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# Analysis of VEGF, PDGF and bFGF Expression by Adipose Stem Cells Incorporated in Bioactive Platelet-Rich Fibrin Scaffolds

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requirements for the degree of

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by

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March 2017

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ABSTRACT

Analysis of VEGF, PDGF and bFGF Production by Adipose Stem Cells Incorporated in Fibrin-Rich Blood Clots

by

Kelsy R. Siegel

Surgical repair of some connective tissues, such as tendon, has a high failure rate. Exploration of surgical adjuvants, including platelet-rich plasma, bioactive scaffolds, and cell delivery, are being explored in an effort to enhance tissue healing. The purpose of this study is to quantify the capture and concentration of platelets (PLTs) during fibrin clot (FC) formation and to evaluate how the immunoreactivity and secretion of growth factors VEGF, PDGF and bFGF from FCs is modulated by the addition of allogeneic adipose stem cells (ASCs). Platelet concentration during fibrin clot formation was determined by quantifying pre-clot and post-clot serum samples. Fibrin clot formation concentrated 92% ($p<0.01$) of the available PLTs in whole blood. Two experimental conditions and one control condition were evaluated over two weeks *in vitro*. FCs made from human whole blood without ASCs, FC(-)ASC, were compared with FCs with ASCs incorporated, FC(+)ASC, and a control group of synthetic polyethylene glycol gels with ASCs incorporated, PEG(+)ASCs. All conditions were examined for secretion and retention of VEGF, PDGF, and bFGF. Analysis of platelet retention for FCs made with this device was performed. ELISA analysis showed significantly higher ($p<0.01$) secretion of VEGF in FC(+)ASC compared to FC(-)ASC or PEG(+)ASC. In contrast, FC(-)ASC produced soluble PDGF, and the addition of ASCs results in decreased soluble PDGF with concomitant increases in PDGF immunoreactivity of

ASCs. Soluble bFGF levels were low in FC(-)ASC, and were found to increase at early time-points in FC(+)ASC. Furthermore, bFGF immunoreactivity could be detected in FC(+)ASC while no bFGF immunoreactivity is present in FC(-)ASC or PEG(+)ASC. These findings provide evidence that FCs made through this described method concentrate a high proportion of available platelets that are known to secrete growth factors over time. The incorporation of ASCs to this system results in increased growth factor production, particularly of VEGF. Enhanced growth factor production localized to a surgical repair site may enhance the healing response and strengthen connective tissue to lower the incidence of re-tear.

## Introduction

Despite recent advances in surgical techniques for connective tissue repair, surgeons continue to explore the use of bioactive factors to enhance tissue healing and improve surgical outcomes[1-3]. The use of biologic agents may be particularly useful and effective for the treatment of compromised tissue. Efforts for this type of augmentation include the use of fibrin clot (FC), platelet-rich plasma (PRP), synthetic scaffolds and stem cells in the effort to increase localized production of beneficial growth factors[4-9].

Fibrin is a biopolymer found in blood that is involved in the coagulation cascade. Fibrin clots can be formed from peripheral blood and are comprised of a fibrin meshwork that can capture and contain platelets, act as a reservoir for cells, growth factors and other bioactive factors, and provide an effective delivery system for cells[10,11]. Fibrin clots have been shown to enhance the surgical repair of tissue with poor healing capacity[12-14]. In 1988, Arnoczky et al. described the application of a fibrin clot to aid healing of meniscal defects in the avascular zone in a canine model. Additional clinical studies have also demonstrated increased healing of meniscal tears with the application of fibrin clot including complete radial tears in the avascular zone and horizontal cleavage tears[13,15-17]. More recently, fibrin clot has been demonstrated in a caprine model to histologically improve healing of the intra-articular ACL reconstruction segment and to decrease the signal intensity on MRI, suggesting possible improvement of graft healing and maturation[18]. Furthermore, the addition of FC to allograft in human ACL reconstruction has been shown to reduce the amount of tunnel widening at one year[14].

The use of PRP as a method to concentrate and deliver bioactive factors also has been explored[3]. The increased concentration of platelets in PRP provides elevated levels of

localized growth factors important for healing of compromised tissue[19]. Some of these growth factors, including vascular endothelial growth factor (VEGF)[19,20], platelet-derived growth factor (PDGF)[19,20], and fibroblast growth factor (FGF)[20] are rapidly degraded and have a short half-life[21], limiting the potential clinical usefulness of PRP. Thus, there is a need to develop sustained delivery of growth factors.

Many types of stem cells express and secrete growth factors. Adipose tissue has proven to be a rich source of multipotent adult stem cells, similar to bone-marrow derived mesenchymal stem cells (BM-MSCs)[22]. Adipose derived stem cells (ASCs) have many of the same differentiation capabilities as BM-MSCs, while being significantly more abundant and readily isolated through standard liposuction procedures[23]. Additionally, ASCs have been found to express growth factors VEGF, PDGF, and bFGF, which have been extensively studied in regards to therapeutic application for damaged tissues[24,25]. Recent studies indicate that allogeneic ASCs have the potential to improve healing in compromised tendon, ligament and other tissues without eliciting an immune response[19-21].

The purpose of this study is to evaluate the secretion and retention of growth factors VEGF, PDGF, and bFGF from human peripheral blood FCs over two weeks in vitro, and to determine how levels are modulated by the addition of allogenic ASCs. It is hypothesized that FC formation will efficiently capture available platelets in whole blood, cells in the clot will secrete growth factors and VEGF, PDGF and bFGF will be retained in the FC over two weeks, and that the incorporation of ASCs into FCs will effectively increase the secretion and retention of these growth factors.

