Published in final edited form as:

Stem Cells. 2013 September ; 31(9): 1932–1941. doi:10.1002/stem.1442.

PDGF signaling is required for primitive endoderm cell survival in the inner cell mass of the mouse blastocyst

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Abstract

At the end of the preimplantation period, the inner cell mass (ICM) of the mouse blastocyst is composed of two distinct cell lineages, the pluripotent epiblast (EPI) and the primitive endoderm (PrE). The current model for their formation involves initial co-expression of lineage-specific markers followed by mutual-exclusive expression resulting in a salt-and-pepper distribution of lineage precursors within the ICM. Subsequent to lineage commitment, cell rearrangements and selective apoptosis are thought to be key processes driving and refining the emergence of two spatially distinct compartments. Here, we have addressed a role for PDGF signaling in the regulation of programmed cell death during early mouse embryonic development. By combining genetic and pharmacological approaches, we demonstrate that embryos lacking PDGF activity exhibited caspase-dependent selective apoptosis of PrE cells. Modulating PDGF activity did not affect lineage commitment or cell sorting, suggesting that PDGF is involved in the fine-tuning of patterning information. Our results also indicate that PDGF and FGF tyrosine kinase receptors exert distinct and non-overlapping functions in PrE formation. Taken together, these data uncover an early role of PDGF signaling in PrE cell survival at the time when PrE and EPI cells are segregated.

Keywords

PDGF signaling; primitive endoderm; blastocyst; mouse embryo; apoptosis

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

INTRODUCTION

Apoptosis or programmed cell death is an essential process for a developing organism. Besides its critical function in removing damaged cells, apoptosis plays important roles during embryogenesis under normal physiological conditions (reviewed in ¹⁻³). However, the temporal and spatial coordination between selective cell death and developmental signals remains an open question. To address this question, we have focused on the first apoptotic events during development, which occur at the end of the preimplantation period in the mouse blastocyst stage embryo ⁴⁻⁶. At this stage, the embryo comprises three molecularly and spatially distinct cell lineages: an outer layer of trophectoderm (TE) cells and an inner cell mass (ICM), which is composed of the epiblast (EPI) and the primitive endoderm (PrE). Later on, the pluripotent EPI will predominantly form the embryo-proper, the TE will give rise to the fetal portion of the placenta and the PrE will contribute to the parietal and visceral yolk sacs and the endoderm of the fetus ⁷⁻¹¹. Formation of these embryonic and extraembryonic cell lineages results from two sequential cell fate decisions (reviewed in ^{12,13}). The first concerns the specification of the TE and the ICM, while the second allows the divergence between EPI and PrE cell lineages within the ICM. The current model of EPI *versus* PrE specification involves three successive steps. Initially, key lineage-specific transcription factors are expressed at various levels in most cells (blastomeres) until the early blastocyst stage ¹⁴. In mid-blastocyst embryos (~64-cell stage), these markers become progressively restricted such that the ICM is a mosaic of cells expressing either PrE or EPI markers that are organized in an apparent salt-and-pepper pattern ^{14,15}. It is thought that this stage corresponds to the period when ICM cells become committed to either a PrE or a EPI fate, a process driven by FGF signaling (reviewed in ¹⁶). The final step concerns the segregation of these two lineages into two distinct tissue layers. Several mechanisms have been shown to regulate the sorting of ICM lineage precursors including actin-dependent cell movements, retention of positional information by sorted PrE cells and epithelialization ^{14,17,18}. It has been proposed that apoptosis could also be involved at these stages where it functions to remove cells that are not properly fated or are mispositioned ^{14,17,19,20}. However, the molecular mechanisms involved in this selective cell death remain unknown.

Here, we provide evidence that the PDGF signaling pathway is involved in the regulation of this process of selective apoptosis. By combining genetic and pharmacological approaches, we demonstrate that absence of PDGF signaling specifically affects the survival of PrE cells in a caspase-dependent manner. Using a null knock-in *Pdgfra*^{H2B-GFP} allele ²¹ which is expressed in the PrE ^{14,22}, we observed that in mutant embryos cell death affected PrE cells independently from their position within the ICM.

