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Mitogen activated protein kinase pathway-dependent effects of platelet-derived growth factor on migration of trophectoderm cells

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ABSTRACT

Successful development of the conceptus and implantation requires an intimate trophic connection between maternal uterus and conceptus mediated by local regulators including growth factors. Platelet-derived growth factor (PDGF) acts as a chemotactic factor for a variety of cell types. Current studies have determined that PDGF participates in rapid growth and development of cleavage stage embryos, but PDGF-induced effects on the growth and development of peri-implantation conceptus remains unknown. In the present study, PDGF induced phosphorylation of ERK1/2, AKT and RPS6 proteins in porcine trophectoderm (pTr) cells in a dose- and time-dependent manner. Addition of U0126 (an inhibitor of ERK1/2) or LY294002 (a PI3K inhibitor) blocked PDGF-induced effects on phosphorylation of signaling proteins. Combinations of PDGF and U0126 decreased PDGF-induced p-ERK1/2 and p-AKT1, but combinations of PDGF and LY294002 blocked only PDGF-induced AKT phosphorylation. Furthermore, PDGF significantly induced pTr cell migration and these stimulatory effects were blocked by U0126 and LY294002. Immunoreactive p-ERK1/2 and p-RPS6 proteins were abundant in pTr cells treated with PDGF, but U0126 reduced PDGF-induced p-ERK1/2 and p-RPS6 levels to basal amounts. Present study suggests that PDGF secreted into the maternal-conceptus microenvironment stimulates pTr cell migration through signal transduction cascades mediated by the ERK1/2 MAPK and AKT1 pathways.

1. Introduction

As in all eutherian mammals, the peri-implantation period of pregnancy in pigs is the most critical points in development of conceptuses (embryo and its associated extra-embryonic membranes) and implantation. There is a well-orchestrated interaction at the maternal-conceptus interface to ensure a suitable environment for implantation [1,2]. This process is especially crucial and unique in pigs that feature a non-invasive, diffuse epitheliochorial type of implantation which requires a protracted 13 day pre-attachment phase compared to implantation in other mammalian species [1,3]. During this peri-implantation period, pig conceptus undergoes dramatic morphological changes which are completely dependent on maternal uterine secretions and direct contact with the uterine luminal epithelium to obtain sufficient nutrients [4].

The maternal uterine secretions called 'histotroph' includes an astonishing amount of molecules such as hormones, nutrients, growth factors and cytokines that are secreted into the uterine lumen to provide a unique environment for growth and development of the conceptus [5–12]. Deficiencies in those factors in uterine luminal fluids can result in failure of conceptus development and implantation [7,13,14]. Emerging evidence emphasizes the importance of growth factors in the maternal-conceptus microenvironment for normal development of the conceptus and implantation. The pig uterus and/or conceptus express various of growth factors specifically during the pre-implantation and peri-implantation periods, and specific receptors for these ligands are expressed by cells of the developing conceptus to provide the synergistic environment required for endocrinological signals for establishment of pregnancy [15].

Platelet-derived growth factor (PDGF) is a mitogen, originally purified from platelets, that exerts mainly pleiotropic effects to growth, proliferation, migration and motility of cells [16–21]. PDGF consists of disulphide-bonded dimers of four different polypeptide chains denoted PDGF-A, -B, -C, and -D [22–26]. The four PDGF ligand subunits can combine as homo- or heterodimers to generate

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5 types of PDGF dimeric isoforms: PDGF-AA, PDGF-BB, PDGF-CC, PDGF-DD, and PDGF-AB [26]. These PDGFs isoforms have distinct binding specificities to different tyrosine kinase receptors consisting of dimmers of α and β subunits (PDGFR-α, and PDGFR-β) in which each α and β subunit interacts with one of the four PDGF polypeptide chains [27].

A large number of growth factors are involved in the implantation process, primarily based on their time-specific and cell-specific expression of uterine- or embryonic growth factors and their specific receptors during the peri-implantation period of pregnancy. The PDGF A chains and PDGFR-α subunit present in pre-implantation mouse and cow blastocysts have been detected at all stages of development [28,29]. And, human PDGF genes are expressed throughout the menstrual cycle, and present in greater amounts in the first trimester of pregnancy than during the menstrual cycle [30]. In cyclic and pregnant pigs, both PDGFR-α and PDGFR-β are present in the developing conceptus and in the endometrium, and their expression levels are high during early pregnancy, indicating steroid hormone-dependent expression of PDGFR during early pregnancy [31].