## Methods and Materials

### *Platelet capture:*

From 10 human volunteers, 35mL of whole blood was obtained; 5mL was evaluated for pre-clot cell count evaluation and the remaining 30mL was placed into a sterile container with a sintered glass cylinder supported by the lid (ClotMaster Hula Cup, Pierce Surgical Corp., Vermont) for FC formation. The FC was removed and 5ml of the post-clot serum was sent for post-clot formation cell count evaluation for platelets, red blood cells (RBCs) and white blood cells (WBCs). The cell counts for pre-clot whole blood and post-clot serum samples were compared using the DxH 800 Coulter Counting Cellular Analysis System (Beckman Coulter Inc, Brea CA). All work with human subjects in this study was approved by the UCSB Human Subjects Committee prior to performing the study (protocol # 4-16-0523). Informed consent was provided to all human subjects who volunteered in this study.

### *Cell lines and culture:*

Human ASCs isolated from lipoaspirate, transduced with a constitutive mCherry–luciferase reporter plasmid and selected for by previously established methods[26] were expanded in culture and frozen in liquid nitrogen for storage. Upon thaw, passage 4-6 cells were suspended in media consisting of 60% Dulbecco's modified Eagle's medium (DMEM; Life Technologies) with 10% fetal bovine serum (FBS;Atlas Biologicals, Fort Collins, CO) and 40% MesenPRO medium (Life Technologies). Cells and media were centrifuged 1200 *g* for 3 minutes and resulting cell pellets were resuspended in 3:2 DMEM +10% FBS:MesenPro media and switched to 100% MesenPRO 24–48 h later. Cells were plated in 225-$cm^2$ cell culture flasks (Sigma-Aldrich; CLS431082

) at a density of $2.2x10^3$cells/$cm^2$ where they were left to expand at 37°C. Media was replenished every two days until cells reached 80% confluence, at which point cells were lifted for seeding in blood clots or PEG gels.

***FC Formation and ASC Incorporation:***

Prior to FC formation, cells were washed with 10mL of phosphate buffered saline (PBS) and dissociated with 10mL TrypLE Select (Life Technologies; A1285901) at 37ºC for 5–10 min. The TrypLE solution was inactivated with a 1:1 dilution of MesenPRO and centrifuged at 1200 *g* for 3 minutes. Cells were resuspended in 2mL DMEM and counted with a hemocytometer to confirm a density of $5x10^6$ cells per mL. Whole human blood was collected from 10 allogeneic volunteers (80mL/volunteer), 20mL of which was deposited into each clotting device. For each volunteer, four FCs were created: two day zero FCs with and without ASCs and two FCs with and without ASCs to continue on for sampling through two weeks. FCs to be seeded with ASCs were each supplemented with $1x10^7$ cells in 2mL DMEM. FCs without ASCs were supplemented with 2mL of DMEM only. Immediately after whole blood was collected and supplemented with cells or media alone, the clot formation cups were placed on a rotator at room temperature for 10 minutes at 125rpm. After 10 minutes, day zero FCs(+/-)ASCs were washed twice with PBS and immediately fixed in 10mL of 4% paraformaldehyde in 0.1M sodium cacodylate and stored at 4ºC until cryo-preservation. FC(+/-)ASCs to be cultured for two weeks were washed twice with PBS and then placed in a 6-well polystyrene plate (Corning Inc., Corning, NY) in 5mL DMEM + 0.1mg/mL normocin (Invivogen; 50mg/mL) and incubated at 37ºC for two weeks. Media (DMEM+normocin) was replenished 24 hours prior to sampling as well as immediately

following sampling. Samples of media were collected at day 1, 3, 7, 9, 12, and 14, snap frozen in liquid nitrogen, and stored at -80°C for future enzyme-linked immunosorbent assay (ELISA) analysis. At day 14, clots were fixed in 4% paraformaldehyde in 0.1M sodium cacodylate and stored at 4°C until cryo-preservation.

***Polyethylene Glycol Gel Casting:***

A solution of 4-arm PEG-Thiol (Creative PEGWorks, Chapel Hill, NC; PSB-440) at 10 wt% was prepared in 37°C cell culture media. A solution of divinyl sulfone (DVS) crosslinker (Sigma-Aldrich) was prepared in 4°C cell culture media. A custom ECM-based peptide was synthesized by BioMatik (VnRGD-CGRCGKGGPQVTRGDVFTMPG-K-biotin) and gels were functionalized with VnRGD at 80mM concentrations before DVS crosslinker addition. After dissociation with TrypLE Select and pelleting, cells were resuspended in PEG/peptide solution ($1x10^7$ cells/gel) and the DVS crosslinker was added at a 10:1 ratio (DVS:PEG) immediately before plating. Approximately 800μL of gel solution was transferred into a 20mm aluminum ring mold. The PEG(+)ASC solution was left to polymerize for 30 min at 37°C, after which each gel was placed directly into 5mL DMEM + 0.1mg/mL normocin in separate wells of a 6-well polystyrene plate. PEG(+)ASCs were incubated at 37°C for two weeks. Media was replenished and samples were taken following the same protocol that was outlined for FC(+/-)ASCs above.

***Immunohistochemistry:***

Day 0 and 14 FC(+/-)ASCs (n=10), and control PEG(+)ASCs (n=5) were removed from 4% paraformaldehyde solution and washed three times with PBS. Samples were placed in 10%

sucrose in PBS for 2 hours, followed by 20% sucrose for 2 hours, 30% sucrose for 2 hours, and 40% sucrose overnight with gentle rotation at 4ºC. Following cryo-protection, samples were placed in plastic disposable base molds (ThermoFisher; 41744) with optimal cutting temperature (O.C.T.; Tissue-Tek, 4583) embedding medium. Once embedded in O.C.T., clots and gels were frozen with liquid nitrogen without full submersion.