Taken together, this study uncovers a role for PDGF signaling in the survival of the PrE lineage at the time when a sub-set of ICM cells have committed to a PrE fate but before their segregation into a distinct layer. Furthermore, we show that FGF and PDGF, two prominent classes of receptor tyrosine kinase (RTK) signaling which regulate similar transduction pathways within the ICM of the mouse blastocyst, exert distinct roles in cell lineage specification and survival during cell sorting, respectively.

MATERIAL AND METHODS

Mouse husbandry

Mice were maintained under a 12-hour light cycle. Mouse strains used were *Pdgfra*^{H2B-GFP/+} 21 and *Fgf4*^{+/-} 23,24 maintained on a CD1 genetic background, wild-type CD1 (Charles River) and ICR (Taconic).

Tamoxifen injection

2–3 month old females were injected intraperitoneally with a mixture of 10 µg Tamoxifen (Sigma) and 2–3 mg progesterone (Abraxis) diluted into corn oil (Sigma) at 2.5 days *post coitum* (dpc). Embryos were recovered 1 day later (referred to as E3.5 + 1 day).

Embryo recovery and culture

Embryos were recovered by flushing uteri or oviducts in M2 (Millipore). Embryos were cultured in 10 µL drops of KSOM (Millipore) under mineral oil (Sigma) for up to 50 hours at 37°C, 5% CO₂. Inhibitors used were Gleevec (gift of P. Besmer, Sloan-Kettering Institute, NY, USA) at concentrations ranging from 1 to 10 µM and Z-VAD-FMK (R&D systems) at 20 µM. Recombinant human PDGF-AA (R&D systems) was used at 500 ng/mL.

Electroporation of blastocyst embryos

Electroporation was performed according to Frankenberg et al ²⁵. Embryos were recovered around noon at 3.5 dpc. The zona pellucida was removed using acidic Tyrode's (Sigma). Embryos were washed in M2 and then G2 (Vitrolife) buffered with 20 mM HEPES (hG2). Embryos were then transferred into 50 µL hG2 with 60 µg pCAGGS ²⁶ or pCAG::Pdgfa vectors and 40 µg pCAG::H2B-mCherry ²⁷. Electroporation was performed in 1 mm gap cuvettes with an ECM 830 (BTX) apparatus. Settings were 4 pulses of 33V and 1 ms duration each with a 100 ms interval. Embryos were subsequently washed through several drops of hG2 and cultured 25–30 hours in 10 µL G2 drops covered by mineral oil at 37°C, 5% CO₂.

Live imaging

For live imaging, a group of 3–4 embryos was transferred into glass bottom microwell dishes (Mat Tek) coated with 2% Agar, 0.9% NaCl. 3D time-lapse data were acquired using Volocity 5.5.1 acquisition software (Perkin-Elmer) and a Perkin-Elmer RS3 Nipkow-type scanhead mounted on a Zeiss Axiovert 200M with Hamamatsu C4742-80-12AG camera. GFP was excited using a 488 nm Argon laser. Images were acquired using a Zeiss plan-Neofluar 25x/0.8 DIC korr objective. 10–20 xy planes were acquired, separated by 3–4 µm every 15 minutes. Embryos were subsequently genotyped as previously described ²².

Immunostaining

Embryos were fixed 10 min in 4% paraformaldehyde at room temperature, washed in PBS 10 mg/mL BSA and permeabilized in 0.25% Triton PBS for 10 min at room temperature. After several washes in PBS-BSA, embryos were preincubated 10 minutes in PBS with 0.1% Tween 20 and 10% fetal bovine serum and then incubated with primary antibodies

overnight at 4°C. The following primary antibodies were used : anti-GATA4 (1/300, Santa Cruz), anti-cleaved Caspase-3 (1/100, Cell Signaling), anti-Nanog (1/700, Cosmo Bio), anti-SOX17 (1/300, R&D Systems). The next day, embryos were washed in PBS-BSA then incubated in presence of secondary antibodies overnight at 4°C. Secondary AlexaFluor-conjugated antibodies (Invitrogen) were used at a dilution of 1/500. DNA was counterstained with Hoechst 33342 (Molecular Probes).