In functional studies, emerging evidence suggests that PDGF improves the development of *in vitro* cultured embryos during the early cleavage stages as it is an important activator of cell division. In mice, PDGF improves trophoblast outgrowth in blastocysts whereas PDGF mutant mouse embryos injected with mRNA encoding mutant PDGF polypeptides to suppress endogenous PDGF cease cellular divisions to blastocysts [28,29,32]. Larson et al. [33] also found that PDGF stimulates development of cleavage-stage bovine embryos by shortening the time to reach the blastocyst stage *in vitro*, while not increasing number of cells in bovine blastocysts [34]. Endometrial cell-derived human PDGF also stimulates proliferation of endometrial cells via an autocrine mechanism, and also act in a paracrine manner on neighboring epithelial cells and the developing conceptus [35]. Only a limited amount of work has been carried out to assess effects of PDGF on developing porcine conceptuses or the reproductive tract. In pigs, PDGF induces a transitory increase in protein synthesis by isolated embryonic disks [36]. Based on detection of PDGF ligand and pattern of expression of its receptor, PDGF is probably implicated in development and implantation of porcine conceptuses as a mediator of the conceptus-maternal dialog. However, PDGF has not been as intensively investigated as the other growth factors such as epidermal growth factor (EGF).

Information is lacking on functions of PDGF in development of conceptuses during the peri-implantation period of pregnancy although possible PDGF signaling pathways have been reported. But, the cell signaling mechanisms activated by PDGF in the porcine conceptus are unknown. This study tested the hypothesis that during early pregnancy in pigs, PDGF of uterine- or conceptus-origin plays a critical role in peri-implantation events by regulating proliferation and migration of porcine trophectoderm (pTr) cells. Therefore, the aims of this study were to investigate 1) inducible effects of PDGF on cell signaling cascades in pTr cells; and 2) functional effects of PDGF on migration and/or proliferation of pTr cells representative of those of pig conceptuses during early pregnancy.

2. Materials and methods

2.1. Experimental animals and cell culture

The porcine trophectoderm (pTr) cell line, established using trophectoderm cells from Day 12 pregnant gilts, were cultured and used in the present *in vitro* studies as described previously [37]. Monolayer cultures of pTr cells were grown in DME/F12 1:1 medium containing 10% FBS. Cells were serum starved for 24 h, and then treated with recombinant porcine PDGF (R&D systems, Minneapolis, MN).

2.2. Western blot analyses

Proteins separated by SDS-PAGE were transferred to nitrocellulose. Blots were developed using enhanced chemiluminescence detection (SuperSignal West Pico, Rockford, IL) and quantified by measuring the light intensity from correctly sized bands using a ChemiDoc EQ system (Bio-Rad, Hercules, CA).

2.3. Migration assay

The serum starved pTr cells (10^5 cells) were seeded on 8-μm pore Transwell inserts (Corning, Inc., Corning, NY) and treatments added to each well for 12 h. For evaluation of cells that migrated onto the lower surface, inserts were fixed in methanol for 10 min. The transwell membranes were then removed, placed on a glass slide with cells facing up, and overlaid with prolong antifade mounting reagent with DAPI (Invitrogen Molecular Probes, Eugene, OR). Migrated cells were counted systematically in five non-overlapping locations using a microscope.

2.4. Proliferation assay

Proliferation assays were conducted using a Cell Proliferation ELISA, BrdU kit (Roche, Basel, Switzerland) according to the manufacturer's recommendations. After 36 h of incubation with treatments, 10 μM BrdU was added for an additional 8 h. After labeling of cells with BrdU and methanol-fixation, cells were incubated with anti-BrdU-POD working solution and the values of the reaction product were quantified by measuring the absorbance at 370 nm and 492 nm (reference wavelength) using an ELISA reader (Bio-Rad, Hercules, CA).

2.5. Immunofluorescence

Serum starved-pTr cells seeded onto Lab-Tek chamber slides (Nalge Nunc International, Rochester, NY) were treated with PDGF and U0126, a highly selective inhibitor of both ERK1 and ERK2. After methanol-fixation, immunofluorescence staining was performed using a rabbit anti-human polyclonal antibody against p-ERK1/2 or p-RPS6. Cells were then incubated with Alexa Fluor 488 goat anti-rabbit IgG secondary antibody (Invitrogen, Carlsbad, CA). Slides were overlaid with prolong antifade mounting reagent with DAPI and images were captured using a Zeiss confocal microscope LSM710 (Carl Zeiss Microimaging, Thornwood, NY).

2.6. Statistical analyses

All quantitative data are presented as least-square means (LSMs) with SEs. A P value less than or equal to 0.05 was considered significant. Western blot data are presented in the figures as measurements of abundance of phosphorylated proteins relative to total proteins.

