Title

Adipose Stem Cells Incorporated in Fibrin Clot Modulate Expression of Growth Factors

Journal

Arthroscopy The Journal of Arthroscopic and Related Surgery, 34(2)

ISSN

0749-8063

Authors

Siegel, Kelsy R
Clevenger, Tracy N
Clegg, Dennis O
et al.

Publication Date

2018-02-01

DOI

10.1016/j.arthro.2017.08.250

Peer reviewed

Adipose Stem Cells Incorporated in Fibrin Clot Modulate Expression of Growth Factors

Kelsy R. Siegel, M.S., Tracy N. Clevenger, Ph.D., Dennis O. Clegg, Ph.D., Duncan A. Proctor, B.S., and Christopher S. Proctor, M.D.

Purpose: To evaluate the platelet capture rate of whole blood fibrin clots and the expression, secretion, and retention of the growth factors vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and basic fibroblast growth factor (bFGF) from fibrin clots and to determine how these levels may be modulated by allogeneic adipose-derived stem cells (ASCs). **Methods:** Whole blood from 10 human volunteers was transferred to a clotting device and the platelet capture rate determined. Two experimental conditions and 1 control were evaluated over 2 weeks in vitro. Clots made from human whole blood without ASCs, clot(−)ASC, were compared with clots with ASCs incorporated, clot(+)ASC, and a control group of synthetic polyethylene glycol gels with ASCs incorporated, control(+)ASCs. All conditions were examined for secretion and retention of VEGF, PDGF, and bFGF via enzyme-linked immunosorbent assay and immunohistochemistry. The analysis of platelet retention for clots made with this device was performed. **Results:** Enzyme-linked immunosorbent assay analysis showed significantly higher ($P < .001$) secretion of VEGF in clot(+)ASC compared with clot(−)ASC or control(+)ASC. In contrast, clot(−)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 clot(−)ASC, and were found to increase at early time points in clot(+)ASC. Furthermore, bFGF immunoreactivity could be detected in clot(+)ASC, whereas no bFGF immunoreactivity is present in clot(−)ASC or control(+)ASC. Control(+)ASC displayed a spike in bFGF secretion at day 0, which may be due to a stress response elicited by the encapsulation process. Approximately 98% of available platelets in whole blood were concentrated in the clot on formation. **Conclusions:** Fibrin clots made by this method retain high concentrations of platelets, and when incorporated with ASCs show modulated secretion and immunoreactivity of VEGF, PDGF, and bFGF. **Clinical Relevance:** Whole blood fibrin clots capture platelets and release growth factors, and the addition of ASCs increases VEGF release for up to 2 weeks after clot formation. This suggests that whole blood fibrin clots may be a viable scaffold and delivery vehicle for future stem cell treatments.

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 clots, platelet-rich plasma, synthetic scaffolds, and stem cells in the effort to increase localized production of beneficial growth factors.[4-9]

Fibrin clots can be formed from peripheral blood and comprise 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.[12] described the application of a fibrin clot to aid healing of meniscal defects in the avascular zone in a

From the Center for Stem Cell Biology and Engineering, Neuroscience Research Institute, and Department of Molecular, Cellular and Developmental Biology, University of California (K.R.S., T.N.C., D.O.C, D.A.P.); and Alta Orthopaedics (C.S.P.), Santa Barbara, California, U.S.A.

The authors report the following potential conflicts of interest or sources of funding: C.S.P. receives $5,000 grant from Pierce Surgical; receives consultancy fees from Arthrex; is a private practitioner at Alta Orthopaedics; and owns stock in Pierce Surgical (less than $10,000, less than 5% of company, no voting or control rights). K.R.S receives grant from U.S. Army Research Office (W911NF-09-0001). Full ICMJE author disclosure forms are available for this article online, as supplementary material.

Received February 9, 2017; accepted August 3, 2017.

Address correspondence to Kelsy R. Siegel, M.S., Department of Molecular, Cellular and Developmental Biology, University of California, Biological Sciences II rm 5166, Santa Barbara, CA 93106, U.S.A. E-mail: kelsy.siegel@lifesci.ucsb.edu

0749-8063/17145/$36.00

http://dx.doi.org/10.1016/j.arthro.2017.08.250

canine model. Additional clinical studies have also shown increased healing of meniscal tears with the application of fibrin clots including complete radial tears in the avascular zone and horizontal cleavage tears.[13,15-17] Fibrin clots have more recently been shown in a caprine model to histologically improve healing of the intra-articular anterior cruciate ligament reconstruction segment and to decrease the signal intensity on magnetic resonance imaging suggesting possible improvement of graft healing and maturation.[18] Furthermore, the addition of a fibrin clot to allograft in anterior cruciate ligament reconstruction has been shown to reduce the amount of tunnel widening at 1 year.[14]

The use of platelet-rich plasma as a method to concentrate and deliver bioactive factors has been explored.[3] The platelets in platelet-rich plasma provide an increase in 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 basic fibroblast growth factor (bFGF),[20] are rapidly degraded and have a short half-life,[21] limiting the potential clinical usefulness of platelet-rich plasma.