Image data acquisition and processing

Image data of immunostained embryos were acquired on a Zeiss LSM510 META confocal microscope. Fluorescence was excited with a 405-nm laser diode (Hoechst), a 488-nm Argon laser (GFP, Alexa Fluor 488), a 543-nm HeNe laser (Alexa Fluor 546, 555, 568) and a 633-nm HeNe laser (Alexa Fluor 633 and 647). Images were acquired using a Plan-apochromat 20x/NA 0.75 objective with optical section thickness ranged from 1 to 2 μ m. Raw data were processed using Zeiss AIM software (Carl Zeiss Microsystems), IMARIS 7.2.2 software (Bitplane AG) and Volocity 5 software (Perkin-Elmer).

RESULTS

Embryos lacking PDGF signaling exhibit increased cell death within the PrE lineage

We previously reported that, in the absence of PDGF signaling, embryonic day (E) 4.5 embryos and tamoxifen-induced diapause embryos exhibited a reduction in the number of PrE cells ²². During the course of this analysis, we noted the presence of picnotic nuclei in mutant embryos raising the possibility that selective cell death may contribute to the reduction in the size of the PrE. To test this possibility, we quantified the number of fragmented nuclei in E3.5 embryos recovered from females one day after tamoxifen injection (E2.5+1 day) (Figure 1A). We noted a significant increase in the number of fragmented nuclei in mutant embryos (3.9 ± 0.8 nuclei in mutant *versus* 1.1 ± 0.3 in heterozygotes and 1.4 ± 0.6 in wild-type). To determine whether cell death was a result of apoptosis, we analyzed the number of cleaved Caspase-3-positive cells in embryos. We noted a significant increase in the number of active Caspase-3-positive cells in mutant embryos (3.6 ± 0.9 apoptotic cells in mutant *versus* 0.7 ± 0.4 in heterozygotes and 1.4 ± 0.8 in wild-type).

To exclude the possibility that the increase of apoptosis in *Pdgfra*-deficient embryos could result from the injection of tamoxifen, we conducted similar analyses on E3.5 embryos recovered from heterozygous intercrosses. We observed a significant increase in fragmented nuclei and Caspase-3-positive cells in mutant embryos (3.6 ± 0.7 nuclei for mutant *versus* 1.4 ± 0.3 for heterozygotes and 1.3 ± 0.4 for wild-type; 2.3 ± 0.8 apoptotic cells *versus* 0.4 ± 0.1 for heterozygotes and 0.5 ± 0.4 for wild-type) (Figure 1B). Interestingly, combining these two sets of experiments, we observed that 82% Caspase-3-positive cells (66/80) in mutants were GFP-positive. We found a comparable situation in heterozygous embryos with 72% Caspase-3-positive cells being GFP-positive (21/29). During this developmental stage which is prior to ICM lineage sorting, and when EPI/PrE cells have acquired mutually-exclusive expression of markers, *Pdgfra* is expressed predominantly in PrE-biased cells ¹⁴. Although EPI or PrE-biased cells retain their developmental plasticity until peri-implantation ²⁸, high

Pdgfra^{H2B-GFP} expression appears to be an accurate predictor of PrE lineage commitment. Therefore, our current findings strongly suggest that in the absence of PDGF signaling PrE cells exhibit increased levels of apoptosis. However, to conclusively rule out that we are not following *Pdgfra*-GFP positive cells that eventually acquire an EPI fate through downregulation of the reporter, we would need to develop an EPI-specific reporter and repeat the analysis. Though concurrent visualization of an EPI reporter would be desirable, it is outside the scope of the present study.

