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Platelet-Derived Growth Factor BB Mimics Serum-Induced Dispersal of Pancreatic Epithelial Cell Clusters

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Abstract

We showed previously that proliferating human islet-derived de-differentiated cells (DIDs) exhibit many characteristics of mesenchymal stem cells. Dispersed DIDs can be induced by serum deprivation to undergo mesenchymal-to-epithelial transition and aggregate into epithelial cell clusters (ECCs). Conversely, ECCs can be induced to disperse and undergo epithelial-to-mesenchymal transition (EMT) by re-addition of mammalian sera. In this study, we show that platelet-derived growth factor BB (PDGF-BB) mimics and mediates serum-induced ECCs' dispersal accompanied by accumulation of cytoplasmic β -catenin and a decrease in the levels of insulin and glucagon mRNAs. Moreover, we show that PDGF-BB-induced dispersal of ECCs is a more general phenomenon that occurs also with bone marrow mesenchymal stem cells (BM-MSCs) and dermal fibroblasts (DFs). In DIDs, BM-MSCs, and DFs, PDGF decreased the levels of DKK1 mRNA, suggesting involvement of the Wnt signaling pathway. PDGF-BB stimulated a significant increase in S473 phosphorylation of Akt and the PI3K specific inhibitor (PIP828) partially inhibited PDGF-BB-induced ECC dispersal. Lastly, the PDGF-receptor (PDGF-R) antagonist JNJ-10198409 inhibited both PDGF-BB—and serum-induced ECC dispersal. Epidermal growth factor (EGF), which shares most of the PDGF signaling pathway, did not induce dispersal and only weakly stimulated Akt phosphorylation. Our data suggest that PDGF-BB mediates serum-induced DIDs dispersal, correlated with the activation of the PI3K-Akt pathway.

A major effort currently underway to treat patients with Type 1 diabetes is directed at the generation of large numbers of insulin-producing cells in vitro from embryonic (Lumelsky et al., 2001; Shi et al., 2005; Jiang et al., 2007; Eshpeter et al., 2008; Mfopou et al., 2010; Higuchi et al., 2011; Jiang et al., 2011), induced pluripotent (Tateishi et al., 2008; Alipio et

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Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web-site.

al., 2010; Thatava et al., 2011) or adult stem cells, adult pancreas (Gu et al., 2002; Gershengorn et al., 2004; Eberhardt et al., 2006; Ouziel-Yahalom et al., 2006; Gallo et al., 2007; Joglekar et al., 2009; Carlotti et al., 2010; Li et al., 2010; Limbert et al., 2010; Noguchi et al., 2010; Smukler et al., 2011), liver cells (Horb et al., 2003; Tuch et al., 2003; Zalzman et al., 2005; Fodor et al., 2007; Kim et al., 2007a; Vorobeychik et al., 2008; Aviv et al., 2009; Yechoor et al., 2009), or other sources, including mesenchymal stromal/stem cells (MSCs) (Timperet al., 2006; Karnieli et al., 2007; Milanesi et al., 2011; Phadnis et al., 2011). We have previously reported that mesenchymal-like cells derived from adult human islets can be expanded and induced to differentiate into insulin-expressing β -like cells, acquiring an epithelial phenotype in vitro (Davani et al., 2007). Maintenance of human islet-derived de-differentiated cells (DIDs) in a defined serum-free medium (SFM) leads to mesenchymal-to-epithelial transition (MET) and aggregation of plastic-adherent cells into islet-like epithelial cell clusters (ECCs) (Gershengorn et al., 2004). Although DID ECCs, when transplanted under the kidney capsules of NOD-SCID mice, may mature into functional cells that secrete human C-peptide in response to glucose (Gershengorn et al., 2004; Davaniet al., 2007), the success of this procedure is often unpredictable. Analysis of xenografts in unsuccessful implants often reveals a mass of undifferentiated, mesenchymal-like cells, suggesting that the implanted epithelial cells undergo reversal to a mesenchymal phenotype in vivo (Davaniet al., 2009). We have recently reported that DID ECCs cultured in mammalian sera undergo epithelial-to-mesenchymal transition (EMT) resulting in cell migration out of ECCs to form a monolayer (Davani et al., 2009). We suggested that the EMT observed in vitro may mimic a similar process in vivo, thus explaining graft failures accompanied by the presence of cells expressing mesenchymal markers.