3. Results and discussion

3.1. PDGF-induced pTr cell migration and cell signal transduction pathways in pTr cells

To determine whether PDGF has dose-dependent stimulatory effects on migration and/-or proliferation of pTr cells, *in vitro* cultured pTr cells were incubated with different concentrations of

PDGF (0, 0.1, 1, 20, 50 ng/ml). PDGF at 1, 20 or 50 ng/ml stimulated increases in pTr cell migration of approximately 160% ($P < 0.05$), 209% ($P < 0.01$) and 211% ($P < 0.01$), respectively (Fig. 1A). On the other hand, no significant effects of PDGF on proliferation of pTr cells were detected at the concentrations tested, indicating that PDGF is not likely to be involved in proliferation of porcine trophectoderm during peri-implantation period of pregnancy (Fig. 1B). Since PDGF increased cell migration, we investigated PDGF-induced cell signaling pathway(s) using the same specific culture conditions. Although PDGF receptors are present on cells of porcine blastocysts during the time of elongation, knowledge of intracellular signaling pathways induced by PDGF-PDGFR is limited [38]. Western blotting showed that PDGF induced phosphorylation of ERK1/2, AKT and RPS6 proteins in pTr cells in a dose-dependent manner (Fig. 1C). Based on results of the dose-response experiments, 20 ng/ml PDGF was used in all experiments in the present study. We next determined levels of phosphorylated (p)-proteins involved in PDGF-mediated cell signal transduction in a time-dependent manner. In pTr cells treated with PDGF, the abundance of p-ERK1/2 proteins increased rapidly by 2.4-fold ($P < 0.001$) within 15 min compared to basal values (Fig. 1D). The abundance of p-ERK1/3 remained higher than basal levels to 60 min post-treatment and then decreased gradually as PDGF-treatment time increased. The abundance of p-AKT, compared to control value, was approximately 2.5-fold ($P < 0.01$) higher in pTr cells treated with PDGF for 15 min and 3.0-fold greater ($P < 0.001$) at 30 min post-treatment, and then returned to basal levels at 60 min post-treatment (Fig. 1E). The abundance of p-RPS6 proteins increased by 3.0-fold ($P < 0.01$) within 15 min, 3.4-fold ($P < 0.001$) at 60 min post-treatment and remained higher than basal levels to 120 min post-treatment (Fig. 1F). These results suggest that PDGF activates various cell signaling pathways including ERK1/2 MAPK and AKT signal transduction in pTr cells, and ERK1/2 and AKT dependent activation of RPS6 likely results in downstream signaling for mRNA translation and protein synthesis required for cell migration.

3.2. Inhibition of PDGF-induced cell signal transduction by blocking ERK1/2 MAPK and AKT signaling pathway in pTr cells

To ensure effects of PDGF on activation of ERK1/2 MAPK cell signal transduction in pTr cells, they were pre-incubated with different concentrations of a pharmacological inhibitor of ERK1/2 MAPK activity (0.1, 1, 10 and 20 μM U0126) 1 h prior to treatment with PDGF (Fig. 2A–C). Stimulatory effects of PDGF on induction of ERK1/2 phosphorylation were blocked by increasing doses of U0126 ($P < 0.05$ or $P < 0.01$). To investigate whether P90RSK and/-or RPS6 proteins were down-stream targets regulated by the PDGF-induced ERK1/2 MAPK cell signaling cascade, we measured abundances of p-P90RSK and p-RPS6 proteins in pTr cells treated with the combination of U0126 and PDGF. PDGF induced phosphorylation of P90RSK by 2.6-fold ($P < 0.05$) which was blocked in the presence of 10 or 20 μM U0126 ($P < 0.01$). Similarly p-RPS6 proteins were present at low basal levels in ERK1/2-blocked pTr cells regardless of PDGF presence as compared to the PDGF-treated group ($P < 0.01$). As illustrated in Fig. 2D–F, we determined effects of PDGF on activation of AKT cell signal transduction in serum starved pTr cells pre-incubated with different concentrations of a pharmacological inhibitor of AKT activity (0.1, 1, 10 and 20 μM