Localization of dying PrE cells in embryos lacking PDGF signaling

The transition between mid-blastocyst (where PrE and EPI have been specified and organized in a salt-and-pepper distribution) and late blastocyst (where ICM cell lineages are segregated) is achieved through coordination of several mechanisms including selective apoptosis of PrE cells located internally within the ICM and not having a major contact with the blastocoel cavity^{14,17}. We therefore hypothesized that absence of PDGF signaling might preferentially affect the viability of inner PrE cells. Unfortunately, our statistical analyses of markers only provided a snapshot of the events occurring within the ICM and we could not determine the position of Caspase-3-positive cells. We therefore used the *Pdgfra*^{H2B-GFP} strain of mice, which functions as a PrE live imaging reporter and in which the nuclear-localized fluorescence allows the tracking of individual cells, as well as the visualization of cell death by nuclear fragmentation (Figure 2A and movie 1). 3D time-lapse imaging revealed a significant increase in GFP+ cell death in mutant embryos (8.2 ± 2.0 dead cells in mutants *versus* 2.5 ± 0.3 in heterozygotes) (Figure 2B). Then, we assigned the position of GFP+ (GFP-positive) cells prior to their death: inside cells (located inside the ICM) are indicated in red, outside cells (located at the surface of the ICM in contact to the blastocoel cavity) in blue (Figure 2C, D and movie 1). In heterozygous embryos, we determined that 54% (42/78) were inner cells, and 41% (32/78) were outside. We found a similar situation in mutant embryos in which 56% (37/66) were inner cells and 45% outside (25/66). From these data we conclude that absence of PDGF signaling affects the survival of GFP+ cells independently from their position within the ICM.

Gleevec, a PDGF signaling inhibitor, specifically affects the PrE lineage

Our results suggested that PDGF signaling might be required at the time of PrE cell sorting. However in *Pdgfra*-null embryos, it was not possible to determine the precise time when PDGF signaling was required during PrE formation. To address this question, we used the pharmacological inhibitor Gleevec (also known as Imatinib), a potent inhibitor of PDGF receptors^{22,29}. We recovered wild-type embryos at various developmental stages (E2.5, early and mid-blastocyst stages) and cultured them until E4.5 in the presence of various concentrations of Gleevec (Figure 3A). Embryos were then immunostained with lineage-specific markers (Supplemental figure S1). We first noticed that Gleevec used at a 10 μ M concentration strongly affected the development of E2.5 and early blastocyst stage embryos (Figure 3B). Indeed, all (5/5) E2.5 embryos died, while cultured early blastocysts were smaller and had a reduced blastocoel cavity. However, at 1 and 5 μ M concentrations, we did not observe any notable morphological differences in inhibitor treated embryos as compared to control embryos (Figure 3B).

We analyzed the number of cells for each of the three blastocyst cell lineages (TE, EPI and PrE) in embryos treated with concentrations 0, 1 and 5 μ M Gleevec (Figure 3C and Supplemental figure. S1). Interestingly, while the numbers of TE and EPI cells were unaffected compared to untreated embryos, the number of PrE cells was severely reduced in E2.5, early and mid blastocyst stage embryos that had been treated with Gleevec. Strikingly, the reduction of PrE cell number was of a similar magnitude in the three categories of embryos at 1 and 5 μ M concentrations (fold reduction compare to control embryos: 1.9 and 2.2 in E2.5; 1.7 and 1.9 in early E3.5; 1.7 and 1.8 in mid E3.5). Thus, at these concentrations, Gleevec exerted a comparable effect at all stages analyzed, but a treatment between E3.5 and E4.5 was sufficient to reduce the number of PrE cells. This indicates that PrE cells are sensitive to Gleevec treatment between E3.5 and E4.5, which corresponds to the period when they are already specified and become sorted to form the PrE layer on the surface of the ICM.

Increased cell death in PrE cells treated with Gleevec

To determine whether the reduction of PrE cell number upon Gleevec treatment was due to cell death, we live imaged the development of early- and mid-blastocyst stage *Pdgfra*^{+/H2B-GFP} embryos cultured with or without 5 μ M Gleevec (Figure 4A and movies 2, 3 and 4). We first observed that early-blastocyst embryos exhibited a significantly higher proportion of cell death when cultured in the presence of Gleevec (8.1 ± 0.6 dead cells, $n=7$ *versus* control 2.9 ± 0.7 dead cells, $n=10$). We next analyzed the position of GFP⁺ cells (GFP-positive) prior to their death (Figure 4A-D). As in control embryos (55% dying cells were localized inside the ICM), cell death was observed both in inner and outer GFP⁺ cells of treated embryos (51% and 48% dying cells localized inside the ICM at early and mid-blastocyst stages, respectively). These data further suggest that, as with the genetic ablation of *Pdgfra*, Gleevec treatment affects the survival of PrE cells independently of their position within the ICM.