For immunohistochemical analysis, all samples were embedded in O.C.T. and cut into 20μm sections using a cryostat (Leica; CM1850), and mounted onto polysine slides (ThermoFisher; P4981-001). Approximately 100 slides were collected from each sample. Tissue samples were subjected to a blocking solution consisting of 0.2% Triton X-100 in 1X PBS and 5% normal donkey serum (Abcam; ab138579) for 1 hour at room temperature. Cells were stained for 2 hours at 4ºC with primary antibodies diluted in the blocking solution (See Table 1). Following primary antibody staining, samples were washed with PBS 3 times and stained with secondary antibodies (See Table 1) and Hoechst 33342 (0.0004 mg/mL; Life Technologies) for 1 h in the dark at room temperature. Secondary antibody-only controls were performed for all conditions to confirm staining specificity. Samples were mounted under glass cover-slips (ThermoFisher) with 4% n-propyl gallate (Sigma) in 90% glycerol mounting media.

| Table 1: A List of Primary and Secondary Antibodies Used in this Study | | | | |
|---|---|---|---|---|
| Antibody | Species | Concentration (mg/mL) | Manufacturer | Catalog Number |
| VEGF-A | Rabbit Polyclonal (1°) | 0.002 | Thermo Fisher Scientific | PA516754 |
| PDGF-BB | Rabbit Polyclonal (1°) | 0.0065 | Abcam | ab23914 |
| FGF-2 | Rabbit Polyclonal (1°) | 0.0005 | Abcam | ab8880 |
| mCherry | Mouse Monoclonal (1°) | 0.004 | Abcam | ab125096 |
| Alexa Fluor® 488 | Donkey anti-Rabbit (2°) | 0.008 | Thermo Fisher Scientific | A-21206 |
| Alexa Fluor® 488 | Donkey anti-Mouse (2°) | 0.008 | Thermo Fisher Scientific | A-21202 |

***Imaging:***

All imaging of immunohistochemical samples was performed on a Spectral-based Laser Scanning Confocal Microscope (Olympus FV1000; Lasers: 405, 440, 488, 514, 559, 633) at 40x magnification. All scale bars were included at the time of imaging (scale=50μm).

***Growth Factor Quantification:***

The culture media from FC(+/-)ASCs and PEG(+)ASCs was sampled at each time point 24 hours after being replenished, and snap frozen in liquid nitrogen for ELISA analysis. ELISA kits for detecting vascular endothelial growth factor (VEGF-A; Life Technologies, KHG0111), platelet-derived growth factor (PDGF-BB; Abcam, ab100624), and basic fibroblast growth factor (FGF-2; Abcam, ab99979), were optimized and used according to the manufacturer's protocol to quantify the concentration of each growth factor present in the

media at each time point. Positive controls were included and implemented for each condition.

***Statistical Analysis:***

Statistical analysis was performed using a paired two-tailed Student's t-test for pre-clot and post-clot cell counts and all subsequent assays. All data are displayed as mean ± standard error. The threshold of statistical significance was set to $p \leq 0.05$ for all comparisons.

**Results**

***Platelet capture:***

The 10 volunteers (5 female and 5 male) had a mean age of 39.8 years. The mean platelet count of whole blood was $187.80 \times 10^3/\mu L$, WBC $5.52 \times 10^3/\mu L$ and RBC $4.47 \times 10^3/\mu L$. The mean platelet count of the post-clot plasma decreased significantly to $4.40 \times 10^6/\mu L$ ($p<0.01$) while neither the post-clot plasma WBC count ($4.79 \times 10^3$) nor the RBC count ($4.59 \times 10^3/\mu L$) changed significantly. By comparing the pre-clot platelet count to the post-clot platelet count it was estimated that FC fibrin clot captured and concentrated an average of 92% of the available platelets (See Table 2).

***Table 2: Platelet Retention Levels From Pre-clot to Post-clot Formation***

| | Age & Gender | | | | | | | | | | Mean | SD |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Subjects* | 52M | 36M | 34F | 51M | 29F | 31F | 29F | 55M | 26F | 55M | | |
| *Whole Blood:* | | | | | | | | | | | | |
| *WBC* | 6.9 | 6.3 | 5.9 | 5.3 | 5.4 | 3.4 | 5.1 | 5.9 | 6.6 | 4.4 | 5.52 | 1.05 |
| *RBC* | 5.1 | 4.8 | 4.4 | 4.6 | 4.2 | 3.6 | 4.2 | 5.0 | 4.1 | 4.8 | 4.47 | 0.46 |
| *PLT* | 119 | 190 | 158 | 186 | 249 | 272 | 177 | 222 | 154 | 151 | 187.8 | 47.43 |
| *Post-clot Plasma:* | | | | | | | | | | | | |
| *WBC* | 5.7 | 5.2 | 5.4 | 4.8 | 4.4 | 2.1 | 5.0 | 5.4 | 6.1 | 3.8 | 4.79 | 1.15 |
| *RBC* | 5.2 | 4.8 | 4.5 | 4.7 | 4.3 | 3.7 | 4.4 | 5.1 | 4.2 | 4.9 | 4.59 | 0.45 |
| *PLT* | 3 | 1 | 1 | 3 | 4 | 20 | 3 | 4 | 2 | 3 | 4.4 | 5.58 |