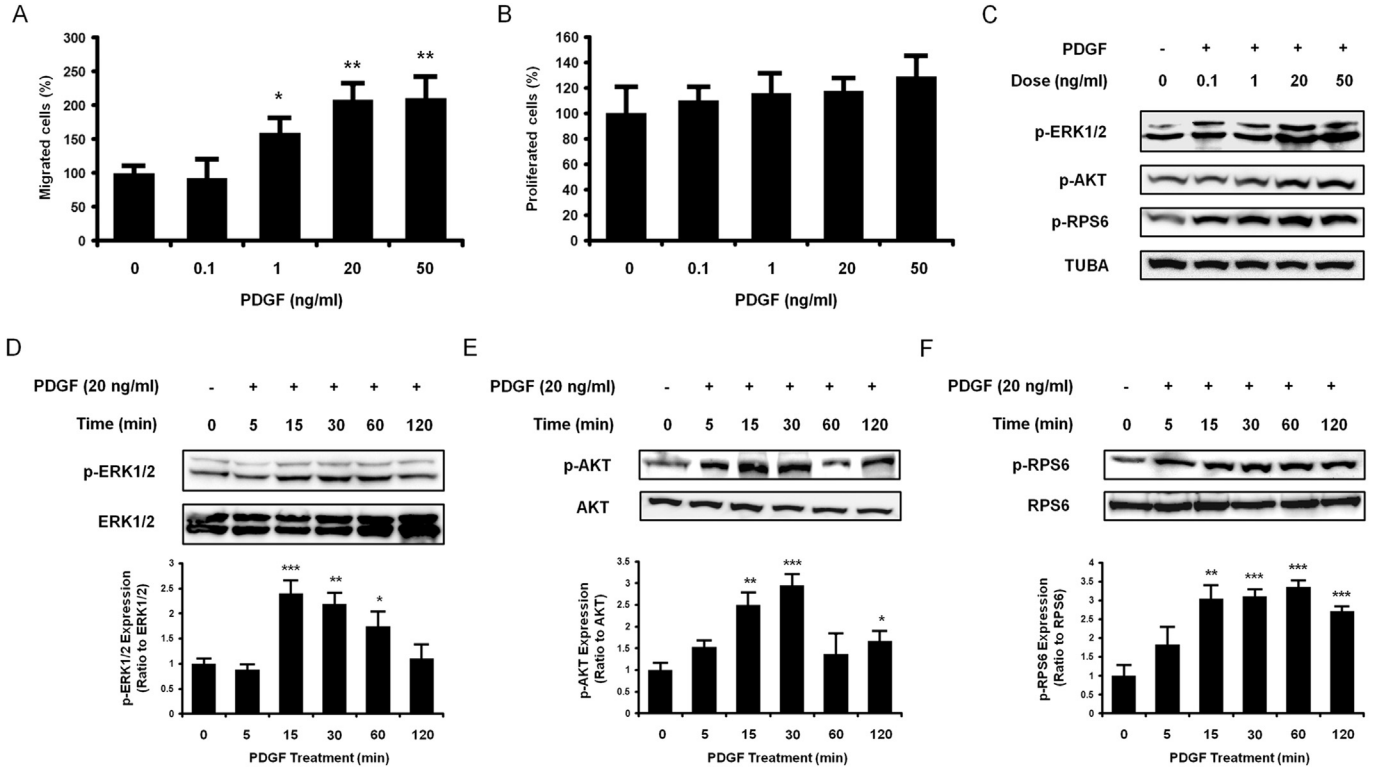

Fig. 1. PDGF stimulates migration of pTr cells and cell signal transduction in a dose-and time-dependent manner. [A] Dose-dependent effects of PDGF on migration of pTr cells *in vitro*. The serum-starved pTr cells were seeded on 8-μm pore Transwell inserts and treated with different doses (0, 0.1, 1, 20 or 50 ng/ml) of PDGF for 12 h. [B] Dose-dependent effects of PDGF on proliferation of pTr cells *in vitro*. Serum-starved 50% confluent pTr cells were treated with different dose of PDGF for 36 h and then re-incubated for an additional 8 h with medium containing 10 μM BrdU. [C] Western blot analyses of dose-dependent effects of PDGF on activation of cell signaling proteins. Serum-starved pTr cells were treated with different dose of PDGF for 30 min. [D] Western blot analyses detected activation of ERK1/2, or [E] AKT, or [F] RPS6 proteins between 0 and 120 min after treatment of pTr cells with PDGF. Serum-starved pTr cells were incubated for the times tested (0, 5, 15, 30, 60 or 120 min) with 20 ng/ml PDGF in the medium. The bars represent the relative abundance of phosphorylated proteins. The asterisks denote statistically significant differences (***$P < 0.001$, **$P < 0.01$ and *$P < 0.05$).

