Characterization of the chemotactic and mitogenic response of SMCs to PDGF-BB and FGF-2 in fibrin hydrogels

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Abstract: The delivery of growth factors to cellularize biocompatible scaffolds like fibrin is a commonly used strategy in tissue engineering. We characterized smooth muscle cells (SMC) proliferation and chemotaxis in response to PDGF-BB and FGF-2, alone and in combination, in 2D culture and in 3D fibrin hydrogels. While both growth factors induced an equipotent mitogenic response in 2D culture, only FGF-2 was significantly mitogenic for SMCs in 3D culture. Only PDGF-BB was significantly chemotactic in a modified Boyden chamber assay. In a 3D assay of matrix invasion, both growth factors induced an invasive response into the fibrin hydrogel in both proliferating and nonproliferating, mitomycin C (MMC) treated cells. The invasive response was less attenuated by the inhibition of proliferation in PDGF-BB stimulated cells compared with FGF-2 stimulated cells. We conclude that SMCs cultured in fibrin hydrogels have a more robust chemotactic response to PDGF-BB compared with FGF-2, and that the response to FGF-2 is more dependent on cell proliferation. Delivery of both growth factors together potentiates the chemotactic, but not mitogenic response to either growth factor alone. © 2010 Wiley Periodicals, Inc.* J Biomed Mater Res Part A: 94A: 988–996, 2010

Key Words: fibrin, PDGF-BB, FGF-2, smooth muscle cells, tissue engineering

INTRODUCTION

While growth factors have been utilized as attractive pharmacologic tools for the augmentation of tissue generation within biocompatible scaffolds, several limitations to their use remain.[1–5] Among these is the ability of a single growth factor to activate broad responses among many different types of differentiated and undifferentiated cells, limiting the specificity and predictability of growth factor activity. This can have detrimental consequences, as many of the regenerative processes targeted by exogenous growth factors thought to be critical for tissue engineering often represent a continuum of host responses, which, if uncontrolled, can counterproductively limit the durability of tissue engineered organs.

The induction of mesenchymal cell recruitment and growth into hydrogels with exogenous growth factors is a critical strategy in tissue engineering, as they comprise the main cellular component of most organ stroma and are significant contributors to organ function. In the development of tissue engineered blood vessels (TEBVs), for example, the deposition of endogenous collagens and elastins, and the synthesis of metalloproteinases by "tunica media" smooth muscle cells (SMCs) is necessary for proper scaffold remodeling and thus TEBV biomechanical functionality and tissue incorporation after implantation.[6–10] However, the promotion of SMC proliferation with growth factors in TEBVs can also lead to uncontrolled SMC growth and synthetic activity which can promote the myointimal hyperplastic response and lead to conduit stenosis. Thus, the characterization of the specific responses of SMCs within hydrogels to growth factors should provide for the future development of more targeted growth factor delivery strategies for tissue and organ engineering.

PDGF-BB and FGF-2 are known regulators of SMC behavior *in vivo*, impacting cell proliferation, migration, and phenotypic differentiation.[11,12] This has made them attractive tools in tissue engineering for a variety of applications.[13–16] While the mitogenic and chemotactic cellular responses to these growth factors have been studied in the context of pathologies such as atherosclerosis and intimal hyperplasia, they have not been extensively and studied in tissue engineering hydrogels.

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In these studies, we utilize 3D *in vitro* culture techniques to determine the relative chemotactic and mitogenic response of SMCs to PDGF-BB and FGF-2, alone and in combination, in fibrin hydrogels. We also investigate SMC proliferation and migration in response to these growth factors in 2D culture to highlight potential differences in cellular behavior which may be attributable to the cell/matrix interactions present in 3D culture.

MATERIALS AND METHODS

Materials

Chemicals, biological reagents, and experimental supplies were obtained as follows: Collagenase (Invitrogen); Human Thrombin (American Red Cross; Rockville, MD); Mitomycin C (MMC), PDGF-BB, FGF-2, L-ascorbic acid, Methylcellulose, Fibrinogen, Transferrin, Insulin, Anti–α-actin Antibody and Aprotinin (Sigma Chemical; St. Louis, MO); Tritiated Thymidine (NEN Life Science Products; Boston, MA); Methanol, Trichloroacetic Acid, Acetic Acid, and Scintillation Fluid (Fisher Scientific; Fair Lawn, NJ); Bovine Lung Heparin (Upjohn; Kalamazoo, MI); Calcein AM (Molecular Probes, Eugene, OR); 0.05% Trypsin/EDTA, HBSS, M199, DMEM, L-nonessential Amino Acids, Sodium Pyruvate, Penicillin, Streptomycin, DMEM-F12 (Gibco, Grand Island, NY); fetal bovine serum (FBS) (Hyclone, Logan, UT); Woven Nylon Mesh Rings (ID = 7.5 mm, OD = 13 mm) (Sefar America; Kansas City, MO); Parafilm M (American National Can, Greenwich, CT); 100-mm and 60 mm Petri Dishes (Fisher Scientific; Pittsburgh, PA); 24 Wells Plates and Tissue Culture Flasks, Costar Transwell Polystyrene Plate (Corning Costar; Cambridge, MA); Round Bottom 96 Well Plates (Greiner Bio-one; NC); 96-Well Polystyrene Plastic Plates (Beckton Dickinson; Lincoln Park, NJ).

Animal care

All animal procedures complied with The Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, Commission of Life Sciences, National Research Council, 1996) and The Principles of Laboratory Animal Care (National Institutes of Health publication no. 85–23, revised 1985).

Cell culture and treatments

Cell isolation. SMCs migrating from explanted canine carotid arteries were harvested and cultured in complete media as previously described.[17] Only cell populations with greater than 95% SMC α-actin expression were used for the assays. Carotid artery explants used as the source of cells for assays were passaged no more than two times.

Cell culture. Experiments were carried out in culture media containing 10% FBS as detailed below. There were two exceptions: (1) Media containing 1% FBS was used for 2D migration assays to minimize basal levels of migration; and (2) 2D proliferation assays were performed by stimulating serum-starved SMCs with growth factors diluted in serum free media as well growth factors diluted in media containing 10% FBS.

MMC treatment. In experiments using MMC treatment to inhibit cell proliferation, 500,000 SMCs were plated onto 60 mm Petri dishes and treated with 10 μg/mL of MMC for 1 hour before use in experiments. SMCs which were plated but not treated with MMC served as controls.