**Table 2.** Cell counts from 5 ml of pre-clot whole blood and 5 ml of post-clot serum samples.

### *VEGF immunoreactivity and secretion:*

Immunohistochemical analysis of VEGF indicates higher expression in FC with ASCs incorporated than in FC without ASCs, and PEG with ASCs. The presence of ASCs was confirmed by the expression of the mCherry marker (Figure 1 A-E). No VEGF immunoreactivity was detected in FC(-)ASC at day 0 or day 14 (Figure 1A, C - green). However, VEGF immunoreactivity was observed in FC(+)ASC at day 0 and day 14 localized to ASCs (Figure 1B, D - green). PEG(+)ASC at day 0 and day 14 showed no VEGF immunoreactivity within ASCs as indicated by a lack of signal in Figure 1E (green). White blood cell nuclei (blue) are also observed in FC(+/-)ASC, and can be distinguished from ASCs based on absence of mCherry signal in addition to being smaller with a more rounded morphology (Figure 1A-E).

VEGF secretion from FC(+)ASC was consistently elevated when compared to FC(-)ASC ($p<0.001$), and PEG(+)ASC ($p<0.001$) (Figure 1F) as determined by ELISA. The average concentrations for FC(+)ASC for days 1, 3, 7, 9, 12, and 14 were 607.10+/-2.29pg/mL to 803.83+/-4.27pg/mL (error=SEM). The average concentrations for FC(-)ASC for days 1, 3, 7, 9, 12, and 14 were 18.75+/-5.96pg/mL to 322.625+/-2.5414pg/mL. The average concentrations for PEG(+)ASC for days 1, 3, 7, 9, 12, and 14 were 0pg/mL to 249.23+/-11.25pg/mL.

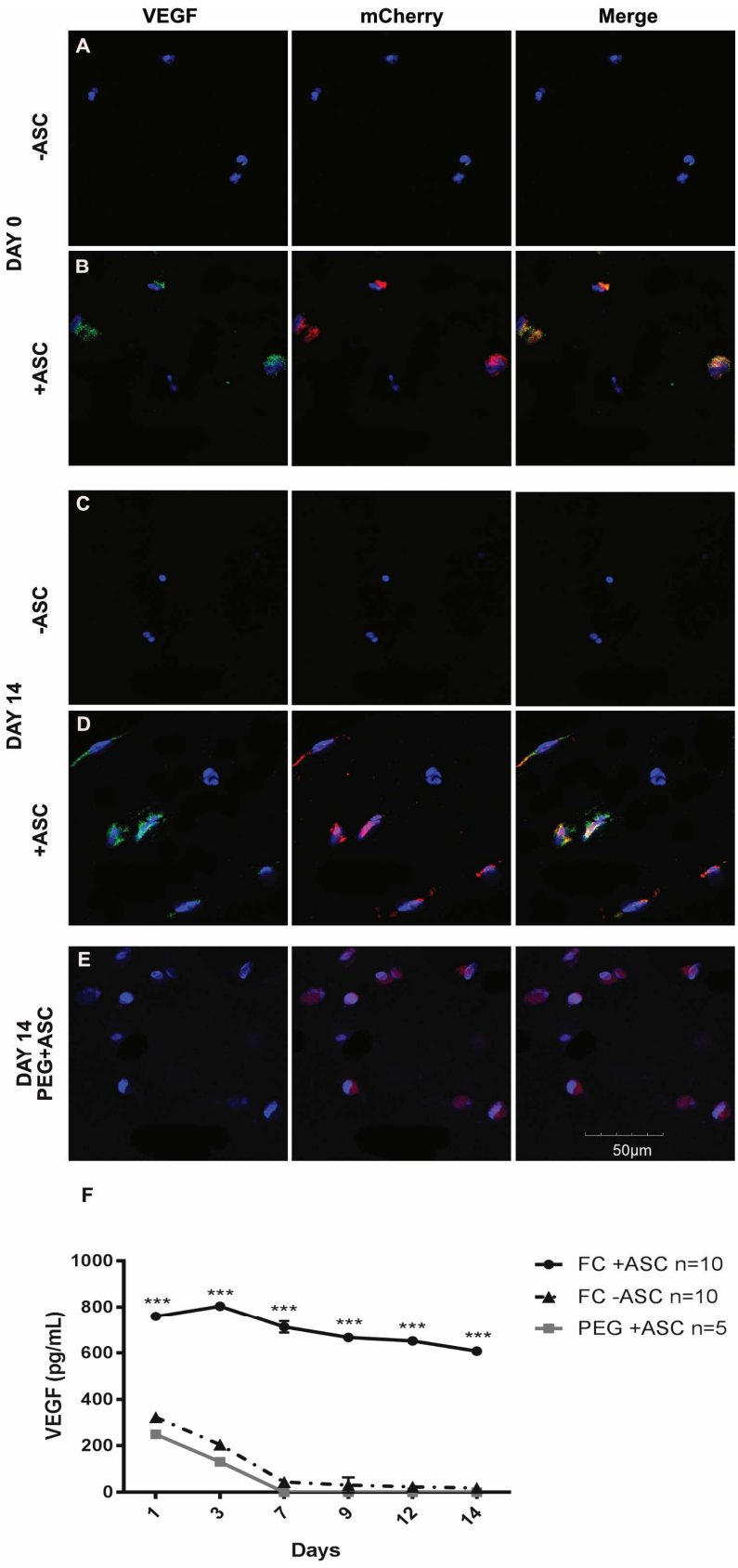


**Figure 1: VEGF immunoreactivity and secretion.** (A) FC(-)ASC at day 0. (B) FC(+)ASC at day 0. (C) FC(-)ASC at day 14. (D) FC(-)ASC at day 14. (E) PEG(+)ASC at day 14. (F) Secretion of VEGF by FC(-)ASC (black solid line), FC(+)ASC (black segmented line), and PEG(+)ASC (gray line) was quantified by ELISA at day 1,3,7,9,12, and 14. Significance compares FC(+)ASC to the control: PEG(+)ASC (***p<0.001;**p<0.01, *p<0.05). Error is displayed as SEM. VEGF, vascular endothelial growth factor; FC, fibrin clot; ASC, adipose stem cell; PEG, polyethylene glycol gel. Scale bar = 50 um for all images.