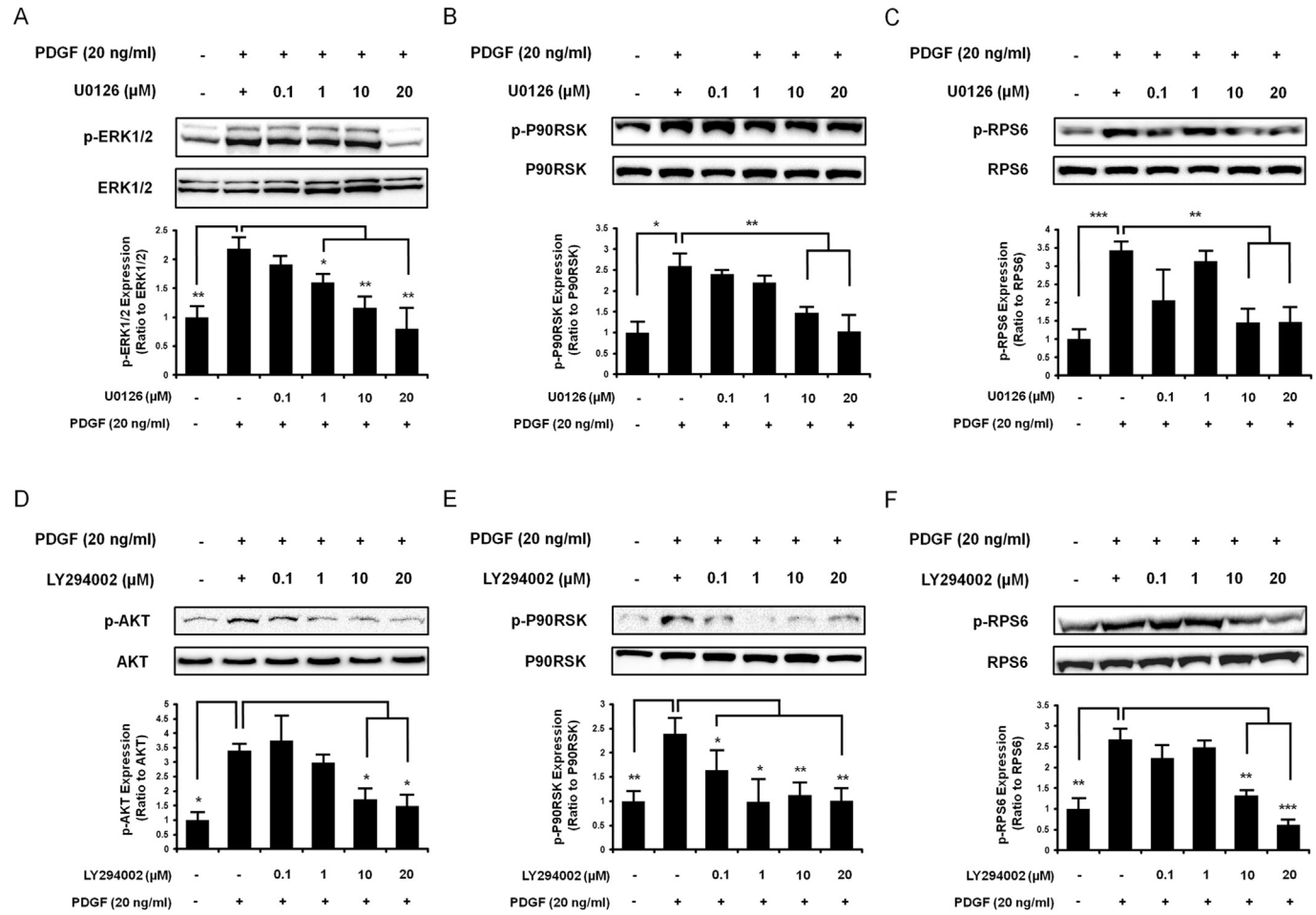

Fig. 2. PDGF activates P90RSK and RPS6 proteins through ERK1/2 MAPK and AKT cell signaling pathways in pTr cells. [A] Western blot analyses of inhibition of PDGF-induced ERK1/2, or [B] P90RSK, or [C] RPS6 phosphorylation in pTr cells by different concentrations of an ERK1/2 specific pharmacological inhibitor (U0126). Serum starved pTr cells were pretreated with different concentrations of U0126 (0.1, 1, 10 or 20 μM) for 1 h and then stimulated with 20 ng/ml PDGF for an additional 30 min. [D] Western blot analyses of inhibition of PDGF-induced AKT, or [E] P90RSK, or [F] RPS6 phosphorylation in pTr cells by different concentrations of AKT specific pharmacological inhibitor (LY294002). Serum starved pTr cells were pretreated with different concentrations of LY294002 (0.1, 1, 10 or 20 μM) for 1 h and then stimulated with 20 ng/ml PDGF for an additional 30 min. The bars presented in the bottom graph represent the relative abundance of phosphorylated proteins. The asterisks denote statistically significant differences (***$P < 0.001$, **$P < 0.01$, and *$P < 0.05$).