Bioassay

2D proliferation assay. Tritiated thymidine incorporation for evaluation of DNA synthesis was used to assay cell proliferation in 2D culture as previously described.[17] Briefly, SMCs were plated onto 96-well polystyrene plastic plates and cultured in complete media for 3 days until they reached 90% confluence. At that point, the media was changed to serum free quiescence media to synchronize cells in G_0 for a period of 48 h. The cells were then stimulated for 24 h by PDGF-BB, FGF-2, or both combined, diluted in invasion assay media consisting of M199, 10% FBS, 100 U/mL penicillin/streptomycin, 0.05 μg/mL gentamicin, 100 KIU/mL aprotinin, 0.25 μg/mL fungisone, and 5 U/mL of heparan sulfate. A 24 h incubation with tritiated thymidine quantified DNA synthesis. Preliminary studies were also carried out in which growth factors were added in the absence of serum. A standard concentration of 10 ng/mL, known to be maximally bioactive in SMC proliferation assays, was used for each growth factor delivered either alone or in combination in this and all subsequent assays. SMCs stimulated with invasion assay media without growth factors served as negative controls. There were four replicate wells per group, and the experiment was performed in triplicate with consistent results. 2D proliferation assay data is presented as mean counts per minute (CPM) ± standard error of the mean (SEM). A representative experiment is presented in the results.

3D proliferation assay. SMCs were mixed in a fibrinogen solution (2.5 μg/mL fibrinogen and M199 at a pH of 8.0) at a concentration of 400,000 cells/mL before polymerization with the addition of thrombin (0.32 U/mL). Calcium content was standardized among hydrogels in all experiments at concentrations found in commercially available formulations of M199 (200 mg/L). Fibrin hydrogels were incubated in invasion assay media with PDGF-BB and/or FGF-2. SMC containing hydrogels incubated in invasion assay media without growth factor served as the negative control. After 72 h of incubation, hydrogels were proteolytically digested for one hour with a combination of 0.05% Trypsin-EDTA and collagenase (2 mg/mL), and recovered cells were counted with a hemocytometer after trypan blue exclusion. Data from two independently performed experiments were normalized to negative controls and pooled results are presented as mean cells recovered per gel ± SEM (n = 10–14).

2D migration assay. SMCs were seeded on the inner membrane of a Costar transwell polystyrene plate (8.0 μm pore size in polycarbonate membrane). After allowing the cells to attach for four hours, a test solution containing PDGF-BB, FGF-2, or the combination of the two diluted in invasion assay media with 1% FBS was placed in the lower chamber.

Cells stimulated with invasion assay media with 1% FBS without growth factor served as the negative control. After two hours of migration, the media in the upper and lower chambers was gently suctioned off and the nonmigratory cells were scraped from the inner membrane with a cotton-tipped applicator. The remaining cells were fluorescently labeled with Calcein AM. Four quadrants of the membrane were visualized under FITC at a magnification of 10× and fluorescent cells were counted. Each group was tested in replicates of three, and the experiment was duplicated, with similar results. Results from each experiment were normalized to negative controls and the pooled results are presented as mean migratory cells ± SEM ($n = 6$).

3D matrix invasion assay. Two thousand two-hundred fifty SMCs were suspended in an aggregating solution consisting of invasion assay media and a 20% methocel solution[18] in round bottom 96-well plates and incubated at 37°C for 24 h until cell aggregates formed. MMC treated or MMC untreated SMC aggregates were then embedded between two 150 μL layers of fibrin that were polymerized on a nylon mesh ring such that the aggregates were completely surrounded by the hydrogel. Once completely polymerized, the disks were transferred into 24 wells plates and cultured in invasion assay media containing PDGF-BB, FGF-2, or both growth factors together at 37°C. Cells stimulated by invasion assay media without growth factor served as negative control. After a period of time, SMCs invade from the central aggregate into the surrounding ECM. Digital images were taken daily for up to 5 days at a magnification of 4× using a Zeiss Axiovert 200M microscope (Carl Zeiss, Oberkochen, Germany) and Axiovision software. The digital photographs were aligned with a grid evenly divided in 36 intervals using Adobe Photoshop for quantification (Fig. 3). For each assay, the distance of invasion (DOI) was determined by measuring the furthest point on each grid line with an invading chain of cells. The presence of cellular debris and discontinuity of previously continuous chains of invading cells is indicative of apoptotic cell death.[18] For this reason, invasion was quantified by measuring only out to the final point of a continuous chain of cells. Experiments were carried out with replicates of four to five and duplicated with consistent results. Data from a representative experiment is presented in the results unless otherwise noted.

Statistical analysis

We used analysis of variance with Tukey posttests or Student's t-tests using SigmaStat (Systat Software, San Jose, CA) at an α of 0.05 to determine statistical significance.

RESULTS

SMC proliferation in 2D culture

In the presence of serum, PDGF-BB, and FGF-2 each demonstrate significantly greater mitogenicity compared with negative controls [Fig. 1(a)]. Thymidine incorporation was 170.7% ± 7.5% and 183.6% ± 11.1% of negative controls in SMCs stimulated by PDGF-BB or FGF-2, respectively ($p < 0.001$ for each growth factor vs. negative control). Simultaneous delivery of PDGF-BB with FGF-2, however, did not induce any significant thymidine incorporation above negative controls (100.9% ± 8.0% of negative controls for PDGF-BB + FGF-2; $p = 0.93$). In the absence of serum, each growth factor, alone and in combination, induced significant thymidine incorporation compared with negative controls ($p < 0.001$ for each treatment group vs. negative control), with FGF-2 demonstrating significantly greater mitogenicity than PDGF-BB ($p < 0.001$) [Fig. 1(b)]. In the absence of serum, thymidine incorporation in response to the combination of both growth factors added simultaneously was not significantly different than PDGF-BB alone ($p = 0.09$), and was significantly less than FGF-2 alone ($p < 0.001$) [Fig. 1(b)].

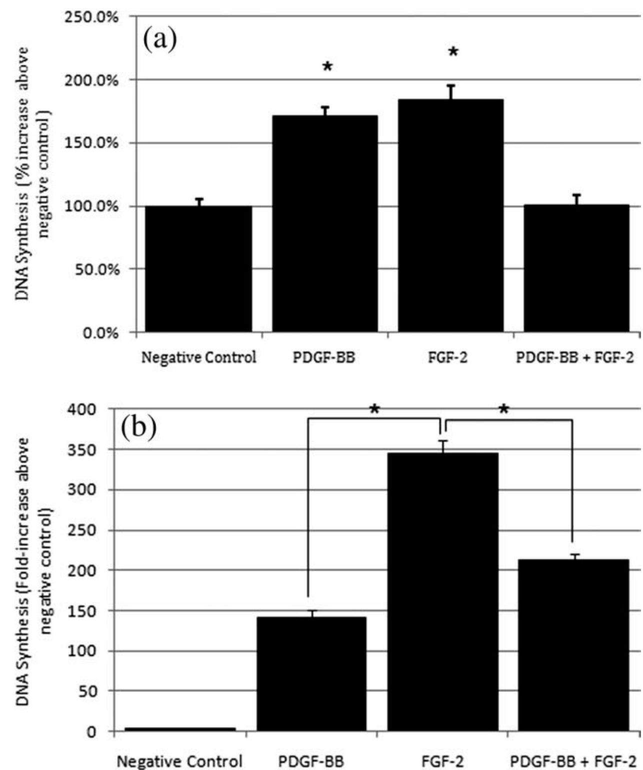

FIGURE 1. SMC proliferation in 2D culture in response to PDGF-BB and FGF-2. Serum starved SMCs seeded on 96 well plates were incubated with indicated growth factors diluted in (a) invasion assay media (10% serum) or (b) serum free media. SMC proliferation was quantified by determining tritiated thymidine incorporation. Results represent the mean CPM ± SEM from a representative experiment ($n = 4$). $^{*}p < 0.001$.