Caspase inhibition rescues the phenotype resulting from loss of PDGF signaling

Since the absence of PDGF signaling correlates with increased apoptosis of PrE cells, we tested whether we could rescue the phenotype in mutant embryos by culturing them in presence of a pan-caspase inhibitor (Z-VAD³⁰) (Figure 5). Z-VAD treatment did not significantly affect the number of TE cell or EPI cells in wild-type, heterozygous and mutant embryos (Figure 5C, D). It also did not affect the number of PrE cells in wild-type and heterozygous embryos (Figure 5E). However in mutants, the number of PrE cells was significantly increased following Z-VAD treatment (4.8 ± 1.0 PrE cells in control *versus* 10.6 ± 1.6 in treated mutant embryos) (Figure 5E). Taken together, these data suggest that *Pdgfra*-deficient PrE cells undergo caspase-dependent apoptosis.

Manipulating PDGF activity does not affect cell sorting

Several hypotheses could explain why in the absence of PDGF signaling, PrE cells exhibited cell survival defects. We favor two, non mutually-exclusive scenarios. Firstly, PDGF may provide survival signals operating during PrE specification. Alternatively, PDGF signaling

might be involved in PrE cell sorting, and so in mutant embryos, PrE cells failing to sort consequently undergo caspase-dependent cell death as proposed previously^{14,17}.

To test these two possibilities, we first analyzed the localization of PrE cells in embryos treated with or without Gleevec. We did not observe any major differences in their localization, such that PrE cells were usually found in contact with the blastocoel cavity (Supplemental figure S1). Thus, Gleevec treatment did not affect PrE cell localization at the time of cell sorting. We next analyzed the localization of PrE cells in embryos treated with Z-VAD³⁰. We did not observe notable defects in PrE cell sorting. Taken together, these data suggest that, rather than being involved in lineage segregation, PDGF signaling provides the necessary survival signals during PrE specification.

To exclude the possibility that PDGF signaling might be involved in PrE segregation, we analyzed the localization of PrE cells in embryos that ectopically expressed the ligand PDGF-A. To do this, we co-electroporated wild-type early blastocysts with two plasmids, one driving constitutive expression of a histone H2B-mCHERRY fusion and a second driving constitutive expression of the PDGF-A ligand. We cultured electroporated embryos for 24 hours (Figure 6A). We detected mCHERRY fluorescence in most of the cohort of electroporated embryos (29 out of 32 embryos) with fluorescence restricted to trophectoderm cells (Figure 6C). We observed an average of 11.2 ± 1.8 and 9.5 ± 1.0 CHERRY-positive cells per embryo (Figure 6B). We noted that in all cases, PrE cells were in contact with the blastocoel cavity indicating that cell sorting had proceeded normally. Thus, an ectopic source of PDGF-A did not affect PrE segregation, confirming the idea that PDGF is not involved in lineage segregation.

Manipulating PDGF activity does not affect cell lineage specification

Cell fate decisions within the ICM are dependent on MAPK/ERK activity such that high levels of activity are required to promote a PrE identity, while acquisition of an EPI identity correlates with low ERK activity^{31,32}. Several lines of evidence demonstrate that this activity is controlled by FGF RTK signaling in a non-cell autonomous manner^{25,33,34}. Whether PDGF RTK signaling could also contribute to regulate MAPK/ERK activity by the time of PrE specification remains an open question.

Previous studies did not report major defects in the distribution of blastocyst cell lineages in *Pdgfra* mutant embryos²². However, we noticed that early blastocyst stage embryos cultured with 10 μ M Gleevec usually exhibited a reduced level of GATA4 (Supplemental figure S1h). This observation could suggest defects in the kinetics of PrE specification program in presence of a high dose of Gleevec, resulting in delayed development and an embryo with an overall decrease in size. We therefore determined whether increasing PDGFR α activity, by providing an excess of its cognate PDGF-A ligand, would affect EPI/PrE specification. We cultured wild-type E2.5 embryos for 2 days in the presence of 500 ng/mL PDGFA and then determined the distribution of cells within the different lineages (Supplemental figure S2 A-B). We failed to detect any significant changes in the proportion of TE (77.4 ± 3.6 cells in control *versus* 67.6 ± 3.4 in treated conditions), PrE (15.9 ± 1.7 *versus* 16.4 ± 1.3) and EPI cells (10.8 ± 1.3 *versus* 9.8 ± 1.4). Thus, the exogenous addition of PDGF-A ligand does not affect the PrE/EPI ratio within the ICM.