LY294002) for 1 h prior to treatment with PDGF. Stimulatory effects of PDGF on induction of AKT phosphorylation were completely inhibited in the presence of 10 or 20 μM U0126 ($P < 0.05$). To investigate whether P90RSK and/-or RPS6 proteins were also regulated by PDGF-induced AKT cell signaling, we measured abundances of p-P90RSK and p-RPS6 proteins in pTr cells treated with the combination of LY294002 and PDGF. PDGF induced phosphorylation of P90RSK by 2.4-fold ($P < 0.01$) as compared to non-treated pTr cells, and this effect was significantly decreased in the presence of all concentrations LY294002 tested ($P < 0.05$ or $P < 0.01$). Additionally, the abundance of PDGF-induced p-RPS6 proteins was significantly reduced by increasing doses of LY294002 ($P < 0.01$ or $P < 0.001$). These results indicate that ERK1/2 MAPK and AKT1 cell signaling is activated by PDGF, and that P90RSK and RPS6 proteins are common downstream targets for those two signaling pathways.

3.3. Stimulatory effect of PDGF on pTr cell migration via ERK1/2 MAPK-AKT signaling pathway

We examined whether PDGF-induced ERK1/2 MAPK pathway and PDGF-induced AKT pathway interact with each other through cross-linking. As compared to naïve pTr cells, treatment of pTr cells with PDGF in the absence of any inhibitors increased p-AKT levels ($P < 0.01$; Fig. 3A). But in the absence of LY294002, treatment of pTr cells with U0126 blocked PDGF induced activation of p-AKT proteins ($P < 0.05$). Phosphorylated-AKT levels were below to basal level in the presence of LY294002. As expected, treatment of pTr cells with PDGF only stimulated ERK1/2 phosphorylation ($P < 0.01$; Fig. 3B). Additional LY294002 treatment had no inhibitory effect on activation of ERK1/2 proteins in response to PDGF. PDGF-induced p-ERK1/2 levels were only blocked in the presence of U0126 ($P < 0.01$), irrespective of LY294002 presence. These results indicate that stimulatory effects of PDGF to activate the AKT cell signaling pathway are enhanced by activation of ERK1/2 cell signaling. On the other hand, PDGF activation of the ERK1/2 MAPK pathway is not affected by status of activation of the AKT pathway. As shown in Fig. 3C and D, PDGF stimulated phosphorylation of P90RSK and RPS6 proteins, and both U0126 and LY294002 blocked those inducible effects of PDGF. Next, we confirmed whether PDGF's functional effects require the ERK1/2 MAPK-AKT signaling pathway (Fig. 3E). As compared to naïve pTr cells, PDGF increased pTr cell migration rate by approximately 203% ($P < 0.01$); however, this stimulatory effect of PDGF was reduced to 140% ($P < 0.05$) or 122%