Adipose-derived stem cells (ASCs) have many of the same differentiation capabilities as bone marrow-derived mesenchymal stem cells, while being significantly more abundant and readily isolated through standard liposuction procedures.[22,23] In addition, ASCs have been found to express growth factors that have been extensively studied in regard 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.[19-21]

The purpose of this study was to determine the platelet capture rate during whole blood fibrin clot formation and to evaluate the expression, secretion, and retention of the growth factors VEGF, PDGF, and bFGF in fibrin clots and to determine how levels may be modulated by ASCs. We hypothesized that fibrin clots would capture platelets and release growth factors, and the incorporation of ASCs would promote growth factor production.

Methods

Platelet Capture

From 10 human volunteers (7 female, 3 male, mean age of 30 years), 35 mL of whole blood was obtained; 5 mL was sent for preclot cell count evaluation and the remaining 30 mL was placed into a sterile container with a sintered glass cylinder supported by the lid (ClotMaster Hula Cup, Pierce Surgical, Waterbury, VT) for fibrin clot formation. Our inclusion criteria were age range 18 to 60 years with normal platelet count, red blood cell count, and white blood cell count, and subjects were excluded if they had a history of coagulopathy, platelet disorders, use of anticoagulants or platelet inhibitors, recent history of infection, or immunocompromised status. The fibrin clot was removed and 5 mL of the postclot serum was sent for postclot formation and cell count evaluation for platelets, red blood cells, and white blood cells. The cell counts for preclot whole blood and postclot serum samples were compared using the DxH 800 Coulter Counting Cellular Analysis System (Beckman Coulter, Brea, CA). All work with human subjects in this study was approved by a Human Subjects Committee before performing the 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 isotyping was performed with flow cytometry to confirm the presence of stem cell markers.[26] On thaw, passage 4 to 6 cells were suspended in media consisting of 60% Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Carlsbad, CA) with 10% fetal bovine serum (Atlas Biologicals, Fort Collins, CO) and 40% MesenPRO medium (Life Technologies). Cells and media were centrifuged 1,200 g for 3 minutes, and resulting cell pellets were resuspended in 3:2 DMEM + 10% fetal bovine serum:MesenPro media and switched to 100% MesenPRO 24 to 48 hours later. Cells were plated in 225 cm^2 cell-culture flasks (Sigma-Aldrich; CLS431082) at a density of 2.2×10^3 cells/cm^2 where they were left to expand at 37°C. Media was replenished every 2 days until cells reached 80% confluence. Cells were then lifted for seeding in blood clots or polyethylene glycol (PEG) gels.

Fibrin Clot Formation and ASC Incorporation

Before fibrin clot formation, cells were washed with 10 mL of phosphate-buffered saline (PBS) and dissociated with 10 mL TrypLE Select (Life Technologies; A1285901) at 37°C for 5 to 10 minutes. The TrypLE solution was inactivated with a 1:1 dilution of MesenPRO and centrifuged at 1,200 g for 3 minutes. Cells were resuspended in 2 mL DMEM and counted with a hemocytometer to confirm a density of 5×10^6 cells/mL. Whole human blood was collected from the same 10 volunteers (80 mL/volunteer), 20 mL of which was deposited into each clotting device. For each volunteer, 4 clots were created: 2-day zero clots with and without ASCs and 2 clots with and without ASCs to continue on for sampling through 2 weeks. Clots to be seeded with ASCs were each supplemented with 1×10^7 cells in 2 mL DMEM. Clots without ASCs were supplemented with 2 mL 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 125 rpm. After 10 minutes, day zero clots(±)ASCs were washed twice with PBS and immediately fixed in 10 mL of 4% paraformaldehyde in 0.1 M sodium cacodylate and stored at 4°C until cryo-preservation. Clots(±)ASCs to be cultured for 2 weeks were washed twice with PBS and then placed in a 6-well polystyrene plate (Corning, Corning, NY) in 5 mL DMEM + 0.1 mg/mL normocin (Invivogen; 50 mg/mL) and incubated at 37°C for 2 weeks. Media (DMEM+normocin) was replenished 24 hours before sampling as well as immediately after sampling. Samples of media were collected at days 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 0.1 M sodium cacodylate and stored at 4°C until cryopreservation.