We next reasoned that under normal conditions a high level of FGF signaling activity may mask a possible role of the PDGF pathway in PrE specification. Since *Fgf4* mutant embryos lack a PrE endoderm layer^{24,35,36}, we compared the distribution of ICM lineages in E4.5 implanting *Pdgfra*^{+/+}, *Pdgfra*^{+/H2B-GFP} and *Pdgfra*^{H2B-GFP/H2B-GFP} blastocyst stage embryos having one or two functional *Fgf4* alleles (Supplemental figure S3). We first noted that, as we previously reported²⁴, removing one *Fgf4* functional allele significantly reduced the number of PrE cells and slightly increased the number of EPI cells. The reduction in PrE cell number in *Fgf4*^{+/-} compared to *Fgf4*^{+/+} was of the same order of magnitude in *Pdgfra*^{+/+}, *Pdgfra*^{+/H2B-GFP} and *Pdgfra*^{H2B-GFP/H2B-GFP} embryos (fold changes: -1.8 in *Pdgfra*^{+/+}, -1.5 in *Pdgfra*^{+/H2B-GFP} and -1.9 in *Pdgfra*^{H2B-GFP/H2B-GFP}). Similarly, the absence of PDGF signaling led to a comparable reduction in the number of PrE cells in *Fgf4*^{+/+} and *Fgf4*^{+/-} embryos (fold changes: -2.7 and -2.9 respectively). Taken together, these data demonstrate that PDGF is not involved in PrE specification and suggest that FGF and PDGF RTK signaling pathways exert distinct functions during PrE formation.

DISCUSSION

In this study we have investigated the role of PDGF RTK signaling during the specification and segregation of embryonic and extraembryonic lineages within the early mouse embryo. *Ex vivo*, PDGF signaling is required for XEN cell maintenance, as well as during the conversion of mouse ES cells to cXEN cells^{22,37}. *In vivo*, we previously reported a significant reduction in the number of PrE cells in implanting mutant embryos, a defect which was exacerbated when implantation was artificially delayed through induction of a period of diapause²². In the present study, we have identified an important role for PDGF signaling in PrE cell survival. We show that genetic inactivation of *Pdgfra* and pharmacological inhibition of PDGF activity specifically induce apoptosis of PrE cells. This process of programmed cell death is mediated by caspase activity, as we noted an increase in the number of Caspase-3-positive cells in *Pdgfra*-deficient embryos. We further demonstrated that inhibition of caspase activity rescued the defect associated with a reduction in PrE cell number observed in *Pdgfra* mutant embryos.

Since apoptosis has been proposed as a mechanism for the removal of cells that are not properly specified and/or positioned within the ICM at the time when EPI and PrE cells are segregating into distinct tissue layers^{14,17}, it was possible that in the absence of PDGF signaling apoptosis might affect a subset of PrE cells based on their position within the ICM. Using live imaging, we noted that both genetic and pharmacological inhibition of PDGF signaling activity affected the survival of PrE cells independently from their position within the ICM. Since Gleevec inhibits not only PDGF receptor activity, but also additional RTKs including KIT and ABL²⁹, whether these RTKs participate in early cell lineage specification remains an open question.

Using a pharmacological approach, we determined more precisely the period when PDGF signaling is critical for PrE cell survival. We first noted that the effect was similar when Gleevec was applied to E2.5 and mid E3.5 embryos. In addition, using live imaging, we observed that PrE cell death occurred earlier in the movies of Gleevec-treated mid-blastocyst stage embryos compared to treated early-blastocyst embryos. These data therefore

suggest that PDGF signaling is required for cell survival at the time when committed PrE cells segregate from EPI cells. Interestingly, a previous study used computational modeling to simulate the process of PrE/EPI segregation and noted that while cell sorting and cell position parameters increased the probability score of the model, changes in the parameter for apoptosis did not improve it¹⁷. It is therefore possible that apoptosis does not function as a key mechanism driving cell sorting *per se*, but that it might act to refine existing patterning information, thereby resulting in the formation of two spatially distinct ICM compartments, a process that we propose is dependent on PDGF signaling.