***PDGF immunoreactivity and secretion:***

PDGF immunoreactivity was detected in both white blood cells and ASCs in both FC without ASCs, FC with ASCs and PEG with ASCs. The FC(-)ASC sample showed decreasing PDGF immunoreactivity from day 0 to day 14 as indicated by an absence of PDGF signal at day 14 (Figure 2A, C - green). In contrast, PDGF immunoreactivity was detected in the day 14 sample of FC(+)ASC, with the PDGF signal localized to ASCs only as indicated by colocalizaton with the mCherry reporter signal (Figure 2B, D - green, red). PEG(+)ASC showed no PDGF immunoreactivity at day 0 and day 14 as indicated by a lack of PDGF signal (Figure 2E - green).

Based on data from ELISA analysis, PDGF secretion from FC(-)ASC was consistently elevated when compared to FC(+)ASC ($p<0.05$), and PEG(+)ASC ($p<0.001$) (Figure 2F). The average concentrations for FC(+)ASC for days 1, 3, 7, 9, 12, and 14 were between 0pg/mL to 123.77+/-1.786pg/mL. The average concentrations for FC(-)ASC for days 1, 3, 7, 9, 12, and 14 were 25.77+/-0.48pg/mL to 130.37+/-1.90pg/mL. The average concentrations for PEG(+)ASC for days 1, 3, 7, 9, 12, and 14 were 0pg/mL to 15.31+/-0.84pg/mL.