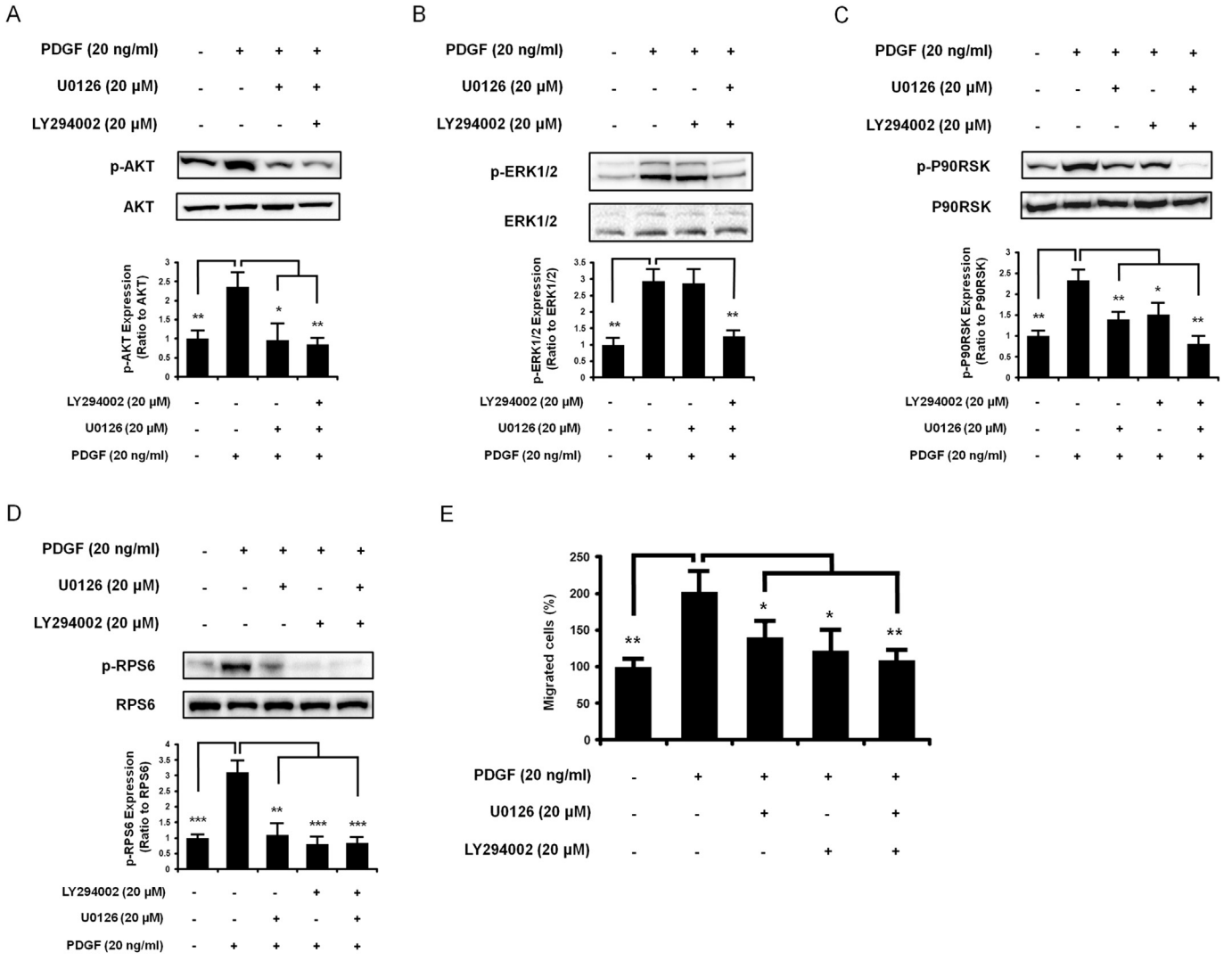

Fig. 3. PDGF induces pTr cell migration through ERK1/2 MAPK-AKT-P90RSK-RPS6 cell signaling pathways in pTr cells. [A] Blocking PDGF-induced AKT, or [B] ERK1/2, or [C] P90RSK, or [D] RPS6 phosphorylation in pTr cells by U0126 and LY294002 combinations. The serum-starved pTr cells were pretreated with 20 μM U0126 or 20 μM LY294002 or their combination for 1 h and then stimulated with 20 ng/ml PDGF for an additional 30 min. [E] Blocking stimulatory effects of PDGF on pTr cell migration by ERK1/2 MAPK-AKT pathway inhibitor. The serum-starved pTr cells were treated with 20 ng/ml PDGF, 20 μM U0126, 20 μM LY294002 or their combination for 12 h. The bars presented in the bottom graph represent the relative abundance of phosphorylated protein. The asterisks denote statistically significant differences (***$P < 0.001$, **$P < 0.01$, and *$P < 0.05$).

($P < 0.05$) by blocking ERK1/2 or AKT signaling pathway, respectively. The number of cells that migrated was not significantly different between the naïve cells, cells treated with both inhibitors, and the PDGF treated cells. Taken together, these results suggest that PDGF induces migration of pTr cells via activation of the ERK1/2-AKT-P90RSK-RPS6 pathway.

3.4. Immunocytochemical localization of p-ERK1/2 and p-RPS6 proteins activated by PDGF-induced ERK1/2 MAPK cell signaling in pTr cells

Immunofluorescence analyses detected phosphorylated-ERK1/2 proteins at low basal levels in nuclei and cytoplasm of naïve pTr cells (Fig. 4A; left panel). But pTr cells treated with PDGF exhibited abundant amounts of immunoreactive p-ERK1/2 proteins in nuclei and cytoplasm (middle panel). As described previously, those PDGF-induced increases of immunoreactive p-ERK1/2 were markedly reduced in the presence of U0126 (right panel). As shown in Fig. 4B, p-RPS6 proteins were localized at basal levels in nuclei and cytoplasm of naïve pTr cells (left panel), whereas immunoreactive p-RPS6 proteins were very abundant in the cytoplasm of PDGF-treated pTr cells (middle panel). This PDGF-induced immunoreactive p-RPS6 decreased in the presence of U0126 (right panel). Many growth factors activate cytoplasmic RPS6 proteins which stimulate mRNA translation [39], and in the present study, RPS6 proteins in the cytoplasm were also activated by PDGF-ERK1/2 MAPK cell signaling in pTr cells.