PEG Gel Casting

A solution of 4-arm PEG-Thiol (Creative PEGWorks, Chapel Hill, NC; PSB-440) at 10 wt% was prepared in cell-culture media at 37°C. A solution of divinyl sulfone (DVS) crosslinker (Sigma-Aldrich) was prepared in cell-culture media at 4°C. A custom extracellular matrix-based peptide was synthesized by BioMatik (VnRGD-CGRCGKGGPQVTRGDVFTMPG-K-biotin) and gels were functionalized with VnRGD at 80 mM concentrations before the addition of the DVS crosslinker. After dissociation with TrypLE Select and pelleting, cells were resuspended in PEG/peptide solution (1×10^7 cells/gel) and the DVS crosslinker was added at a 10:1 ratio (DVS:PEG) immediately before plating. Approximately 800 μL of gel solution was transferred into a 20-mm aluminum ring mold. The PEG(+)ASC (control) solution was left to polymerize for 30 minutes at 37°C, after which each gel was placed directly into 5 mL DMEM + 0.1 mg/mL normocin in separate wells of a 6-well polystyrene plate. All controls were incubated at 37°C for 2 weeks. Day 0 controls + ASCs were not fixed for immunohistochemical (IHC) analysis because of the expense of the materials. Media was replenished and samples were taken following the same protocol that was outlined for clots(±)ASCs above.

Immunohistochemistry

Day 0 and 14 clots(±)ASCs (n = 10), and controls(+) ASCs (n = 5) were removed from 4% paraformaldehyde solution and washed 3 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 4°C. After cryoprotection, samples were placed in plastic disposable base molds (ThermoFisher; 41744) with the 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 IHC analysis, all samples were embedded in O.C.T. and cut into 20-μm 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 4°C with primary antibodies diluted in the blocking solution. After primary antibody staining, samples were washed with PBS 3 times and stained with secondary antibodies and Hoechst 33342 (0.0004 mg/mL; Life Technologies) for 1 hour 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.

Imaging

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

Growth Factor Quantification

The culture media from clots(±)ASCs and controls(+) 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 VEGF-A (Life Technologies, KHG0111), PDGF-BB (Abcam, ab100624), and bFGF-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 2-tailed Student's *t*-test for preclot and postclot cell counts and all subsequent assays. All data are displayed as mean ± standard error. The threshold of statistical significance was set to $P \leq .05$ for all comparisons.

Results

Platelet Capture

The 10 human volunteers (7 female and 3 male) had a mean age of 30 years and all met the inclusion and exclusion criteria. The mean platelet count of whole blood was $187.80 \times 10^3/\mu L$, white blood cell $5.52 \times 10^3/\mu L$,