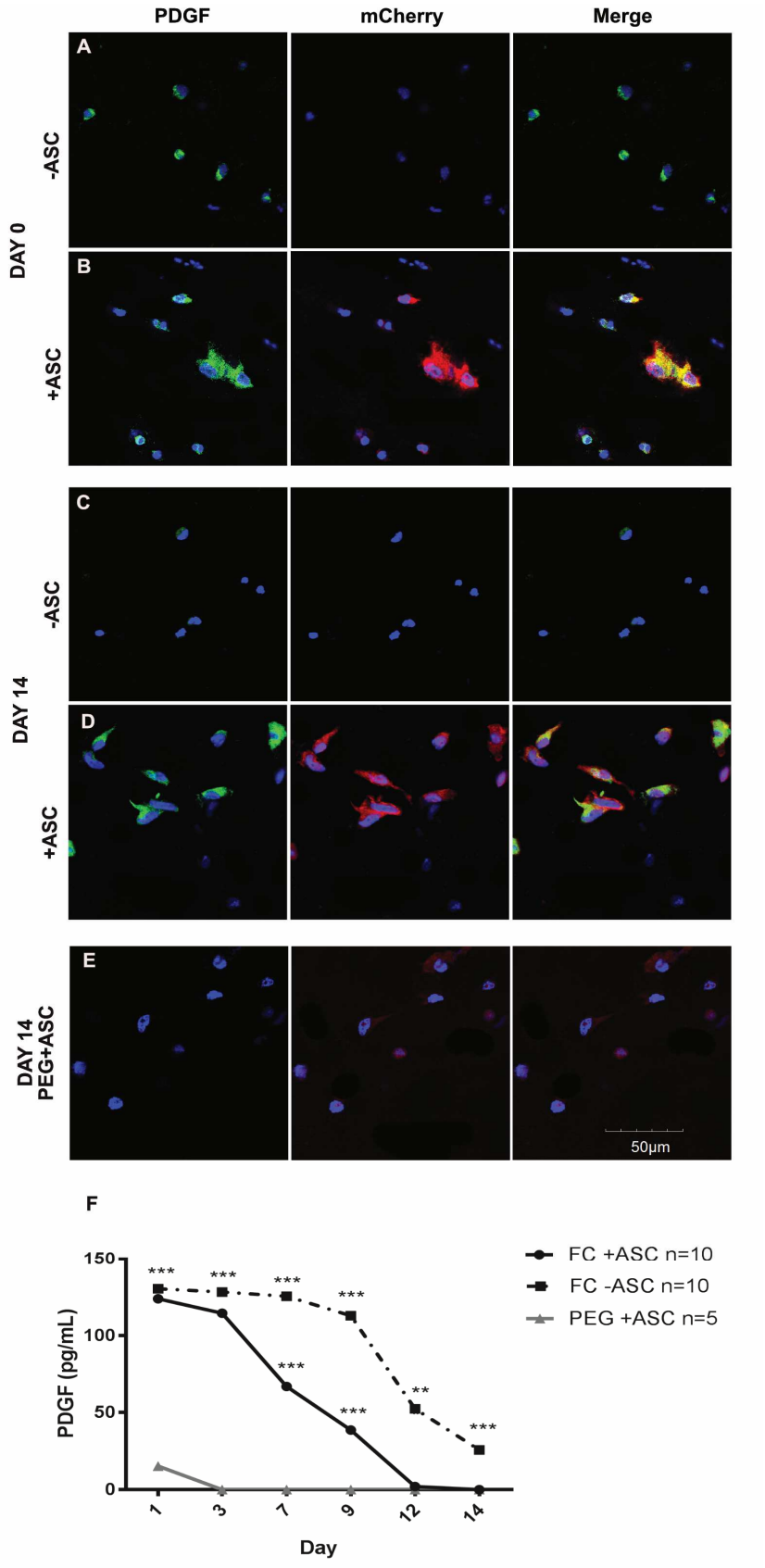


**Figure 2: PDGF immunoreactivity and secretion**. (A) FC(-)ASC at day 0. (B) FC(+)ASC at day 0. (C) FC(-)ASC at day 14. (D) FC(+)ASC at day 14. (E) PEG(+)ASC at day 14. (F) Secretion of PDGF by FC(+)ASC (black solid line), FC(-)ASC (black segmented line), and PEG(+)ASC (gray line) was quantified by ELISA at day 1,3,7,9,12, and 14. Significance compares FC(+)ASC, and FC(-)ASC to the control: PEG(+)ASC (***$p<0.001$;**$p<0.01$, *$p<0.05$). Error is displayed as SEM. PDGF, platelet-derived growth factor; FC, fibrin clot; ASC, adipose stem cell; PEG, polyethylene glycol gel. Scale bar = 50 um for all images.

***bFGF immunoreactivity and secretion:***

Immunohistochemical (IHC) analysis of bFGF indicated higher immunoreactivity in FC with ASCs incorporated than in FC without ASCs or in PEG with ASCs. FC(+)ASC showed bFGF immunoreactivity at day 0 and day 14 as indicated by bFGF signal that is localized to ASCs only as indicated by colocalization with the mCherry reporter signal (Figure 3B, D – green, red). FC(-)ASC show no bFGF immunoreactivity at day 0 or day 14 as indicated by a lack of bFGF signal (Figure 3A, C - green). PEG(+)ASC also showed no bFGF immunoreactivity at day 0 or day 14 from ASCs as indicated by a lack of bFGF signal (Figure 3E - green).

Based on ELISA analysis, bFGF secretion from FC(+)ASC was elevated when compared to FC(-)ASC at days 1, 3 and 9 ($p<0.05$), but not at days 12 and 14 (Figure 3F). PEG(+)ASC show a significant spike in bFGF ($p<0.001$) secretion compared to FC(+)ASC and FC(-)ASC between days 0 and 3, and then drop to levels at or below those of FC(+)ASC by day 7. The average concentrations for FC(+)ASC for days 1, 3, 7, 9, 12, and 14 were 379.88+/-13.64pg/mL to 1599.21+/-14.68pg/mL. The average concentrations for FC(-)ASC for days 1, 3, 7, 9, 12, and 14 were 189.35+/-15.03pg/mL to 587.15+/-9.47pg/mL. The average concentrations for PEG(+)ASC for days 1, 3, 7, 9, 12, and 14 were 510.55+/-12.23pg/mL to 5667.72+/- 11.25pg/mL.