4. Conclusion

Results of this study are the first to indicate that PDGF activates P90RSK-RPS6 via PI3K-AKT and ERK1/2 MAPK cell signaling pathways in pTr cells, and that PDGF-induced ERK1/2 MAPK signal transduction cascades activate AKT1 to transduce PDGF signals. Further, we found that these PDGF-stimulated signaling pathways induce migration of pTr cells during the peri-implantation period. In conclusion, overall results of the present study provide new evidence for important roles of PDGF in development of the peri-implantation porcine conceptus and how signaling events regulate it. Further understanding of intracellular processes in PDGF-

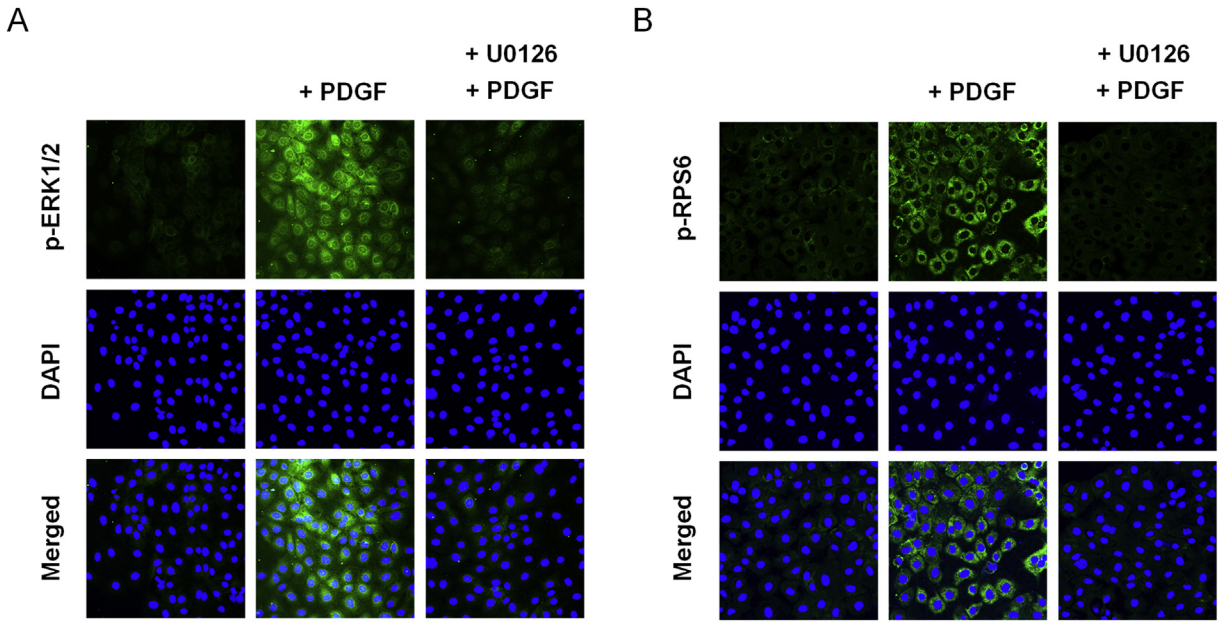

Fig. 4. Effects of blocking PDGF-induced increase of p-ERK1/2 and p-RPS6 proteins in pTr cells treated with U0126 (ERK1/2 inhibitor) were determined by immunofluorescence microscopy. [A] Immunocytochemical localization of PDGF-induced p-ERK1/2 proteins in *in vitro* cultured pTr cells. The serum starved pTr cells were incubated with 20 ng/ml PDGF for 30 min (middle panel) or pre-incubated with 20 μM U0126 for 1 h prior to treatment with PDGF (right panel). After treatment, immunofluorescence staining was performed using anti-p-ERK1/2 first antibody and Alexa Fluor 488 secondary antibody. [B] Immunocytochemical localization of PDGF-induced p-RPS6 proteins in *in vitro* cultured pTr cells. After processing on cells in specific treatments, immunofluorescence staining was performed using anti-p-RPS6 first antibody and Alexa Fluor 488 secondary antibody. Cell nuclei were stained with DAPI (blue). All images were captured at 20X objective magnification. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

mediated conceptus development will ultimately identify mechanisms responsible for failure of conceptus development and implantation during early pregnancy.

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