In embryonic development, platelet-derived growth factor (PDGF) is required for the spreading of various populations of cells, including oligodendrocyte precursors in the spinal cord (Finzsch et al., 2008), neural crest mesenchymal cells (Richarte et al., 2007), and pericytes (Abramsson et al., 2007). Analysis of various gene-targeted and transgenic models for PDGFs and PDGF receptors (PDGFRs) demonstrates the importance of PDGFs as epithelium-derived factors promoting proliferation and spreading of PDGFRs-expressing mesenchymal cells in a paracrine fashion as in the case of the lung epithelium (Hirota et al., 2011), developing skin (Utoh et al., 2003), tubular epithelial cells during testicular development (Gnessiet al., 2000), and in vascular endothelium (Zhang et al., 2009).

In the present report we demonstrate that PDGF-BB alone mimics serum-induced DID ECCs' dispersal and suggest that it is a mediator of serum-induced EMT. This action of PDGF-BB appears to be correlated with the activation the PI3K/Akt cascade.

Materials and Methods

Cells

DIDs (formerly called hIPCs) were derived from donated human islets as described elsewhere (Hardikar et al., 2003; Gershengorn et al., 2004). Human dermal fibroblasts were from the ATCC (Manassas, VA). Passage 2 bone marrow-derived MSCs (BMMSCs) were purchased from Cambrex Bio Science (Walkersville, MD).

Media and supplements.—DMEM, CMRL 1066, antibiotics, Hank's balanced salt solution, trypsin solutions (0.05%), and heat-inactivated fetal bovine serum were all purchased from Biological Industries (Beit-Haemek, Israel). Basal growth medium for bone-marrow MSCs was obtained from Cambrex Bio Science. Fatty acid-free bovine serum albumin (BSA) was from Gemini Bioproducts (West Sacramento, CA). ITS-A was from Invitrogen (Carlsbad, CA).

Antibodies.—Akt(pan)-HRP and phosphorylated Akt(S473)-HRP conjugated antibodies were purchased from Cell Signaling Technology (Danvers, MA).

Other materials.—JNJ-10198409 and Sigmacote[®] were obtained from Sigma–Aldrich (St. Louis, MO). Human recombinant PDGF-BB was purchased from ProSpec-Tany (Ness-Ziona, Israel). PIP828 was from Tocris (Ellisville, MO). Proteome Profiler Array kit was from R&D Systems (Minneapolis, MN). 24-well Transwell[®] dishes were from BD Falcon (Bedford, MA). SuperSignal West Pico Chemiluminescence substrate was from Pierce Chemical (Rockford, IL). Superfrostplus glass slides were from Menzel (Brunschweig, Germany). All other chemicals were of analytical quality.

Cell culture

DIDs were maintained in 150-mm tissue culture dishes in growth medium (SCM) composed of CMRL-1066 supplemented with 10% foetal bovine serum (FBS), 2 mM L-glutamine, penicillin (50 U/ml), and streptomycin (50 µg/ml). Cells were passaged every 3–4 days at 80–90% confluence. Bone marrow-derived MSCs were maintained in appropriate growth media according to the manufacturer's protocols (Cambrex). Dermal fibroblasts were grown in DMEM with 10% FBS, supplemented with 2 mM L-glutamine, penicillin (50 U/ml), and streptomycin (50 µg/ml). Twice a week cells were harvested and re-seeded in SCM at 25% confluence. For cluster formation, 25–50% confluent cells were washed X3 with Hank's solution and cultured in SFM (DMEM/F12 1:1, 1% BSA, L-glutamine and antibiotics).

Clusters of either DIDs, BM-MSCs, or dermal fibroblasts were formed following seeding cells at $3.5 \times 10^4/\text{cm}^2$ in induction media (SFM, CMRL-1066 for DIDs, DMEM for dermal fibroblast, or mesenchymal stem cell basal medium for BM-MSCs) supplemented with 2 mM L-glutamine, penicillin (50 U/ml), and streptomycin (50 µg/ml), ITS-A supplement and 1% BSA.