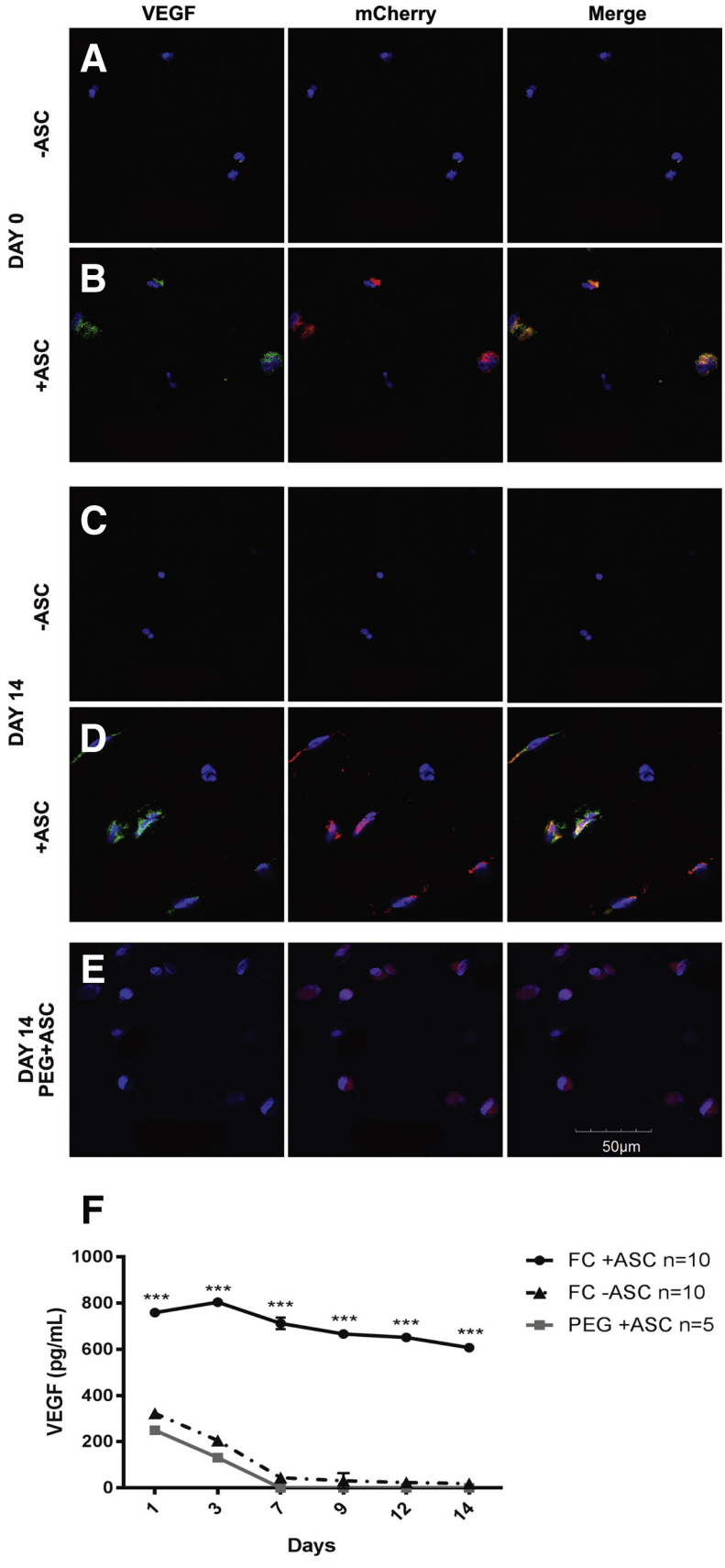

Fig 1. VEGF retention and secretion. (A) FC(−) ASC at day 0. (B) FC(+)ASC at day 0. (C) FC(−) ASC at day 14. (D) FC(+)ASC at day 14. (E) PEG(+)ASC at day 14. (F) Secretion of VEGF by FC(−)ASC (black solid line), FC(+)ASC (black segmented line), and PEG(+)ASC (gray line) was quantified by enzyme-linked immunosorbent assay at days 1, 3, 7, 9, 12, and 14. Significance compares FC(+)ASC with the control: PEG(+)ASC ($^{***}P < .001$). Average P values for FC(+)ASC at days 1, 3, 7, 9, 12, and 14 are all less than .001 when compared with FC(−)ASC and PEG(+)ASC. Error is displayed as standard error of the mean. Scale bar = 50 μm for all images. (ASC, adipose stem cell; FC, fibrin clot; PEG, polyethylene glycol gel; VEGF, vascular endothelial growth factor.)

and red blood cell 4.47 × 10^6/μL. The mean platelet count of the postclot plasma decreased significantly to 4.40 × 10^3/μL ($P < .001$), whereas neither the postclot plasma white blood cell count (4.79 × 10^3/μL, $P = .16$) nor the red blood cell count (4.59 × 10^6/μL, $P = .57$) changed significantly. By comparing the preclot platelet count with the postclot platelet count, it was estimated that fibrin clot captured and concentrated an average of 98% of the available platelets. We believe that the small increase in the mean red blood cell count in the postclot plasma is a result of an acceptable counting error of the cellular analysis system.

VEGF-A Immunoreactivity and Secretion

IHC analysis of VEGF-A indicated expression in fibrin clots with ASCs incorporated when compared to fibrin clots without ASCs, and controls with ASCs. No VEGF immunoreactivity was detected in clot(−)ASC at day 0 or day 14 (Fig 1 A and C—green). However, VEGF immunoreactivity was observed in clot(+)ASC at day 0 and day 14 localized to ASCs (Fig 1 B and D—green). Control(+)ASC at day 0 and day 14 showed no VEGF immunoreactivity within ASCs as indicated by a lack of signal in Figure 1E (green). Although we did not quantitatively determine the rate that the ASCs were captured, our study did show (with m-Cherry and Hoescht colocalization) that ASCs were being captured by the clot. White blood cell nuclei (blue) are also observed in clot(±)ASC, and can be distinguished from ASCs based on the absence of mCherry signal in addition to being smaller with a more rounded morphology (Fig 1A-E).

VEGF secretion from clot(+)ASC was consistently elevated when compared with clot(−)ASC ($P < .001$) and control(+)ASC ($P < .001$) (Fig 1F) as determined by ELISA. Refer to Table 1 for ELISA concentrations.

PDGF Immunoreactivity and Secretion

PDGF immunoreactivity was detected in both white blood cells and ASCs in fibrin clots without ASCs, fibrin clots with ASCs, and controls with ASCs. The clot(−)ASC sample showed decreased PDGF immunoreactivity from day 0 to day 14 as indicated by an absence of the PDGF signal at day 14 (Fig 2 A and C—green). In contrast, PDGF immunoreactivity was detected in the day 14 sample of clot(+)ASC, with the PDGF signal localized to ASCs only as indicated by colocalizaton with the mCherry reporter signal (Fig 2 B and D—green, red). Control(+)ASC showed no PDGF immunoreactivity at day 0 and day 14 as indicated by a lack of the PDGF signal (Fig 2E—green).