SMC chemotaxis in 2D culture

PDGF-BB, but not FGF-2, induced a significant chemotactic response in SMCs compared to negative controls (353.7% ± 47.9% of negative controls for PDGF-BB, $p < 0.001$; and 170.8% ± 57.2% of negative controls for FGF-2, $p = 0.28$) (Fig. 2). Simultaneous stimulation with PDGF-BB and FGF-2 induced a synergistic migratory response in comparison to either growth factor alone (659.2% ± 86.9% of negative controls; $p \leq 0.01$ vs. either PDGF-BB or FGF-2 alone).

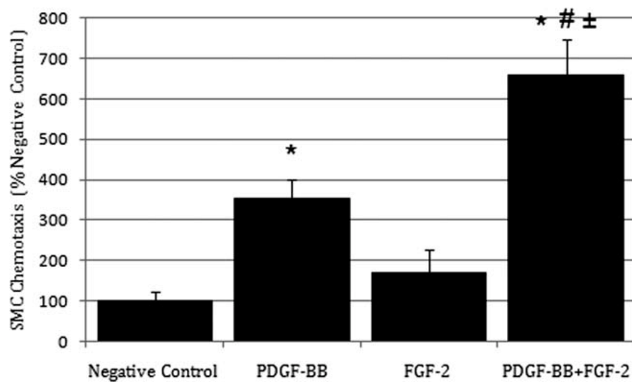

FIGURE 2. SMC chemotaxis in response to PDGF-BB and FGF-2. SMCs were seeded on the inner surface of an 8 μm porous membrane and allowed to migrate in response to respective growth factor gradients. Each column represents the mean number of migratory cells ± SEM of pooled results from two independently performed experiments ($n = 6$). *p <0.001 vs. negative control; #$p < 0.01$ vs. PDGF-BB; ±$p < 0.001$ vs. FGF-2.

SMC matrix invasion in 3D culture

Both PDGF-BB and FGF-2 induced significant invasion into fibrin hydrogels compared to negative controls (Fig. 3). At 48 h, the average DOI in SMCs stimulated by PDGF-BB or FGF-2 alone was 200.1 ± 22.2 μm, and 231.0 ± 14.6 μm, respectively, vs. 64.1 ± 5.9 μm in negative controls ($p < 0.05$ for each growth factor vs. negative controls). Simultaneous stimulation with PDGF-BB and FGF-2 induced an additive invasive response (367.3 ± 28.8 μm; $p < 0.05$ vs. either growth factor delivered alone) (Fig. 3). Similar results were seen at 72 h, which was the last time point accurately quantifiable due to the invasion of cells beyond the limits of the camera lens field at a magnification of 4×. No significant differences were observed between the groups treated with PDGF-BB or FGF-2 alone at any time point.

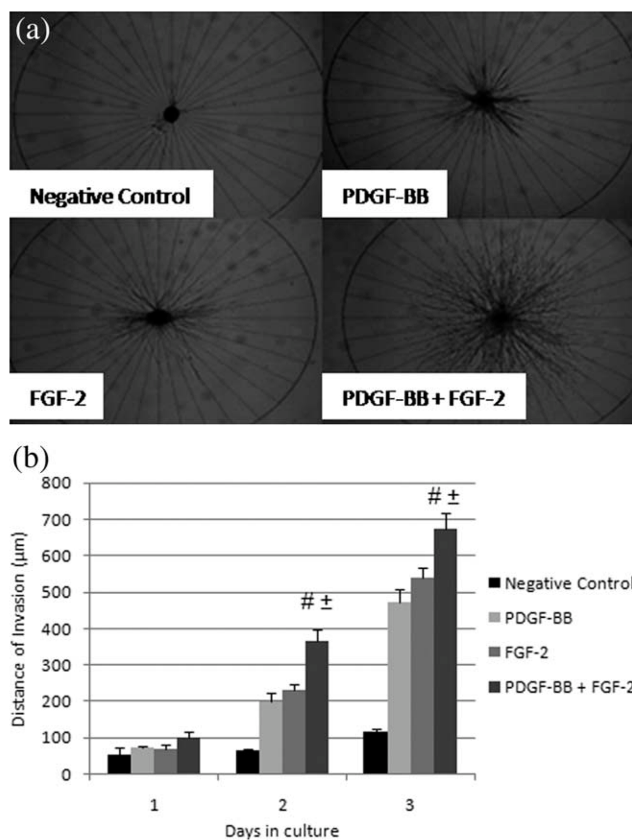

FIGURE 3. SMC invasion into fibrin hydrogels in 3D culture in response to PDGF-BB and FGF-2. (a) SMC invasion after 48 h of 3D culture (Magnification - 4×). (b) Quantification of SMC invasion into fibrin hydrogels. Results represent the mean DOI ± SEM from a representative experiment ($n = 4$–5). #$p < 0.05$ vs. PDGF-BB; ±$p < 0.05$ vs. FGF-2.

Proliferation and chemotaxis in 3D matrix invasion

To investigate the relative role for proliferation vs. chemotaxis in the invasive response to PDGF-BB and FGF-2 in 3D culture, we first quantified their mitogenicity on SMCs homogenously distributed in fibrin hydrogels (Fig. 4). All 3D culture experiments were carried out in the presence of serum in order maximize nutrient delivery to cells embedded within ECM, and to most accurately model the culture conditions likely required for *ex vivo* or *in vivo* tissue development. After 72 h of culture, there were 231.3% ± 19.7% more cells in the PDGF-BB stimulated group compared to negative controls ($p = 0.07$), 509.5% ± 69.3% more cells in the FGF-2 stimulated group compared to negative controls ($p < 0.001$), and 392.6% ± 32.0% more cells in the PDGF-BB plus FGF-2 stimulated group compared to negative controls ($p < 0.001$). FGF-2 delivered alone was significantly more mitogenic in 3D than PDGF-BB delivered alone ($p < 0.001$). The combination of PDGF-BB and FGF-2 delivered simultaneously was significantly more mitogenic than PDGF-BB delivered alone ($p = 0.01$), and nonsignificantly less mitogenic than FGF-2 delivered alone ($p = 0.10$).