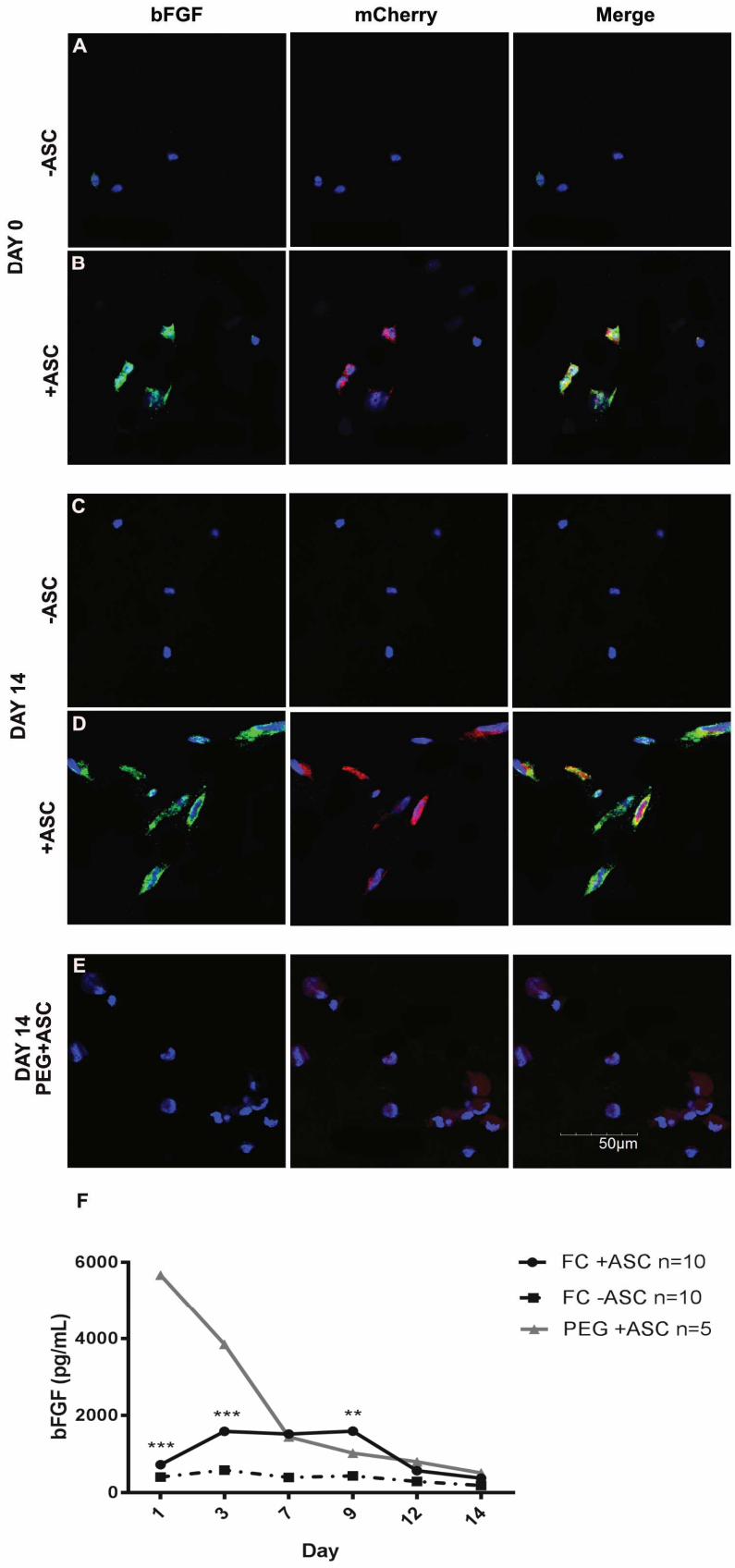


**Figure 3: bFGF immunoreactivity and secretion.** (A) FC(-)ASC at day 0. (B) FC(+)ASC at day 0. (C) FC(-)ASC at day 14. (D) FC(+)ASC at day 14. (E) PEG(+)ASC at day 14. (F) Secretion of bFGF by FC(+)ASC (black solid line), FC(-)ASC (black segmented line), and PEG(+)ASC (gray line) was quantified by ELISA at day 1,3,7,9,12, and 14. Significance compares FC(+)ASC to the control: PEG(+)ASC (***$p<0.001$;**$p<0.01$, *$p<0.05$). Error is displayed as SEM. bFGF, basic fibroblast growth factor; FC, fibrin clot; ASC, adipose stem cell; PEG, polyethylene glycol gel. Scale bar = 50 um for

**Discussion**

Despite the long history of FC being used to augment tissue healing in the orthopedic literature, to our knowledge, this is the first study to determine platelet capture during FC formation. Our finding of a platelet capture rate averaging 92% compares favorably to prior studies that have demonstrated platelet capture rates of traditional PRP ranging from 17-80%[27]. Additionally, FC formation using this technique did not significantly change the WBC count in the post clot serum indicating that the FC did not selectively capture and concentrate available WBCs. These findings indicate that this technique for fibrin clot formation creates a platelet rich fibrin scaffold (PRFS) without concentrating WBCs. Since FCs capture platelets, they produce similar growth factors to PRP but also provide a structural support and a scaffold for cellular ingrowth, while serving as a reservoir for growth factor release over time from incorporated cells[18,28]. The dense nature of the PRFS also provides a structural integrity that allows for arthroscopic suturing over a repair site[29].

The use of biologic and synthetic scaffolds to deliver stem cells for therapeutic benefit has become increasingly widespread. A major challenge faced when seeding cells in a synthetic scaffold is mimicking an environment that will provide an adequate niche for cell survival and proliferation. Common synthetic scaffolds including polypropylene, polyurethane, and polytetrafluoroethylene can produce mechanical stresses, illicit host response, and inhibit integration into recipient tissue[16,30]. In contrast to synthetic materials, biologic materials may provide a more suitable environment to adipose stem cells by promoting sustained proliferation, in addition to growth factor recruitment and secretion[9]. The use of biomaterials such as decellularized ECM and PRP has been explored for tissue healing, but have been found to be sub-optimal in regards to cell survival, localization, and

growth factor retention[9,31]. Our findings demonstrate that *in vitro*, FC secretes VEGF, PDGF and bFGF over 14 days. Additionally, VEGF expression from ASCs was increased substantially when incorporated in a FC. This underscores the possibility that FCs may be an enhanced method for delivering cells and their bioproducts to a localized area. Our findings indicate that the FC may act as an effective well for cells to attach and express growth factors that may provide therapeutic benefit to biologically compromised tissue.

Previous studies have shown that in varying conditions, ASCs have receptors for VEGF, PDGF, and bFGF *in vitro* as well as *in vivo,* and can effectively express these growth factors through intra- and extra-cellular signalling[24,25]. The interaction between the fibrin matrix of different materials and endothelial cells has been investigated thoroughly in studies to characterize how the expression of VEGF, PDGF, and bFGF is modulated[32-34]. To our knowledge, this is the first study to characterize the secretion and retention of VEGF, PDGF, and bFGF from ASCs incorporated in the fibrin matrix of an allogeneic FC.

***Interaction between VEGF, PDGF, and Fibrin***

ASCs have been found to express VEGF without exogenous supplementation in vitro[35]. We found significantly higher secretion of soluble VEGF in FC(+)ASC compared to FC(-)ASC or PEG(+)ASC. In addition, IHC analysis indicates that VEGF immunoreactivity of FC(+)ASC is elevated compared to the other conditions. These results may be suggestive of a synergistic interaction between ASCs and the fibrin matrix of the FC. Factors within the FC may increase ASC expression of VEGF, which could initiate a positive feedback loop that in turn promotes increased cell surface VEGF receptors and subsequent enhancement of VEGF immunoreactivity in ASC[32]. The VEGF secretion data collected here supports

previous findings that ASCs secrete levels of VEGF that are comparable to BM-MSCs and endothelial cells upon interaction with a synthetic fibrin matrix[36]. Based on significantly elevated VEGF secretion from FC(+)ASC when compared to FC alone (FC-ASC) and ASC alone (PEG+ASC), the environment of the FC may be conducive to VEGF production from ASCs.