Based on data from ELISA analysis, PDGF secretion from clot(−)ASC was consistently elevated when compared with clot(+)ASC ($P = .039$) and control(+)ASC ($P < .001$) (Fig 2F). Refer to Table 1 for ELISA concentrations.

Basic FGF Immunoreactivity and Secretion

IHC analysis of bFGF indicated expression in fibrin clots with ASCs incorporated compared with fibrin clots without ASCs or in controls with ASCs. Clot(+)ASC showed bFGF immunoreactivity at day 0 and day 14 as indicated by the bFGF signal that is localized to ASCs only as indicated by colocalization with the mCherry reporter signal (Fig 3 B and D—green, red). Clot(−)ASC showed no bFGF immunoreactivity at day 0 or day 14 as indicated by a lack of the bFGF signal (Fig 3 A and C—green). Control(+)ASC also showed no bFGF immunoreactivity at day 0 or day 14 from ASCs as indicated by a lack of the bFGF signal (Fig 3E—green).

Based on ELISA analysis, bFGF secretion from clot(+)ASC was elevated when compared with clot(−)ASC at days 1, 3, and 9 ($P = .026$), but not at days 12 and 14 (Fig 3F). Control(+)ASC showed a significant spike in bFGF ($P < .001$) secretion compared with clot(+)ASC and clot(−)ASC between days 0 and 3, and then dropped to levels at or below those of clot(+)ASC by day 7. Refer to Table 1 for ELISA concentrations.

Table 1. Enzyme-Linked Immunosorbent Assay Concentrations

	Average Secretion (ng/mL) ± Standard Error of the Mean					
Growth Factor	Day 1	Day 3	Day 7	Day 9	Day 12	Day 14
VEGF-A						
Clots + ASC (N = 10)	758.80 ± 17.34	803.83 ± 4.27	711.67 ± 25.21	666.03 ± 6.23	650.95 ± 11.30	607.09 ± 2.29
Clots − ASC (N = 10)	322.63 ± 2.54	205.12 ± 14.93	44.42 ± 9.22	31.09 ± 33.79	23.84 ± 12.14	18.75 ± 5.96
Control + ASC (N = 5)	249.23 ± 11.25	131.08 ± 7.68	0	0	0	0
PDGF-BB						
Clots + ASC (N = 10)	123.77 ± 1.78	114.50 ± 1.15	66.88 ± 1.90	38.74 ± 0.99	2.07 ± 0.72	0
Clots − ASC (N = 10)	130.37 ± 1.90	128.24 ± 1.95	125.46 ± 0.93	112.75 ± 2.46	52.48 ± 1.54	25.77 ± 0.48
Control + ASC (N = 5)	15.31 ± 0.84	0	0	0	0	0
FGF-2						
Clots + ASC (N = 10)	725.96 ± 17.53	1593.92 ± 15.01	1524.92 ± 23.19	1599.21 ± 14.68	573.64 ± 17.65	379.88 ± 13.64
Clots − ASC (N = 10)	406.36 ± 22.14	587.15 ± 9.47	399.24 ± 22.48	436.73 ± 12.41	293.57 ± 14.27	189.35 ± 15.03
Control + ASC (N = 5)	5667.72 ± 11.25	3845.01 ± 7.68	1448.07 ± 21.45	1025.44 ± 23.88	801.60 ± 13.08	510.55 ± 12.23

ASC, adipose stem cell; FGF, fibroblast growth factor; PDGF, platelet-derived growth factor; VEGF, vascular endothelial growth factor.