To quantify the nonproliferation dependent component of SMC invasion into fibrin hydrogels in response to these growth factors, cells were pretreated with MMC and

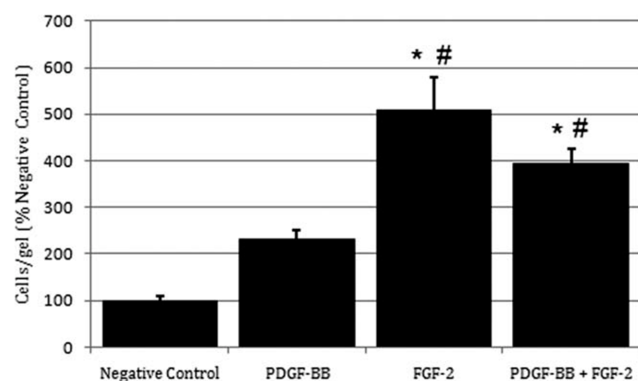

FIGURE 4. SMC proliferation in 3-D fibrin culture in response to PDGF-BB and FGF-2. SMCs were distributed homogenously in fibrinogen solution before gel polymerization with the addition of thrombin. 3D cell cultures were incubated in media ± respective growth factors. After 72 h, the gels were digested and trypan blue excluding cells were counted with a hemocytometer. Each column represents the mean number of cells/gel ± SEM of pooled results from two independently performed experiments ($n = 10$–14). *$p < 0.001$ vs. negative control; #$p \leq 0.01$ vs. PDGF-BB.

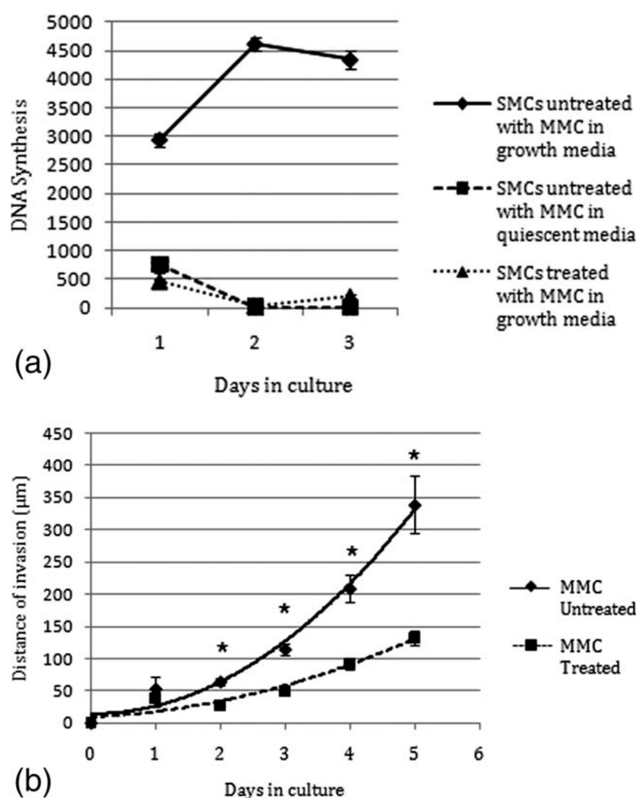

FIGURE 5. The effects of MMC treatment on SMC proliferation and matrix invasion in the absence of growth factors. (a) SMC proliferation. SMCs either treated or untreated with MMC were seeded onto 96 well plates and cultured in indicated media. SMC proliferation was determined by quantifying tritiated thymidine incorporation. Results are mean CPM ± SEM from a representative experiment ($n = 4$). (b) SMCs either treated or untreated with MMC were embedded within fibrin hydrogels and matrix invasion was quantified. Results represent the mean distance of invasion ± SEM from a representative experiment ($n = 4$–5). $^*p < 0.05$ vs. MMC untreated control.

compared to MMC untreated SMCs. Significant inhibition of tritiated thymidine incorporation within 24 h of MMC treatment confirmed the inhibition of cell proliferation (468 ± 36 CPM vs. 2927 ± 116 CPM in MMC treated cells vs. MMC untreated cells, respectively at 24 h, $p < 0.001$; and 37 ± 21 CPM vs. 4624 ± 109 CPM in MMC treated cells vs. MMC untreated cells, respectively at 48 h, $p < 0.001$) [Fig. 5(a)]. Cell viability studies using trypan blue exclusion along with the persistence of viable phenotypic characteristics of cells in 2D and 3D culture confirmed that MMC treatment was not cytotoxic (data not shown). In addition, no differences in cell morphology under light microscopic examination in MMC treated cells were noted in comparison to MMC untreated cells. In the absence of any growth factors, MMC treatment attenuated the distance of SMC invasion compared to MMC untreated controls [Fig. 5(b) and Table I]. MMC treatment decreased the average distance of SMC invasion to a maximal reduction of 40.0% ± 2.2% of MMC untreated controls ($p < 0.001$) at Day 5, with significant reductions in the DOI during the entirety of the experiment (Table I).

In MMC treated SMCs, all growth factor groups induced significantly greater matrix invasion compared to MMC treated, growth factor unstimulated negative controls ($p < 0.001$ in all groups vs. negative controls) (Fig. 6). By 48 h, the simultaneous stimulation of MMC treated SMCs with PDGF-BB and FGF-2 induced a significantly greater invasive response compared to either growth factor alone (317.7 ± 24.0 μm vs. 143.0 ± 8.5 μm for PDGF-BB + FGF-2 vs. FGF-2, $p < 0.001$; and 317.7 ± 24.0 μm vs. 188.1 ± 11.5 μm for PDGF-BB + FGF-2 vs. PDGF-BB, $p < 0.005$) (Fig. 6). Similar statistically significant results were seen at the 72 h time point.

After 48 h of 3D culture, MMC treatment significantly diminished the SMC invasive response to FGF-2, but not to PDGF-BB or PDGF-BB plus FGF-2 [Fig. 7(a,c)]. By 72 h, MMC treatment significantly decreased the SMC invasive response to all three growth factor stimulated groups to 76.0% ± 3.0%, 56.1% ± 2.1%, and 81.9% ± 4.5% of respective MMC untreated controls when stimulated by PDGF-BB, FGF-2, and PDGF-BB plus FGF-2, respectively [Fig. 7(b,c)]. At 72 h, the overall attenuation of SMC matrix invasion by MMC treatment was significantly greater in the FGF-2 stimulated groups in comparison to the PDGF-BB or PDGF-BB plus FGF-2 stimulated groups ($p < 0.05$) [Fig. 7(c)].