Upon clot formation, the alpha granules of platelets are activated and quickly release anabolic cytokines like PDGF[37]. Exogenous PDGF has been found to bind with high affinity to PDGF receptors on the surface of ASCs[25]. Fibrin matrices have been shown to slowly release PDGF and VEGF over time[38], suggesting that once these anabolic cytokines are released from platelets, free PDGF may be steadily supplemented to ASCs that are attached to the FC, which can readily be bound by cell surface receptors. This conclusion is supported by the enhanced immunoreactivity of PDGF in FC(+)ASC than in FC(-)ASC and PEG(+)ASC conditions. Lower levels of PDGF in the surrounding media of FC(+)ASC, as confirmed by ELISA analysis, further supports that ASCs may bind and retain free PDGF that is being released from the platelets in the FC.

Furthermore, when PDGF is bound to its cell surface receptor on ASCs, intracellular signaling with VEGF is activated[39]. VEGF is known to be the primary initiator of angiogenesis, but has been shown to be insufficient in establishing mature and complex vasculature[40]. PDGF is known to initiate blood vessel maturation by recruiting smooth muscle cells to developing vasculature, but is incapable in initiating nascent vessels[40]. These characteristics led to the finding that dual delivery of VEGF and PDGF to a synthetic scaffold results in a complex and mature vascular network[40]. Studies have found that PDGF bound to human cells significantly up-regulates VEGF expression within the cell[39,41,42].

While this study does not evaluate the angiogenic potential of the FC(+)ASC construct, it is an intriguing possibility for the future investigation of the therapeutic application of this novel cell delivery system.

***Interaction between VEGF, bFGF, and Fibrin***

Previous studies have found that undifferentiated and differentiated ASCs express bFGF receptors without exogenous supplementation[25,43]. This study found that the concentration of bFGF may be modulated by the interaction between ASCs and a fibrin matrix. Secreted bFGF from endothelial cells has been found to bind to fibrinogen and fibrin with high affinity, allowing for a large degree of retention in a synthetic fibrin matrix[32,44]. The observed bFGF immunoreactivity in ASCs incorporated in a FC may be due to an interaction with the fibrin matrix of the FC.

The secretion data provides evidence that the fibrin matrix of the FC is providing attachment and eventual release of bFGF, which may be available to then bind to ASCs and activate expression of endogenous bFGF. The process of embedding ASCs into PEG can be harsh, and may be sufficient to illicit a stress response from the cells. It has been shown that bFGF is secreted by cells immediately following injury[45], which may explain the initial spike in secretion of bFGF from PEG(+)ASC at day 0. The immediate drop in secretion and lack of bFGF immunoreactivity in PEG(+)ASC could be evidence that PEG does not retain adequate amounts of bFGF to promote subsequent up-take by ASCs. Ultimately, the fibrin matrix of the FC may be retaining and potentially enhancing bFGF up-take by ASCs. This binding of soluble bFGF could effectively increase bFGF receptor formation on ASCs, which has been found to promote cellular stability when combined with VEGF receptors[34,46].

Previous studies have found that growth factors are more effective when expressed in combination, and in some instances, their expression is inhibited in the absence of certain growth factors[39,47,48]. VEGF and bFGF have been studied extensively for their synergistic relationship[39,44,46,47]. When supplemented exogenously or endogenously, bFGF has been shown to up-regulate VEGF mRNA expressed by endothelial cells[49]. Moreover, quiescent endothelial cells could not produce VEGF unless supplemented with exogenous or endogenous bFGF, which acts both intra- and extracellularly to promote VEGF expression. The extracellular signaling of bFGF with VEGF membrane receptors acts to stimulate cell proliferation, migration, and up-regulate VEGF mRNA production[49,50]. Intracellular bFGF signaling is localized to the nucleus and modulates cell proliferation while up-regulating VEGF mRNA production from within the cell[49,50]. The high bFGF immunoreactivity of ASCs when incorporated in a FC may therefore be interacting with cellular VEGF, resulting in increased expression of VEGF as confirmed by significant increases in ELISA levels of VEGF in FC(+)ASC.

***Interaction between PDGF, bFGF, and Fibrin***

Synergism is also evident between PDGF and bFGF[41,51]. PDGF has been found to stimulate proliferation and migration of smooth muscle cells that could be beneficial in tissue healing[51]. PDGF expression is activated by the ERK pathway, which is initiated by tyrosine kinase receptors, FGFR-1/2, which in turn are activated by endogenous bFGF[51]. ASCs have been found to produce bFGF endogenously[43]. Exogenous bFGF is also available for cellular uptake through heparanase activity in the extracellular matrix of an in vivo environment[52], or as demonstrated here, from the fibrin matrix of the FC. In the absence of bFGF, PDGF has

been found to be significantly down-regulated[41], which could subsequently lower VEGF expression. The findings from this study demonstrate that ASCs may exhibit increased expression of VEGF when incorporated in a FC due to the presence of endogenous bFGF and exogenous PDGF. This conclusion supports previous findings that indicate a synergistic behavior exists between these three growth factors, which may be promoted by the environment of the FC.

**Conclusions**

This study has demonstrated FC formation binds platelets and releases VEGF, PDGF and bFGF over 14 days *in vitro*. Further, VEGF levels were enhanced in FCs containing ASCs. The addition of ASCs in a FC results in decreased soluble PDGF with concomitant increases in PDGF immunoreactivity in ASCs. Soluble bFGF increased when FCs were combined with ASCs, and bFGF immunoreactivity was detected in ASC encapsulated in FCs. Whole blood fibrin clots may provide a hospitable environment for ASC attachment and expression of VEGF, and bFGF, along with retention of PDGF. Enhanced expression of VEGF, PDGF, and bFGF that is localized to a surgical repair site has the potential to augment tissue healing.

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