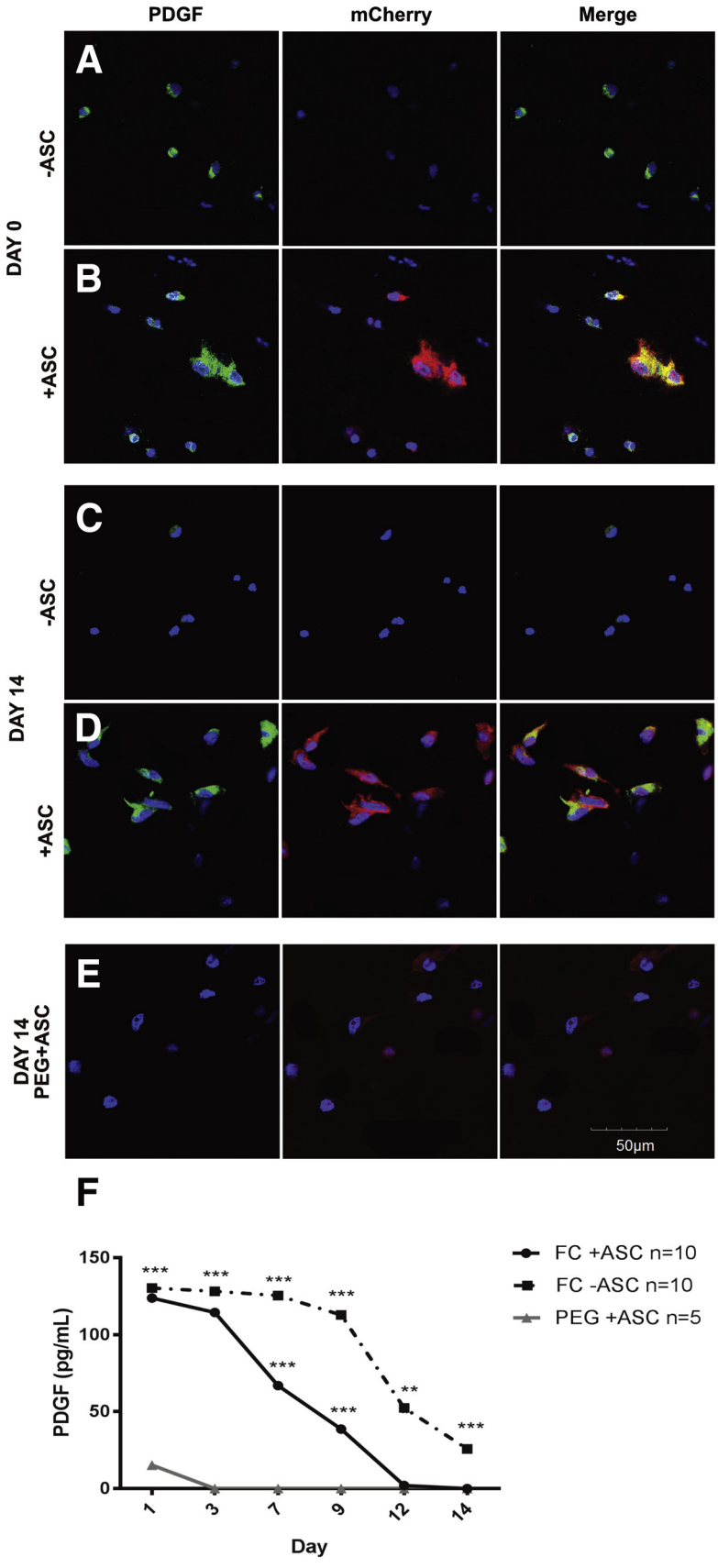

Fig 2. PDGF retention and secretion. (A) FC(−) ASC at day 0. (B) FC(+)ASC at day 0. (C) FC(−) ASC at day 14. (D) FC(+)ASC at day 14. (E) PEG(+)ASC at day 14. (F) Secretion of PDGF by FC(+)ASC (black solid line), FC(−)ASC (black segmented line), and PEG(+)ASC (gray line) was quantified by enzyme-linked immunosorbent assay at days 1, 3, 7, 9, 12, and 14. Significance compares FC(+)ASC, and FC(−)ASC with the control: PEG(+)ASC ($^{***}P < .001$; $^{**}P < .01$). Average P values for FC(+)ASC and FC(−)ASC are less than .001 for days 1, 3, 7, and 9 when compared with PEG(+)ASC. FC(−)ASC displayed average P values of .0031 and .0047 for days 12 and 14, respectively, compared with PEG(+)ASC. Error is displayed as standard error of the mean. Scale bar = 50 μm for all images. (ASC, adipose stem cell; FC, fibrin clot; PDGF, platelet-derived growth factor; PEG, polyethylene glycol gel.)