DISCUSSION

In cardiovascular tissue engineering, the use of acellular biomaterials for valves, stents, and TEBVs is dependent on the eventual population of the scaffold with mesenchymally derived stromal cells. These cells provide vasomotor and tonal functions, deposit endogenous extracellular matrix (ECM), and remodel the biomaterial scaffold.[19] While the strategic delivery of growth factors may be beneficial in the promotion of scaffold cellularization, the uncontrolled growth of SMCs and other mesenchymal cells can result in the early proliferative process key to the pathogenesis of stenotic neointimal and atherosclerotic lesions. For this reason, the identification of growth factors which promote the chemotactic recruitment of surrounding stromal cells into the biomaterial scaffold without promoting an excessive proliferative capacity of these cells can minimize unwanted effects of growth factor therapy, and potentially provide for synergistic benefits with the development of designer growth factors.[20,21]

TABLE I. Matrix Invasion of MMC Treated SMCs as % of MMC Untreated Controls

	Day 1	Day 2	Day 3	Day 4	Day 5
Distance of invasion (μm)	68.7 ± 14.2	50.1 ± 4.2[a]	51.6 ± 3.6[a]	42.1 ± 3.5[a]	40.0 ± 2.2[a]

Data are means ± SEM of pooled results from two independently performed experiments ($n = 8$–10).
[a] $p < 0.001$ vs. MMC untreated controls.

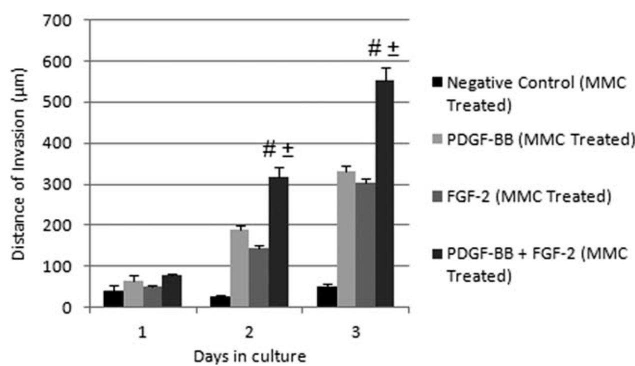

FIGURE 6. Invasion of MMC treated SMCs into fibrin hydrogels in 3D culture in response to PDGF-BB and FGF-2. SMCs were treated with MMC, embedded in fibrin hydrogels, and then incubated in invasion assay media with or without indicated growth factors. Results represent the mean distance of invasion ± SEM from a representative experiment (n = 4–5). $^{\#}p < 0.005$ vs. PDGF-BB; $^{\pm}p < 0.001$ vs. FGF-2.

Differential mitogenic and chemotactic responses by SMCs to PDGF-BB and FGF-2 have been suggested in studies *in vivo*.[22–25] This observation provides potential utility for the targeting of specific SMC activities for tissue engineering applications. However, the relative chemotactic and mitogenic response of SMCs to these growth factors in fibrin hydrogels, a commonly used scaffold and growth factor delivery biomaterial, has not been investigated. In these studies, we demonstrate that both PDGF-BB and FGF-2 are potent inducers of SMC invasion into fibrin hydrogels *in vitro*. The mechanism of this effect, however, differs between the two growth factors, with PDGF-BB functioning primarily as a chemotactic agent demonstrating limited mitogenicity in 3D culture, and FGF-2 functioning primarily as a mitogenic agent, with less pronounced chemotactic effects. Thus, while matrix invasion into fibrin in the presence and absence of each of these growth factors is a function of both migration and proliferation, we show that the invasive response to PDGF-BB is less dependent on proliferation than FGF-2. These results indicate that PDGF-BB would be an attractive growth factor for the induction of SMC invasion into tissue engineered constructs in situations where the minimization of cell proliferation is desirable. This might be especially useful for growth factor delivery strategies in devices or constructs which utilize antiproliferative drugs for the inhibition of myointimal hyperplasia as used in drug eluting stents.[26] The relative dependence of SMC invasion on cell proliferation in response to FGF-2 suggests that it would be a less effective choice in those circumstances.

Several caveats to the interpretation of these results should be considered. First, we should note that these experiments were carried out at a single concentration of 10 ng/mL, a concentration known to have mitogenic and chemotactic potency, and to mediate interactions between both growth factors.[27,28] The results in these studies should be interpreted with the understanding that the responses demonstrated here could be dependent on the specific concentrations used.[29] Additionally, we observed that the mitogenic response to these growth factors was partially dependent on the presence of serum. Observations from other groups which demonstrated altered PDGF receptor expression in cells cultured in the absence of serum factors may offer an explanation for these results.[30] Second, the replicability of these results in other cell types, as well as to other species should be a consideration before application in other animal or human studies. Finally, while fibrin is a commonly used hydrogel in tissue engineering for many applications, the possibility that these responses are specific to fibrin compared to other extracellular matrices should be considered.[8,9,31–36] Recent evidence has demonstrated that fibrinogen alone can augment the proliferation rates of specific carcinoma cells and endothelial cells in response to

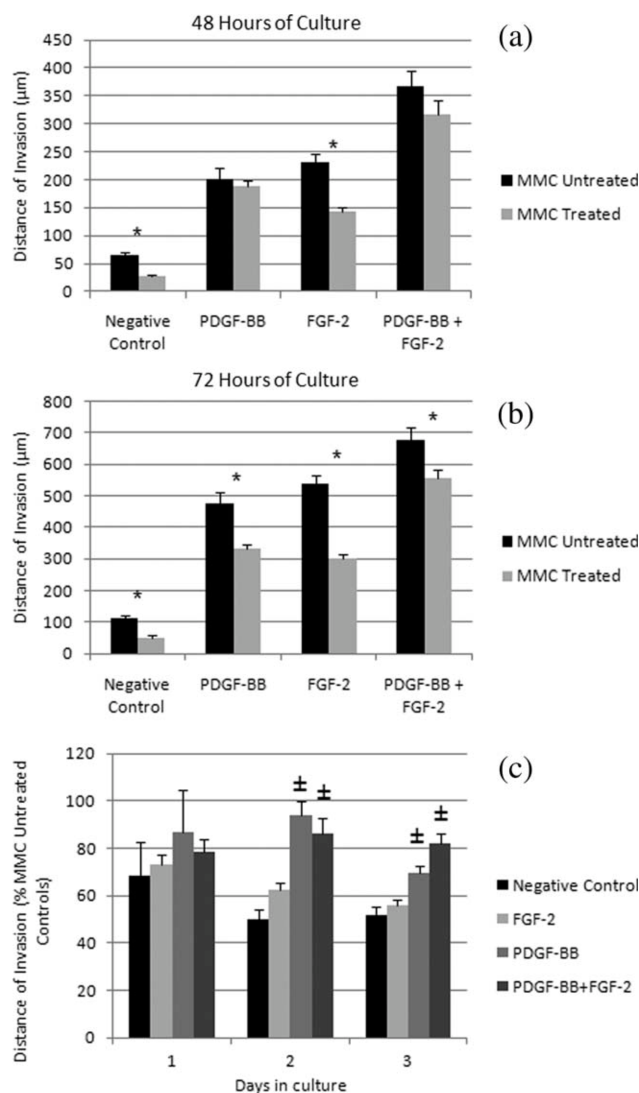

FIGURE 7. Invasion of MMC treated and untreated SMCs into fibrin hydrogels in response to PDGF-BB and FGF-2. (a) Invasion quantified after 48 h of culture. (b) Invasion quantified after 72 h of culture. (c) Matrix invasion of MMC treated SMCs as % of MMC untreated controls in the presence of indicated growth factors. Results represent the mean distance of invasion ± SEM from a representative experiment (n = 4–5). $^{*}p < 0.05$ compared to respective MMC untreated controls; $^{\pm}p < 0.05$ vs. FGF-2.