Mitomycin-C treatment

DIDs were incubated with 10 µg/ml mitomycin C for 4 h at 37°C. Cells were trypsinized and frozen. After thawing, mitomycin C-treated cells were maintained in SCM and incubated for 96 h in SFM to form ECCs before being treated. We have previously shown, using cell counting and BrdU methods, that Mitomycin C-treated cells do not proliferate (Davani et al., 2009).

Clusters dispersal

DID ECCs formed for 96 h were collected by gentle aspiration and size selected (40–100 µm) before seeding in 24-well plate in SFM, supplemented with growth factors as indicated.

Migration assay

Migration assays were performed in 24-well Transwell® plates with 8-mm-apertures. Overnight serum-starved DIDs (30,000) were added to the upper chamber of a Transwell® and migration medium with chemotactic factors was added to the lower chamber. After 6 h incubation at 37°, 5% CO₂, the media were removed from the upper chambers and the upper surfaces of the filters were carefully wiped with a cotton-swab. Inserts were transferred to a second 24-well plate containing 4% paraformaldehyde in DPBS and stored overnight at 4°. Inserts were washed three times in DPBS and placed in 1.5% crystal violet in DPBS for 1 h at 37°. The cells that migrated to the undersurface of the filters were imaged by Orca camera mounted on Zeiss 200 Axiovert microscope and counted. When putative chemotactic factors were added to both upper and lower chamber, the results represented migration due to chemokinesis.

Cell proliferation

Monolayer DIDS were seeded in 6-well plate at 30% confluence in SCM for 6 h, washed (X3) with Hanks' balanced salt solution and cultured in SFM or 1% human serum and FBS, PDGF or epidermal growth factor (EGF) at the indicated concentrations. The 1% human serum does not induce proliferation, but is required for cell adhesion and survival (not shown, see Fig. S1). Cells were trypsinized and counted by Vi-Cell at the indicated times.

Morphometry

Universal Imaging MetaMorph 6.1 software was used to acquire digital micrographs and analyze the results. Greyscale frames were thresholded, binarized, and the total pixel area of the objects was measured by the Integrated Morphometric Analysis module of the software. The analysis of ECCs' dispersal was performed as follows: The pixel area occupied by the ECCs and the dispersed cells was determined. This area increased with ECCs dispersal and the net increase was obtained by subtracting the area of the original ECCs. The net pixel area of ECCs treated with 5% FBS (usually full dispersal) was set as 100% and the various treatments were normalized relative to the FBS-treated ECCs. The percent inhibitory effects of JNJ-10198409, PIP828 and PDGF-R α/β antibodies were calculated according to the equation:

$$\text{Inhibition(\%)} = 100 \times (1 - (\text{treated pixel area})/(\text{control pixel area}))$$

Immunostaining

ECCs formed for 24 h were cultured on super-frost-plus glass slides.

Slides were fixed with 4% formaldehyde. Whereas ECCs cultured with EGF or in SFM did not adhere, clusters were fixed with 25% ethanol, paraffin embedded and sections were prepared. Immunocytochemical staining was performed on BenchMark XT (Ventana Medical Systems, Tucson, AZ) using a standard protocol (60 min pretreatment with CC1, blocking with I-View-Inhibitor (40 min, 37°C), primary Ab and detection with SA-HRP/DAB).

Confocal immunofluorescence microscopy

Cells were fixed with fresh 4% paraformaldehyde in phosphate-buffered saline, and permeabilized with 0.15% Triton x100 in a blocking buffer containing 4% normal donkey serum. Cells were incubated with 1:1,000 mouse anti- β -catenin (BD Biosciences, San Jose, CA) in blocking buffer for 1 h at 37°C, washed three times with PBS, and then incubated with 1:2,000 Alexa 488 goat anti mouse antibody (Life Technologies, Grand Island, NY) in blocking buffer for 1 h at room temperature. Coverslips were washed, mounted in Mowiol® (Sigma–Aldrich) with DAPI (Sigma–Aldrich) and images captured on a Zeiss META NLO confocal microscope. Figures are 3D projection of z-stacks, 1 μ M interval-6 slices each of the DIDs cluster.

