.

Model for FGF and PDGF signaling in PrE development. At the mid-blastocyst stage, FGF4 is produced by a subpopulation of the ICM cells and signals in a paracrine fashion through ERK1/2 to induce PrE cells. In expanded blastocysts, PDGFA signaling to PDGFRα, and potentially FGF4 signaling to FGFR2, activates PI3K in newly forming PrE cells, which controls their survival.

Table 1.

Cell lineage composition in *Fgfr2*; *Pdgfra* compound mutants

		Number of cells		
	#	**Epi**	**PrE**	**Total**
Fgfr2$^{+/+}$; *Pdgfra*$^{+/+}$	11	15 ± 6	28 ± 3	156 ± 14
Fgfr2$^{+/-}$; *Pdgfra*$^{+/-}$	14	16 ± 2	26 ± 2	161 ± 11
Fgfr2$^{-/-}$; *Pdgfra*$^{+/-}$	11	19 ± 1	30 ± 3	156 ± 9
Fgfr2$^{+/+}$; *Pdgfra*$^{-/-}$	4	18 ± 4	22 ± 7	163 ± 31
Fgfr2$^{+/-}$; *Pdgfra*$^{-/-}$	11	19 ± 3	18 ± 2*	138 ± 16
FgfrZ$^{-/-}$; *Pdgfra*$^{-/-}$	4	13 ± 4	9 ± 1**	133 ±15

Notes:

*, p=0.02;

**, p=0.006 when compared to wild-type control

Table 2.

Number of pyknotic nuclei in *Fgfr2*; *Pdgfra* compound mutants

	#	Epi
Fgfr2$^{+/+}$; *Pdgfra*$^{+/+}$	11	1.3 ± 0.4
Fgfr2$^{+/+}$; *Pdgfra*$^{-/-}$	4	1.8 ± 0.6
Fgfr2$^{-/-}$; *Pdgfra*$^{+/-}$	11	2.1 ± 0.7
Fgfr2$^{+/-}$; *Pdgfra*$^{-/-}$	11	2.4 ± 0.6
Fgfr2$^{-/-}$; *Pdgfra*$^{-/-}$	4	2.8 ± 0.3

Table 3.

Cell lineage composition in *Fgfr1*; *Pdgfra* compound mutants

		Number of cells		
	#	**Epi**	**PrE**	**Total**
Fgfr1$^{+/+}$; *Pdgfra*$^{+/+}$	6	14 ± 2	30 ± 5	156 ± 14
Fgfr$^{-/-}$; *Pdgfra*$^{+/+}$	5	23 ± 4	20 ± 6	161 ± 11
Fgfr1$^{+/+}$; *Pdgfra*$^{-/-}$	10	13 ± 1	20 ± 3	156 ± 9
Fgfr1$^{+/-}$; *Pdgfra*$^{-/-}$	5	22 ± 4	19 ± 2	163 ± 31
Fgfr1$^{-/-}$; *Pdgfra*$^{+/-}$	5	21 ± 4	8 ± 3*	148 ± 17
Fgfr1$^{-/-}$; *Pdgfra*$^{-/-}$	3	19 ± 8	0 ± 0**	51 ± 12

Notes:

*, p=0.002;

**, p=0.003 when compared to wild-type control