FGF-2, and that this appears to be attributable to fibrinogen-FGF-2 binding.[37,38] Similarly, thrombin promotes endogenous FGF-1 expression in endothelial cells[39] and FGF-2 in SMCs,[40] upregulates VEGF expression in SMCs via endogenous PDGF and FGF-2 activity,[41] can itself be a significant SMC mitogen and chemoattractant *in vitro* at concentrations of 0.5 U/mL in a process likely involving FGF-2 release and FGFR-1 transactivation,[42] and can significantly impact fibrin gel structural and mechanical properties which could affect cellular behavior.[43] While thrombin can affect SMC proliferation rates in 2D culture systems, the role of thrombin in modulating SMC proliferation in 3D culture systems is less established. Previous studies by Rowe et al., for example, have suggested that thrombin may have no significant effect on the proliferation, phenotype, or actin and cellular alignment of SMCs cultured within fibrin hydrogels.[43] Due to the low concentrations of thrombin (0.32 U/mL) in our model, it is unlikely that a significant amount of free thrombin is present after gelation and likely plays a minimal role in modulating cellular responses. However, observations that individual components of fibrin can significantly impact cellular responses within the hydrogel as well as their response to growth factors do imply that the responses seen in these experiments may not extrapolate directly to other extracellular matrix configurations.

In our studies, the simultaneous delivery of PDGF-BB with FGF-2 completely eliminated the mitogenic response to either growth factor alone in 2D culture, and appeared to attenuate the mitogenic response to FGF-2 in 3D culture. In contrast, the combination of both growth factors induced a synergistic chemotactic response in 2D culture, and an additive invasive response in 3D culture, both in the presence and absence of proliferation. Inhibiting proliferation had little to no effect on SMC matrix invasion induced by the codelivery of both growth factors. These results were similar to those that were seen in SMCs stimulated by PDGF-BB alone. Thus, our results suggest that the codelivery of PDGF-BB with FGF-2 in fibrin hydrogels potentiates the invasive SMC response by a preferentially greater chemotactic signal, rather than increased mitogenesis.

The mechanism of this effect is yet unclear. There is known interaction between PDGF-BB and FGF-2 and growing evidence that PDGF-BB and FGF-2 are intimately involved in the regulation of one another's activity.[44] PDGF in the arterial wall is thought to interact with FGF-2 to promote SMC proliferation in a rat carotid artery filament injury model.[25] It has been demonstrated that PDGF-BB-induced SMC proliferation and late activation of ERK in humans depends at least in part on FGF-2 release and FGFR-1 activation,[27] and that PDGF-BB-induces upregulation of the high molecular weight form of FGF-2 in a mechanism dependent on ERK-1/2 kinase activity.[45] Given the role of ERK as a cell cycle progression factor, it is notable that the codelivery of both growth factors in our studies did not result in any additive mitogenic effects. The effects on SMC chemotaxis by the codelivery of both growth factors can be equally confounding based on contrary finding in the literature. Consistent with our results, Pickering et al. had demonstrated in 2D migration assays that pretreatment of human SMCs with 48 h of exposure to FGF-2 potentiates their chemotactic response to PDGF-BB on type I collagen coated surfaces in a mechanism dependent on the upregulation of the integrin receptor $\alpha 2\beta 1$.[46] In contrast, other studies have suggested that FGF-2 inhibits PDGF-BB induced migration in rat SMCs via a PDGF-α receptor dependent mechanism.[28] We speculate that alterations in growth factor or integrin receptor expression, differential expression of competence factors, alterations in cellular phenotype and growth factor responsiveness, or a combination of all may account for the synergistic chemotactic effects observed after growth factor codelivery in our studies. Specifically, investigating ERK expression and activation, integrin receptor expression, and PDGF receptor expression and activation in fibrin based 3D culture models are warranted in understanding the mechanism of PDGF-BB and FGF-2 interactions seen in our studies.

Finally, differences in the mitogenic response of SMCs to PDGF-BB, with or without FGF-2, in 2D vs. 3D culture are notable. First, PDGF-BB and FGF-2 demonstrated near equipotent mitogenicity in 2D culture, while the mitogenicity of PDGF-BB in 3D culture was insignificant. Second, while the codelivery of both growth factors eliminated the mitogenic response to either growth factor alone in 2D culture, this was not observed in 3D culture. While these discrepancies may be attributable to incomplete penetration of growth factors into the ECM altering effective growth factor concentrations, it is more likely due to cell/matrix/growth factor interactions which may modulate the growth factor response, either by altering cell signaling or cellular FGF or PDGF receptor expression and occupancy.[47–50] Alterations in cellular phenotype by the 3D ECM conformation may also account for these changes, leading to differential expression of competence factors and cellular organelles, growth factors and growth factor and integrin receptors, and altered synthetic capacity in response to exogenous growth factor delivery.[51] Stegemann had demonstrated that SMCs in 3D collagen matrices demonstrate less expression of α-SMA, a marker of the "contractile" SMC phenotype compared to cells cultured on a 2D collagen lattice. They further demonstrated that exogenous PDGF-BB decreased α-SMA expression in SMCs cultured in 3D collagen gels, but not in 2D culture.[52] In our studies, SMCs appeared to maintain a spindled, elongated shape under light microscopy indicating a "contractile" phenotype in 3D culture, but we did not further characterize markers indicative of a "contractile" vs. "synthetic" phenotype as this was not the primary purpose of these studies.[53] Future studies investigating the phenotypic characteristics of SMCs in 3D vs. 2D culture and its impact on SMC chemotaxis and proliferation in response to PDGF-BB and FGF-2 are required to provide further insight.