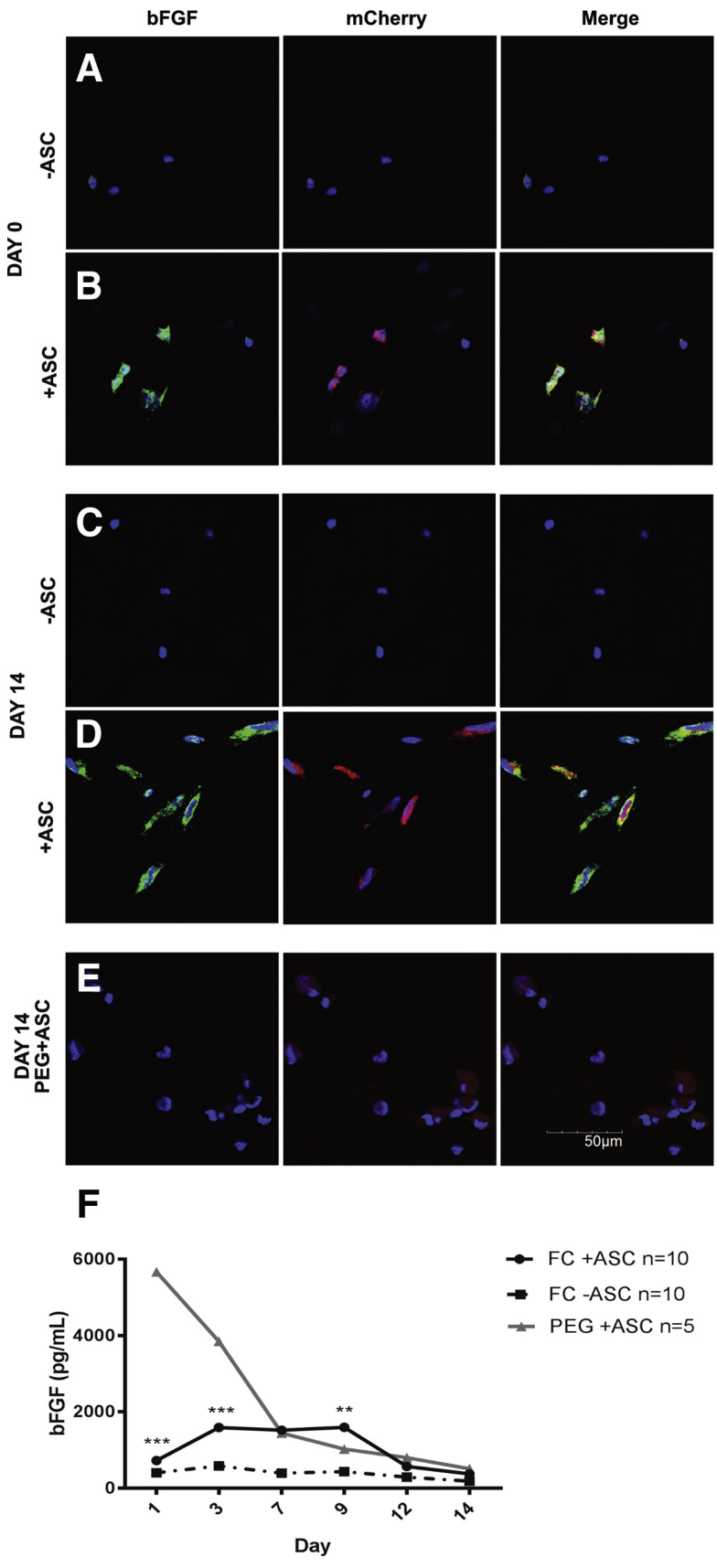

Fig 3. Basic FGF retention and secretion. (A) FC(−) ASC at day 0. (B) FC(+)ASC at day 0. (C) FC(−) ASC at day 14. (D) FC(+)ASC at day 14. (E) PEG(+)ASC at day 14. (F) Secretion of bFGF by FC(+)ASC (black solid line), FC(−)ASC (black segmented line), and PEG(+)ASC (gray line) was quantified by enzyme-linked immunosorbent assay at days 1, 3, 7, 9, 12, and 14. Significance compares FC(+)ASC with the control: PEG(+)ASC ($^{***}P < .001$; $^{**}P < .01$). Average P values for FC(+)ASC are less than .001 for days 1 and 3, while displaying no significance on days 7, 12, and 14, and a P value of .0062 on day 9 when compared with PEG(+)ASC. Error is displayed as standard error of the mean. Scale bar = 50 µm for all images. (ASC, adipose stem cell; bFGF, basic fibroblast growth factor; FC, fibrin clot; PEG, polyethylene glycol gel.)

Discussion

Our findings show that fibrin clot formation captures 98% of the platelets and that in vitro fibrin clots secrete VEGF, PDGF, and bFGF over 14 days. In addition, VEGF expression from ASCs was increased when incorporated in a fibrin clot, indicating that fibrin clots may be an enhanced method of biomaterial cell delivery. Our findings indicate that the fibrin clot may act as an effective well for cells to attach and express growth factors that may provide therapeutic benefit to biologically compromised tissue. Our finding of a platelet capture rate averaging 98% compares favorably with previous studies that have shown platelet capture rates of traditional platelet-rich plasma ranging from 17% to 80%.[27] Because fibrin clots capture platelets, they produce similar growth factors to platelet-rich plasma 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 platelet-rich fibrin scaffold 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 ASCs by promoting sustained proliferation, in addition to growth factor recruitment and secretion.[9] The use of biomaterials such as decellularized extracellular matrix and platelet-rich plasma has been explored for tissue healing, but they have been found to be suboptimal in regard to cell survival, localization, and growth factor retention.[9,31]

The question of whether a whole blood fibrin clot is sufficient for cell survival was recently evaluated using human muscle-derived stem cells.[32] This study showed that for up to 2 weeks, stem cells captured by a whole blood fibrin clot were able to proliferate in the clot matrix and were not actively undergoing cell-programmed death. Although ASC viability was not specifically evaluated in our study, the immunoreactivity of growth factors at day 14 being stronger in most cases than at day 0 is an indication of cell proliferation and overall cell health. Cell binding of soluble PDGF, which is potentially enhancing the secretion of VEGF that we see through 14 days, is also an indicator of good cell health. Although the structural integrity of the whole fibrin clot with and without ASCs was not evaluated here, a previous study has shown the ability to use whole fibrin clots formed with the same technique in augmenting rotator cuff repair.[29]