Western blotting

Untreated or EGF- or PDGF-BB-treated cells were lysed directly in plates with R&D lysis buffer for 5 min (in accordance with the manufacturer's instructions). Protein extracts (15 μ g) were solubilized in sample buffer, separated on 10% SDS–PAGE and electroblotted onto nitrocellulose membranes (BioRad, Hercules, CA). After transfer, membranes were blocked for 1 h (5% BSA, 0.1% Tween-20 in Tris-buffered saline), incubated overnight (4°C) on rocking plate with primary rabbit antibody (HRP conjugated, 1:1,000) against phospho-Akt (S473). After detection, membranes were incubated for 1 h with 5% BSA, 0.1% Tween-20 in Tris-buffered saline, followed by 1 h incubation with blocking buffer and incubated overnight at 4°C with primary Akt(pan) mouse pAb (HRP conjugated, 1:1,000). For actin immunoblotting, membranes were stripped with a buffer containing 100 mM 2-mercaptoethanol, 0.1 M glycine and 2% SDS for 1 h at room temperature. After washing, the membranes were blocked and reprobed with primary anti-actin mouse mAb antibody (1:10,000) for 30 min and followed by incubation with the appropriate HRP-conjugated secondary antibody (1:10,000, 1 h). For detection, membranes were incubated in ECL for 5 min, and developed films were scanned and analyzed with TINA 2.07d software.

Phospho-kinase antibody array (R&D Systems) experiments were performed on cells lysates as described in the manufacturer's manual.

RNA extraction and quantitative reverse transcription-polymerase chain reaction (qRT-PCR) determination

RNA from approximately 0.5×10^6 cells was extracted using the RNeasy mini kit (Qiagen, Germantown, MD). cDNA was generated from 1 μ g total RNA using the ABI High capacity reverse transcription kit. Quantitative PCR was performed on cDNAs. All reactions were conducted in 96 well plates in 25 μ l with the Universal PCR Master Mix (Life Technologies), and Primers/Probe mix. All primers and probes are from Applied Biosystems Assay-on-Demand.

Statistics

All assays were performed at least in two independent experiments, each assayed in duplicates or triplicates. Data are presented as mean $\pm$ SE. Students' *t*-test was used and differences were considered significant when $P < 0.05$.

Results

We reported that addition of sera to DID ECCs formed in SFM induces EMT, manifested morphologically by cell migration out of ECCs to disperse and form a monolayer. We proposed that this in vitro phenomenon may mimic the in vivo failure of epithelial cell xenografts (Davani et al., 2009). To identify the factor(s) responsible for serum-induced dispersal, we screened a number of potential growth factors including, fibroblast growth factor 2, transforming growth factor β , insulin-like growth factor 1, and vascular endothelial growth factor. None of the above factors affected ECC morphology (not shown). However, PDGF-BB alone mimicked the effect of serum. EGF, which shares many of the PDGF signaling pathways, had a marginal effect (not shown). PDGF-BB dose-dependently induced cell migration out of ECCs (Fig. 1A and B) to form a monolayer of fibroblast-like cells resembling the original DIDs in morphology. Partial ECC dispersal was apparent after 24 h (not shown) and full dispersal after 96 h of PDGF-BB stimulation (Fig. 1B), resembling the effect of FBS (Fig. 1B-insert). There was an absolute requirement for the presence of PDGF throughout the de-differentiation process. PDGF washout at any time before full dispersal arrested the process (not shown).

We found a modest effect of PDGF-BB (50% increase in cell number after 4 days of culture) on DID proliferation (Fig. S1). Hence, the possibility that the observed PDGF-BB-induced migration of cells out of the ECCs could be explained by PDGFBB-stimulated proliferation of residual mesenchymal cells overgrowing the epithelial cells appears unlikely. To definitely exclude this possibility we used the cell division/proliferation inhibitor, mitomycin C, which had been shown to completely abolish the proliferation of DIDs (Davani et al., 2009). ECCs derived from mitomycin C-treated DIDs (Fig. 1C—inset) exhibited a similar response to PDGF-BB stimulation as untreated DID-ECCs (Fig. 1C). Hence, the PDGF-BB-induced changes in morphology of DIDs reflected a bona fide behavior of the majority of the ECC population (see Fig. 2).