In addition to its role in cell survival, PDGF signaling has been implicated in chemotaxis and cell migration in various cellular contexts^{38–44}. It was therefore possible that the reduction of PrE cell number was a secondary consequence of an impairment of ICM cells to properly sort. Indeed, in a model where PDGF signaling could be involved in PrE cell sorting, absence of PDGF signaling would impair proper PrE segregation and, as a consequence, mislocalized PrE cells would undergo apoptosis. In early mouse development three observations argue against this being the case : (i) the presence of exogenous PDGFA proteins, or (ii) ectopic source of PDGFA ligand in TE cells do not affect PrE cell sorting, and (iii) rescued PrE cells lacking PDGF signaling by inhibition of caspase activity properly locate at the surface of the ICM. We therefore propose, that PDGF signaling is involved in PrE cell survival at the time of cell sorting, but it does not play a role in the process of cell sorting itself.

It is interesting to note that *Pdgfra* is, together with *Gata6*, an early marker of PrE identity but seems required only when ICM cells have committed to a PrE fate. As FGF RTK signaling is a prominent determinant in ICM cell fate decisions (for review, see¹⁶), it was possible that an earlier role of PDGF RTK in PrE specification could have been masked by FGF. Our results indicate that this is unlikely to be the case, since embryos cultured in presence of high doses of exogenous PDGF-A develop similarly to control embryos. In addition, we observed a similar reduction of PrE cells in embryos lacking one functional allele of *Fgf4* in wild-type and *Pdgfra* mutant embryos. Taken together, we propose that the successive steps in PrE formation (cell lineage specification and survival, sorting and expansion) are dependent on MAPK activity which in turn is controlled by FGF and PDGF RTK signaling activities respectively. It remains to be determined whether additional RTKs operate in these successive events.

We and others previously reported an increase of apoptosis at the transition from mid-to-late blastocyst stages which has been proposed to participate to the formation of two distinct EPI and PrE lineages within the ICM^{6,14,17,45}. Interestingly, *Pdgfra* is a target of GATA transcription factors and we have previously proposed that initiation and maintenance of *Pdgfra* expression might be regulated by GATA6 and GATA4 respectively^{22,46}. As such, it is tempting to speculate that failure to properly specify a PrE fate might directly affect the levels of PDGF activity leading to cell death. This mechanism would facilitate the removal not only of cells that failed to properly activate the PrE program, but also of PrE cells that are incorrectly positioned within the ICM. Therefore, it will be important to test this model in various situations where the PrE program is initiated, but fails to progress, and where committed PrE cells fail to properly sort.

CONCLUSION

In this study, we have uncovered a role for PDGF signaling in the survival of PrE cells at the time when they segregate from to the pluripotent epiblast to form a distinct compartment in contact with the blastocoel cavity. Absence of PDGF signaling activity using genetic and pharmacological approaches specifically affects the PrE lineage and induces a caspase-dependent selective apoptosis. In addition, we demonstrate that FGF and PDGF receptor tyrosine kinase signaling exert distinct roles in PrE lineage specification and survival, respectively.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

We thank Peter Besmer for Gleevec and Claire Chazaud for technical advice on embryo electroporation. We thank Sigolène Meilhac for critical reading of the manuscript. Work in AKH's laboratory is supported by the Human Frontier Science Program, National Institutes of Health (RO1-HD052115 and RO1-DK084391). JA is supported in MCT's laboratory by the European programme Marie Curie (International Incoming Fellowship, 7th European Community Framework Programme). MCT's laboratory is supported by the Institut Pasteur, the CNRS and the ANR "Laboratoire d'Excellence" programme (REVIVE, ANR-10-LABX-73-01).

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Figure 1. Increased apoptosis in blastocyst embryos lacking *Pdgfra*

Analysis of cell death in E2.5 embryos recovered one day after tamoxifen injection (A) and in E3.5 embryos (B). Embryos were recovered from *Pdgfra*^{H2B-GFP/+} intercrosses, processed for immunostaining, imaged and subsequently genotyped, as previously described²². Upper panels represent 3D-rendering of a *Pdgfra*^{H2B-GFP/H2B-GFP} embryo. Lower panels represent distribution of fragmented nuclei and Caspase-3 positive cells in *Pdgfra*^{+/+} (WT), *Pdgfra*^{H2B-GFP/+} (HET) and *Pdgfra*^{H2B-GFP/H2B-GFP} (HOM) embryos at E2.5+1 day after tamoxifen injection and E3.5. Only E3.5 blastocysts with a total cell

number ranging from 60 to 120 cells were analyzed. This corresponds to the time when ICM cells are committed towards PrE and EPI cell fates. GFP, green; active Caspase-3, red; Hoechst, blue. Scale bar: 20 μ m. Statistical Mann-Whitney tests are indicated when significant (*, $P < 0.05$; **, $P < 0.01$). Error bars indicate s.e.m. n, number of embryos analyzed.