CONCLUSIONS

In summary, we demonstrate that both PDGF-BB and FGF-2 are potent growth factors for the induction of SMC invasion into fibrin hydrogels *in vitro*. PDGF-BB functions primarily as a chemotactic agent demonstrating limited mitogenicity

in 3D culture, and FGF-2 functions primarily as a mitogenic agent with less pronounced chemotactic effects. Thus, PDGF-BB appears to be a superior choice over FGF-2 for growth factor delivery systems which aim to promote SMC invasion into fibrin hydrogels with limited cell proliferation. The delivery of both growth factors added in combination with one another can promote an invasive response superior to either growth factor alone, and this appears to be primarily a function of augmented chemotaxis rather than mitogenesis. The observation that the simultaneous delivery of both growth factors at these concentrations can increase the mitogenic response above that of PDGF-BB alone, however, should be considered when choosing this strategy. Finally, the presence of serum and 3D extracellular matrix conformations can alter SMC responsiveness to PDGF-BB and FGF-2 alone, or in combination, confirming previous studies demonstrating the importance of considering the extracellular environment as a regulator or cellular behavior. Further studies are required to elucidate the mechanism of these differences, as well as the applicability of these studies to other cell types within other biomaterials used in tissue engineering.

REFERENCES

1. Zisch AH, Lutolf MP, Hubbell JA. Biopolymeric delivery matrices for angiogenic growth factors. Cardiovasc Pathol 2003;12:295–310.
2. Gray JL, Kang SS, Zenni GC, Kim DU, Kim PI, Burgess WH, Drohan W, Winkles JA, Haudenschild CC, Greisler HP. FGF-1 affixation stimulates ePTFE endothelialization without intimal hyperplasia. J Surg Res 1994;57:596–612.
3. Ehrbar M, Djonov VG, Schnell C, Tschanz SA, Martiny-Baron G, Schenk U, Wood J, Burri PH, Hubbell JA, Zisch AH. Cell-demanded liberation of VEGF121 from fibrin implants induces local and controlled blood vessel growth. Circ Res 2004;94: 1124–1132.
4. Ehrbar M, Zeisberger SM, Raeber GP, Hubbell JA, Schnell C, Zisch AH. The role of actively released fibrin-conjugated VEGF for VEGF receptor 2 gene activation and the enhancement of angiogenesis. Biomaterials 2008;29:1720–1729.
5. Boontheekul T, Mooney DJ. Protein-based signaling systems in tissue engineering. Curr Opin Biotechnol 2003;14:559–565.
6. Gao J, Crapo P, Nerem R, Wang Y. Co-expression of elastin and collagen leads to highly compliant engineered blood vessels. J Biomed Mater Res Part A 2008;85:1120–1128.
7. Ross JJ, Tranquillo RT. ECM gene expression correlates with in vitro tissue growth and development in fibrin gel remodeled by neonatal smooth muscle cells. Matrix Biol J Int Soc Matrix Biol 2003;22:477–490.
8. Long JL, Tranquillo RT. Elastic fiber production in cardiovascular tissue-equivalents. Matrix Biol J Int Soc Matrix Biol 2003;22: 339–350.
9. Grassl ED, Oegema TR, Tranquillo RT. A fibrin-based arterial media equivalent. J Biomed Mater Res Part A 2003;66:550–561.
10. L'Heureux N, Paquet S, Labbe R, Germain L, Auger FA. A completely biological tissue-engineered human blood vessel. FASEB J 1998;12:47–56.
11. Clowes AW. Regulation of smooth muscle cell proliferation and migration. Transplantation Proc 1999;31:810–811.
12. Mitra AK, Del Core MG, Agrawal DK. Cells, cytokines and cellular immunity in the pathogenesis of fibroproliferative vasculopathies. Can J Physiol Pharmacol 2005;83:701–715.
13. Lin H, Chen B, Sun W, Zhao W, Zhao Y, Dai J. The effect of collagen-targeting platelet-derived growth factor on cellularization and vascularization of collagen scaffolds. Biomaterials 2006;27: 5708–5714.
14. Zhao W, Chen B, Li X, Lin H, Sun W, Zhao Y, Wang B, Han Q, Dai J. Vascularization and cellularization of collagen scaffolds incorporated with two different collagen-targeting human basic fibroblast growth factors. J Biomed Mater Res A 2007;82: 630–636.
15. Chen RR, Silva EA, Yuen WW, Mooney DJ. Spatio-temporal VEGF and PDGF delivery patterns blood vessel formation and maturation. Pharm Res 2007;24:258–264.
16. Atluri P, Woo YJ. Pro-angiogenic cytokines as cardiovascular therapeutics: Assessing the potential. BioDrugs 2008;22:209–222.
17. Brewster LP, Brey EM, Tassiopoulos AK, Xue L, Maddox E, Armistead D, Burgess WH, Greisler HP. Heparin-independent mitogenicity in an endothelial and smooth muscle cell chimeric growth factor (S130K-HBGAM). Am J Surg 2004;188:575–579.
18. Uriel S, Brey EM, Greisler HP. Sustained low levels of fibroblast growth factor-1 promote persistent microvascular network formation. Am J Surgery 2006;192:604–609.
19. Pang Y, Ucuzian AA, Matsumura A, Brey EM, Gassman AA, Husak VA, Greisler HP. The temporal and spatial dynamics of microscale collagen scaffold remodeling by smooth muscle cells. Biomaterials 2009;30:2023–2031.
20. Shireman PK, Xue L, Maddox E, Burgess WH, Greisler HP. The S130K fibroblast growth factor-1 mutant induces heparin-independent proliferation and is resistant to thrombin degradation in fibrin glue. J Vasc Surg 2000;31:382–390.
21. Brewster LP, Washington C, Brey EM, Gassman A, Subramanian A, Calceterra J, Wolf W, Hall CL, Velander WH, Burgess WH, Greisler HP. Construction and characterization of a thrombin-resistant designer FGF-based collagen binding domain angiogen. Biomaterials 2008;29:327–336.
22. Jawien A, Bowen-Pope DF, Lindner V, Schwartz SM, Clowes AW. Platelet-derived growth factor promotes smooth muscle migration and intimal thickening in a rat model of balloon angioplasty. J Clin Invest 1992;89:507–511.
23. Lindner V, Olson NE, Clowes AW, Reidy MA. Inhibition of smooth muscle cell proliferation in injured rat arteries. Interaction of heparin with basic fibroblast growth factor. J Clin Invest 1992;90: 2044–2049.
24. Reidy MA. Neointimal proliferation: The role of basic FGF on vascular smooth muscle cell proliferation. Thromb Haemostasis 1993; 70:172–176.
25. Lewis CD, Olson NE, Raines EW, Reidy MA, Jackson CL. Modulation of smooth muscle proliferation in rat carotid artery by platelet-derived mediators and fibroblast growth factor-2. Platelets 2001;12:352–358.
26. Vaina S, Serruys PW. Progressive stent technologies: New approaches for the treatment of cardiovascular diseases. Expert Opin Drug Deliv 2006;3:783–797.
27. Millette E, Rauch BH, Defawe O, Kenagy RD, Daum G, Clowes AW. Platelet-derived growth factor-BB-induced human smooth muscle cell proliferation depends on basic FGF release and FGFR-1 activation. Circ Res 2005;96:172.
28. Facchiano A, De Marchis F, Turchetti E, Facchiano F, Guglielmi M, Denaro A, Palumbo R, Scoccianti M, Capogrossi MC. The chemotactic and mitogenic effects of platelet-derived growth factor-BB on rat aorta smooth muscle cells are inhibited by basic fibroblast growth factor. J Cell Sci 2000;113:2855–2863.
29. Schollmann C, Grugel R, Tatje D, Hoppe J, Folkman J, Marme D, Weich HA. Basic fibroblast growth factor modulates the mitogenic potency of the platelet-derived growth factor (PDGF) isoforms by specific upregulation of the PDGF alpha receptor in vascular smooth muscle cells. J Biol Chem 1992;267:18032–18039.
30. Zimmermann O, Zwaka TP, Marx N, Torzewski M, Bucher A, Guilliard P, Hannekum A, Hombach V, Torzewski J. Serum starvation and growth factor receptor expression in vascular smooth muscle cells. J Vasc Res 2006;43:157–165.
31. Hall H. Modified fibrin hydrogel matrices: Both, 3D-scaffolds and local and controlled release systems to stimulate angiogenesis. Curr Pharm Des 2007;13:3597–3607.
32. Mol A, Van Lieshout MI, Dam-De Veen CG, Neuenschwander S, Hoerstrup SP, Baaijens FP, Bouten CV. Fibrin as a cell carrier in cardiovascular tissue engineering applications. Biomaterials 2005; 26:3113–3121.
33. Cummings CL, Gawlitta D, Nerem RM, Stegemann JP. Properties of engineered vascular constructs made from collagen, fibrin, and collagen-fibrin mixtures. Biomaterials 2004;25:3699–3706.