To quantify the dose-dependence of the PDGF-BB-induced effect, the degree of dispersal of ECCs was evaluated by morphometric analysis (see Material and Methods Section). The percentage of dispersed ECCs as a function of PDGF-BB concentration is shown in Figure 2. The effect of PDGF-BB was biphasic, with the maximal dispersal of ECCs (~80%) between 10 and 30 ng/ml. It should be emphasized that in most experiments the effect of 5% FBS was more rapid and often more extensive than that of PDGF-BB.

FBS- and PDGF-BB-induced dispersal of DID- ECCs was dose-dependently inhibited by a selective inhibitor of PDGFR, JNJ-10198409 (Fig. 3). JNJ-10198409 at 0.1 μ M partially (by $52 \pm 15\%$ and $51 \pm 17\%$) inhibited and at 0.4 μ M more completely (by $84 \pm 10\%$ and or $91 \pm 6\%$) inhibited the effect of PDGF-BB FBS, respectively. This strongly suggests that the effect of FBS is mediated mainly by PDGFRs.

To test whether the PDGF-BB-induced dispersal is general, we tested its effect on two additional cell cultures exhibiting mesenchymal stem cell characteristics—human dermal fibroblasts and human bone marrow mesenchymal stem cells (BM-MSCs). Both cell types formed cell clusters in SFM. Like serum, PDGF-BB (30 ng/ml) induced dispersal of dermal

fibroblasts and BM-MSCs though to a lesser extent (Fig. 4). These findings suggest that reversal of clustering, mediated at least partially by PDGF-BB, may be a general phenomenon of cultured cells.

Since the transition of cells from compact spherical ECCs to a monolayer of dispersed fibroblast-like cells involves migration, we tested whether FBS or PDGF-BB were chemotactic for DIDs. FBS and PDGF-BB produced robust responses, while EGF was only a modest chemotactic agent (Fig. 5A). PDGF-BB and EGF chemotactic effects were dose-dependent and biphasic, with maximal responses observed between 10 and 30 ng/ml (Fig. 5B). Neither PDGF-BB or EGF stimulated chemokinesis. These data are also consistent with the idea that PDGF-BB is one of the mediators of DID migration in serum.

We previously showed that serum-induced dispersal of DID ECCs was a component of serum-induced EMT (Voulgari and Pintzas, 2009). Indeed, PDGF caused a decrease in the levels of PDX1, insulin and glucagon mRNAs, the two hormones characteristic of differentiated endocrine pancreas (Fig. 6). Moreover, PDGF-BB, but not EGF, stimulated the expression of two proteins that are considered markers of EMT- α -smooth muscle actin (α SMA) and β -catenin (Fig. 7). ECCs exhibited weak expression α SMA in 22% of the cells and β -catenin in 37%. Dispersal induced by FBS or PDGF-BB, markedly enhanced the expression of α SMA and β -catenin in virtually all of the cells, whereas EGF did not increase α SMA or β -catenin expression and may have even decreased them. The increased expression of β -catenin was also detected using immunofluorescence and confocal microscopy (Fig. S2). The mRNA level of β -catenin did not, however, change significantly (Fig. 6) and the increase in its expression was cytoplasmic, with little if any nuclear translocation.

The changes in the expression of β -catenin suggested the involvement of Wnt signaling pathway in the transduction of the PDGF signal. We tested, therefore, the expression levels of DKK1, a known inhibitor of the Wnt pathway, mRNA. PDGF caused a decrease in DKK1 mRNA levels in DIDs, BM-MSCs, and DFs (Fig. S3), consistent with activation of the Wnt pathway. However, the levels of the frizzled receptors for Wnt signaling proteins-FZD1, 7 or 8 and the matrix metalloproteinase MMP2 mRNAs decreased in all three cell types (except for MMP2 mRNA in DFs, Figs. S3B-E).

The above-described results demonstrated that the increased migration, dispersal and expression of α SMA and β -catenin by DIDs were much greater with PDGF-BB than EGF.