Figure 2. Live imaging reveals increased cell death of inner PrE cells in *Pdgfra*-deficient embryos
 (A) Single frames from 3D time-lapse sequence of ICM of a *Pdgfra*^{H2B-GFP/H2B-GFP} E3.5 mid-blastocyst embryo. Cell death events occurring in GFP-positive cells are depicted either with a red (inner PrE cell) or blue arrowhead (outer PrE cell). Annotated movie sequence is provided as Movie 1. GFP, green. Scale bar: 10 μ m. (B) Numbers of cell death events in HET and HOM embryos. Statistical Mann-Whitney test is indicated when significant (***, $P < 0.001$). Error bars indicate s.e.m. n, number of analyzed embryos. (C, D) Localization of

PrE cells prior to death within ICM of HET (C) or HOM (D) embryos. IN, inside; OUT, outside; ND, not determined. Numbers indicate number of cells analyzed.

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Figure 3. Gleevec treatment affects preimplantation development

(A) Schematic representation of timeline of Gleevec treatment. (B) Single optical sections of embryos cultured at indicated concentrations of Gleevec. bf, brightfield; Hoechst, blue. Scale bar: 20 μ m, n, number of analyzed embryos. (C) Distribution of TE (green), PrE (blue) and EPI (red) cells in E2.5, early and mid E3.5 embryos cultured with or without Gleevec until E4.5. Statistical Mann-Whitney tests are indicated when significant (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). Error bars indicate s.e.m.

Figure 4. Gleevec treatment phenocopies *Pdgfra* inactivation

(A) Single frames from 3D time-lapse sequence of ICM of a *Pdgfra*^{+/H2B-GFP} mid E3.5 blastocyst embryo cultured in 5 μ M Gleevec. Cell death events occurring in GFP-positive cells are depicted either with a red (inner PrE cell), a blue (outer PrE cell) or a grey arrowhead (undetermined localization). Annotated movie sequence is provided as Movie 4. GFP, green. Scale bar: 10 μ m. (B-D) Localization of PrE cells prior to cell death within the ICM of control early E3.5 (B), Gleevec-treated early (C) and mid (D) E3.5 blastocyst

embryos. IN, inside; OUT, outside; ND, not determined. Numbers indicate number of cells analyzed.

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Figure 5. Caspase inhibition rescues the phenotype resulting from the loss of *Pdgfra*
 (A) 3D rendering of *Pdgfra*^{+/H2B-GFP} (HET) and *Pdgfra*^{H2B-GFP/H2B-GFP} (HOM) early blastocyst embryos cultured 24 hours in DMSO or 20 μ M Z-VAD. GFP, green; GATA4, red; Hoechst, blue. Scale bar: 20 μ m. (B-E) Distribution of total (B), TE (C), EPI (D) and PrE (E) cells in wild-type (WT), heterozygous (HET) and mutant (HOM) embryos cultured in DMSO or 20 μ M Z-VAD. n, number of embryos analyzed. Statistical Mann-Whitney tests are indicated when significant (*, $P < 0.05$; **, $P < 0.01$). Error bars indicate s.e.m.

Figure 6. Overexpression of PDGF-A ligand does not affect sorting of PrE cells

(A) Schematic representation of plasmid DNA electroporation. (B) Average number of mCHERRY-positive cells per embryo. n, number of electroporated embryos. Error bars indicate s.e.m. (C) 3D-rendering of blastocyst embryos co-electroporated with p*CAG::H2B-mCherry* and p*CAGGS* or p*CAG::Pdgfa* vectors. GATA4, green; mCHERRY, red; Hoechst, blue. Scale bar: 20 μ m.