34. Syedain ZH, Weinberg JS, Tranquillo RT. Cyclic distension of fibrin-based tissue constructs: Evidence of adaptation during growth of engineered connective tissue. Proc Natl Acad Sci USA 2008;105:6537–6542.
35. Shaikh FM, Callanan A, Kavanagh EG, Burke PE, Grace PA, Mcgloughlin TM. Fibrin: A natural biodegradable scaffold in vascular tissue engineering. Cells Tissues organs 2008;188:333–346.
36. Swartz DD, Russell JA, Andreadis ST. Engineering of fibrin-based functional and implantable small-diameter blood vessels. Am J Physiol Heart Circ Physiol 2005;288:H1451–H1460.
37. Sahni A, Simpson-Haidaris PJ, Sahni SK, Vaday GG, Francis CW. Fibrinogen synthesized by cancer cells augments the proliferative effect of fibroblast growth factor-2 (FGF-2). J Thromb Haemost 2008;6:176–183.
38. Sahni A, Khorana AA, Baggs RB, Peng H, Francis CW. FGF-2 binding to fibrin(ogen) is required for augmented angiogenesis. Blood 2006;107:126–131.
39. Duarte M, Kolev V, Kacer D, Mouta-Bellum C, Soldi R, Graziani I, Kirov A, Friesel R, Liaw L, Small D, Verdi J, Maciag T, Prudovsky I. Novel cross-talk between three cardiovascular regulators: Thrombin cleavage fragment of Jagged1 induces fibroblast growth factor 1 expression and release. Mol Biol Cell 2008;19:4863–4874.
40. Cao H, Dronadula N, Rao GN. Thrombin induces expression of FGF-2 via activation of PI3K-Akt-Fra-1 signaling axis leading to DNA synthesis and motility in vascular smooth muscle cells. Am J Physiol Cell Physiol 2006;290:C172–182.
41. Bassus S, Herkert O, Kronemann N, Gorlach A, Bremerich D, Kirchmaier CM, Busse R, Schini-Kerth VB. Thrombin causes vascular endothelial growth factor expression in vascular smooth muscle cells: Role of reactive oxygen species. Arterioscler Thromb Vasc Biol 2001;21:1550–1555.
42. Rauch BH, Millette E, Kenagy RD, Daum G, Clowes AW. Thrombin- and factor Xa-induced DNA synthesis is mediated by transactivation of fibroblast growth factor receptor-1 in human vascular smooth muscle cells. Circ Res 2004;94:340.
43. Rowe SL, Lee S, Stegemann JP. Influence of thrombin concentration on the mechanical and morphological properties of cell-seeded fibrin hydrogels. Acta Biomater 2007;3:59–67.
44. Millette E, Rauch BH, Kenagy RD, Daum G, Clowes AW. Platelet-derived growth factor-BB transactivates the fibroblast growth factor receptor to induce proliferation in human smooth muscle cells. Trends Cardiovasc medice 2006;16:25–28.
45. Pintucci G, Yu PJ, Saponara F, Kadian-Dodov DL, Galloway AC, Mignatti P. PDGF-BB induces vascular smooth muscle cell expression of high molecular weight FGF-2, which accumulates in the nucleus. J Cell Biochem 2005;95:1292–1300.
46. Pickering JG, Uniyal S, Ford CM, Chau T, Laurin MA, Chow LH, Ellis CG, Fish J, Chan BM. Fibroblast growth factor-2 potentiates vascular smooth muscle cell migration to platelet-derived growth factor: Upregulation of alpha2beta1 integrin and disassembly of actin filaments. Circ Res 1997;80:627–637.
47. Thie M, Harrach B, Schonherr E, Kresse H, Robenek H, Rauterberg J. Responsiveness of aortic smooth muscle cells to soluble growth mediators is influenced by cell-matrix contact. Arterioscler Thromb 1993;13:994–1004.
48. Thie M, Schlumberger W, Semich R, Rauterberg J, Robenek H. Aortic smooth muscle cells in collagen lattice culture: Effects on ultrastructure, proliferation and collagen synthesis. Eur J Cell Biol 1991;55:295–304.
49. Griffith LG, Swartz MA. Capturing complex 3D tissue physiology in vitro. Nature reviews. Mol Cell Biol 2006;7:211–224.
50. Stegemann JP, Hong H, Nerem RM. Mechanical, biochemical, and extracellular matrix effects on vascular smooth muscle cell phenotype. J Appl Physiol 2005;98:2321–2327.
51. Rensen SS, Doevendans PA, Van Eys GJ. Regulation and characteristics of vascular smooth muscle cell phenotypic diversity. Nerls Heart J 2007;15:100–108.
52. Stegemann JP, Nerem RM. Altered response of vascular smooth muscle cells to exogenous biochemical stimulation in two- and three-dimensional culture. Exp Cell Res 2003;283:146–155.
53. Rzucidlo EM, Martin KA, Powell RJ. Regulation of vascular smooth muscle cell differentiation. journal of vascular surgery: Official publication, the society for vascular surgery [and] international society for cardiovascular surgery. North Am Chapter 2007; 45(Suppl A):A25.