We hypothesized, therefore, that the action of PDGF-BB is transduced by the PI3K/Akt pathway, since it is the only major pathway directly activated by PDGF and not by EGF (Yarden and Sliwkowski, 2001; Schoeberl et al., 2009). Indeed, the PI3K specific inhibitor, PIP828, at a concentration of 50 μ M caused a modest decrease in PDGF-BB- or FBS-induced effect (45% or 26%, not shown). Higher concentrations of the inhibitor were toxic to the cultures.

We therefore tested the effects of PDGF-BB and EGF on phosphorylation of receptor tyrosine kinase substrates using a phospho-kinase antibody array. We identified Akt as the most responsive (approximately fivefold increase) phosphorylation substrate in DIDs

exposed to PDGF-BB (30 ng/ml) for 30 min (Fig. 8A). To directly monitor Akt and its phosphorylation at Ser473, which reflects its activation (Feng et al., 2004b), we performed Western analysis with anti-phospho(S473)Akt and total Akt (pan) antibodies (Fig. 8B). PDGF-BB-stimulated ECCs showed a rapid and robust increase of Akt phosphorylation (Fig. 8B, left panel and Fig. 7C) of approximately sevenfold ($P < 0.001$) at 15 min and 1 h. At 24 h of PDGF-BB exposure, Akt phosphorylation was still elevated. By comparison, EGF treated ECCs exhibited a much weaker stimulation of Akt phosphorylation (Fig. 8B, right panel and Fig. 8C) that was maximal at only 2.2-fold at 15 min of exposure ($P < 0.01$) and decayed to pre-stimulation levels by 1 h.

Discussion

During normal development, undifferentiated precursor cells move to their designated sites and differentiate by a finely orchestrated process of neighbor-dependent extracellular signaling and controlled gene expression. In contrast, cells/tissues implanted in adults are placed into a fully differentiated environment where undifferentiated or partially differentiated cells may have a lesser chance to simulate the normal developmental/differentiation processes.

DIDs were shown to possess characteristics of mesenchymal stem cells (Davani et al., 2007; Gallo et al., 2007; Carlotti et al., 2010). We demonstrated that serum deprivation resulted in cell aggregation into spherical ECCs, comprised of cells that exhibit an epithelial phenotype and express neuroendocrine markers characteristic of endocrine pancreas (Gershengorn et al., 2004). These results have been confirmed by other studies (Zulewski et al., 2001; Eberhardt et al., 2006; Ouziel-Yahalom et al., 2006; Gallo et al., 2007; Joglekaret al., 2009; Carlotti et al., 2010). However, transplantation of DID ECCs under the kidney capsule in immunodeficient mice produced variable results. While in some animals the cells continued to differentiate and ultimately express and secrete pancreatic hormones, the grafts often appeared to revert to a less differentiated, mesenchymal phenotype (Davani et al., 2009). We described an in vitro model in which serum induced dispersal of ECCs and changed the expression profile of characteristic markers from epithelial-like to mesenchymal-like in a bona fide EMT (Davani et al., 2009).

The goal of this study was to identify a growth factor(s) that can mimic the above-described action of serum. To identify putative serum components that might be responsible for its effects on ECCs, we tested a number of growth factors that activate receptor tyrosine kinase pathways. PDGF-BB best mimicked the serum effect by activating PDGFRs. Similarly to FBS, PDGF-BB proved to be an efficient chemoattractant. Although PDGF-BB also stimulated DIDs proliferation, its effect on ECCs was independent of proliferation, suggesting bona fide change of phenotype for the majority of cells within the ECCs. We attribute the effect of FBS mainly to its ability to stimulate PDGFRs, since the PDGFR specific inhibitor, JNJ-10198409, almost fully blocked the effects of PDGF-BB and FBS.

Hence, the data reported here support the idea that PDGF, similarly to FBS, is a factor causing reversal of the mesenchymal-to-epithelial transition induced by serum deprivation (Gershengorn et al., 2004). The PDGF-BB- and FBS-induced dispersals were both

accompanied by marked increases in α SMA expression, a hallmark of the mesenchymal phenotype (Kinner et al., 2002). Additionally, there was a decrease in mRNA levels of PDX1, insulin and glucagon, markers of differentiated endocrine pancreas, confirming the de-differentiating effect of PDGF. During EMT, β -catenin accumulates in the cytoplasm and translocates to the nucleus where it functions to activate key genes (Kalluri, 2009). In proliferating DIDs, β -catenin was predominantly nuclear. β -catenin translocated to the cell membrane when DIDs formed ECCs (Ikonomou et al., 2008). In DIDs migrating out of ECCs following PDGF-BB stimulation expressed we observed no changes in β -catenin mRNA level and high levels of cytosolic β -catenin. Little if any of it membrane associated, unlike the weakly staining β -catenin in ECCs, which also exhibited modest membranal association. Using confocal microscopy and Z-stack analysis, no significant nuclear translocation was seen, as only a negligible percentage of the cells exhibited nuclear localization in the SFM condition, where the cells express a more mesenchymal phenotype. However, similar to FBS, PDGF increased the expression of cytoplasmic β -catenin, as evidenced by both IHC and confocal microscopy. The dynamics of β -catenin nuclear translocation reflects the activation of the Wnt pathway. Following treatment with PDGF, we observed a decrease in the level of DKK1 mRNA, a secretable inhibitor of the Wnt pathway, which was compatible with cytoplasmic β -catenin stabilization. However, the decrease in the DKK1 mRNA level was also accompanied by a decrease in FZD1, 7 and 8 transcripts and a decrease in MMP2 mRNA, which is one of the down-stream targets of the pathway. Interestingly, this was also observed in BM-MSCs and in DFs, suggesting that this mode of partial Wnt canonical pathway activation (Kato, 2007) maybe specific to PDGF. In other studies, the expression of β -catenin has been associated with epithelial tissue repair (Douglas et al., 2006) and, in a non-canonical Wnt pathway, in reduced osteogenic differentiation (Liu et al., 2011).

The role of receptor tyrosine kinases in general, and of PDGFR in particular, in EMT has been extensively studied in the context of development (Patel et al., 2010; Mima et al., 2011; Smith et al., 2011) and cancer therapy (Fischer et al., 2006; Jechlinger et al., 2006; Yang et al., 2006), and in reference to pathological states (Xiong et al., 2010; Mima et al., 2011), for example, the role of PDGF in normal and pathological fibrosis (Jechlinger et al., 2006; Yang et al., 2006; Patel et al., 2010; Sethi et al., 2011; Smith et al., 2011). PDGF signaling was shown to target PI3K as well as nuclear β -catenin accumulation (Fischer et al., 2006). However, little is known about PDGF effects in non-transformed cells. It is thus difficult to compare our results with the existing literature. The role of PDGFs and their receptors in cancer is controversial. For example, while one study demonstrated a decreased growth and metastasis of colorectal and pancreatic cancer cells over-expressing PDGFb (McCarty et al., 2007), another study reported diametrically opposite effects (Fjallskog et al., 2007).

The specificity of PDGFRs is underlined by the fact that activation of EGFR, the tyrosine kinase receptor sharing almost all of its downstream signaling pathway (Schlessinger, 2000), was largely ineffective. We concluded, therefore, that the mechanism of PDGF-induced dispersal of DID ECCs, is likely to be a PDGFR-specific pathway, notably not shared with EGFR, perhaps mediated by direct activation of PI3K and its down-stream effector Akt (Kratchmarova et al., 2005; Kim et al., 2007b; Moon et al., 2009; Xiong et al., 2010; Veevers-Lowe et al., 2011); EGF receptor has been shown to activate PI3K and Akt only

indirectly (Yarden and Sliwkowski, 2001; Schoeberl et al., 2009). Akt (S473) phosphorylation by members of the PI3K family was shown to be required to achieve full kinase activity (Feng et al., 2004a). Indeed, our phospho-kinase antibody array and Western analysis experiments demonstrated a marked increase in phosphorylated Akt (S473) caused by PDGF-BB that was much larger than the weak and transient effect of EGF. Both the PI3K inhibitor, PIP828, and the PDGFR specific inhibitor, JNJ-10198409, inhibited the effects of both PDGF and FBS. Treatment with a PI3K inhibitor only partially reversed PDGF-BB induced dispersal. Therefore, other signaling pathways might be mediating the dispersal induced by PDGF-BB.

It is interesting that PDGF-BB caused dispersal of DIDs, DFs, and BM-MSCs-ECCs. These three cellular models, only one of pancreatic origin, conform to the mesenchymal stem/stromal cell phenotype. If future experiments in vivo confirm the de-differentiating action of PDGF, PDGFRs should be considered as putative targets to maintain a differentiated phenotype of mesenchymal stem/stromal cells transplanted into animals, including pancreatic islets and in vitro expanded β -cell precursors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. PDGF-BB induces DID ECC dispersal in the absence of proliferation. Photomicrographs (10 × objective) of DIDs ECCs in SFM (A) and PDGF-BB (30 ng/ml) following stimulation for 96 h either in control ECCs (B, insert-96 h in FBS) or ECCs formed in SFM from mitomycin C-pretreated DIDs (C, insert-Mitomycin-treated ECCs, t = 0).

Fig. 2. PDGF-BB-induced ECCs dispersal is concentration-dependent. DID ECCs were exposed to PDGF-BB at the indicated concentrations or 10% FBS for 96 h. Dispersal was calculated as described in Materials and Methods Section. Data are the average $\pm$ SE in three experiments, performed in duplicate.

Fig. 3. PDGF-R inhibitor (JNJ) blocks PDGF-BB- and FBS-induced dispersal. ECCs pretreated with JNJ (0.1 or 0.4 μM) were stimulated with 30 ng/ml PDGF-BB or 10% FBS for 96 h. Images were analyzed with Universal Imaging MetaMorph 6.1 software and the inhibitory effect (%) was calculated as described in Materials and Methods Section. Data show average $\pm$ SE of five experiments.

Fig. 4. PDGF-BB induces dispersal of DF and BM-MSCs. Dermal fibroblasts or BM-MSCs were cultured for 48 h in SFM to promote aggregation into clusters (upper panel), followed by exposure to PDGF-BB (30 ng/ml) or 10% Human serum for 48 h (lower panel).

Fig. 5. PDGF-BB and FBS are more efficacious chemoattractants than EGF. (A) Effects of 10 ng/ml PDGF-BB or EGF or 10% FBS on migration of DIDs. Chemokinesis: factors in both upper and lower Transwells chambers. Chemotaxis: factors present only in lower chamber. (B) Chemotaxis dose responses to PDGF or EGF. Data presents average $\pm$ SE of two experiments performed in duplicate.

Fig. 6.

The effects of PDGF on insulin, glucagon, or β -catenin mRNA levels. DIDs were stimulated with 30 ng/ml PDGF for 72 h. RNA was extracted from control and PDGF-stimulated cells and qRT-PCR performed as described in Materials and Methods Section. Results are mean $\pm$ SE values of three independent determinations. $P < 0.01$ for PDX1, insulin, and glucagon.

Fig. 7.

Cells dispersed from DID ECCs by PDGF-BB or FBS exhibit increased expression of a smooth muscle actin (α SMA) and β -catenin. Immunocytochemical staining for α -smooth muscle actin (α SMA) or β -catenin in ECCs prior to and subsequent to 24 h stimulation with EGF, PDGF-BB (30 ng/ml), or 5% FBS. Mean percent of positive cells multiple fields acquired in two independent experiments are shown.

Fig. 8.

PDGF-BB stimulates a marked increase in Akt1 phosphorylation. (A) Representative section of human phospho-kinase antibody array of untreated DIDs maintained in SFM (48 h) and following 30 min treatment with PDGF-BB (30 ng/ml). Duplicate spots of phosphorylated Akt1(S473) (white) and internal control (black) are marked with rectangles. (B) Western analysis of untreated DID-ECCs and following stimulation with 30 ng/ml PDGF-BB (left panel) or EGF (right panel) for the indicated times. (C) Actin-normalized ratios of pAkt(S473)/Akt(pan) of untreated DID-ECCs (0 h) or following stimulation with 30 ng/ml PDGF-BB (black bars) or EGF (open bars) for the indicated times. $P < 0.01$, $P < 0.001$. Mean $\pm$ SE values of two independent experiments performed in duplicate are shown.