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 extracellular signaling.[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.[33-35]

Interaction Between VEGF, PDGF, and Platelets

ASCs have been found to express VEGF without exogenous supplementation in vitro.[36] In addition, IHC analysis indicates that VEGF immunoreactivity of clot(+)ASC is elevated compared with the other conditions. These results may be suggestive of a synergistic interaction between ASCs and the platelets trapped in the fibrin matrix of the clot. This interaction may promote a positive feedback loop that in turn increases cell surface VEGF receptors and subsequent enhancement of VEGF immunoreactivity in ASC.[33] The VEGF secretion data collected here support the previous findings that ASCs secrete levels of VEGF that are comparable to bone marrow-derived mesenchymal stem cells and endothelial cells on interaction with a synthetic fibrin matrix.[37] We found significantly higher secretion of soluble VEGF in clot(+)ASC compared with clot(−)ASC or control(+)ASC. Based on significantly elevated VEGF secretion from clot(+)ASC when compared with fibrin clot alone (clot(−)ASC) and ASC alone (control(+)ASC), the environment of the fibrin clot may be conducive to VEGF production from ASCs.

On clot formation, the alpha granules of platelets are activated and quickly release anabolic cytokines such as PDGF.[38] 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,[39] suggesting that once these anabolic cytokines are released from platelets, soluble PDGF may be steadily supplemented to ASCs that are attached to the fibrin clot. This conclusion is supported by the enhanced immunoreactivity of PDGF in clot(+)ASC than in clot(−)ASC and control(+)ASC conditions. Lower levels of PDGF in the surrounding media of clot(+)ASC, as confirmed by ELISA analysis, further support that ASCs may be binding and retaining soluble PDGF released from platelets.

Furthermore, when PDGF is bound to its cell surface receptor on ASCs, intracellular signaling with VEGF is activated.[40] Studies have found that PDGF bound to human cells significantly upregulates VEGF expression within the cell.[40-42] These findings, along with our own, provide evidence of cross-talk between PDGF and VEGF

that may be beneficial in sustained growth factor production for therapeutic application.

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.[33,44]

The secretion data provide evidence that the fibrin matrix of the fibrin clot provides 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 (control) can be harsh, and may be sufficient to elicit a stress response from the cells. It has been shown that bFGF is secreted by cells immediately after injury,[45] which may explain the initial spike in secretion of bFGF from control(+)ASC at day 0. The immediate drop in secretion and lack of bFGF immunoreactivity in control(+)ASC could be evidence that PEG does not retain adequate amounts of bFGF to promote subsequent uptake by ASCs. Ultimately, the fibrin matrix of the fibrin clot can retain and potentially enhance bFGF uptake 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.[35,46]

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.[40,47,48] VEGF and bFGF have been studied extensively for their synergistic relationship.[40,44,46,47] When supplemented exogenously or endogenously, bFGF has been shown to upregulate 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.[49,50] The high bFGF immunoreactivity of ASCs when incorporated in a fibrin clot may therefore be interacting with cellular VEGF, resulting in increased expression of VEGF as confirmed by significant increases in ELISA levels of VEGF in clot(+)ASC.

Limitations

The platelet capture rate during fibrin clot formation did not directly measure platelets bound within the fibrin. However, based on the change in platelet concentration in the preclot whole blood and the postclot serum, our method aligns with previous studies that have shown that platelets are trapped inside the fibrin matrix with clot formation.[10,11] The in vitro release of growth factors from the fibrin clot and the alteration of this growth factor release with the addition of ASCs may be different than the in vivo release of these growth factors. An additional limitation is that we did not measure catabolic growth factor release such as matrix metalloproteinase 9 and interleukin-1β. Also, this study did not directly evaluate ASC viability in the fibrin clot; however, continued cell viability can be inferred by soluble PDGF binding to ASCs and by the sustained increase in VEGF production in clots(+)ASCs.

Conclusions

Fibrin clots made by this method retain high concentrations of platelets, and when incorporated with ASCs show modulated secretion and immunoreactivity of VEGF, PDGF, and bFGF.

References

1. Creaney L, Hamilton B. Growth factor delivery methods in the management of sports injuries: The state of play. *Br J Sports Med* 2008;42:314-320.
2. Cummins CA, Murrell GA. Mode of failure for rotator cuff repair with suture anchors identified at revision surgery. *J Shoulder Elbow Surg* 2003;12:128-133.
3. Foster TE, Puskas BL, Mandelbaum BR, Gerhardt MB, Rodeo SA. Platelet-rich plasma: From basic science to clinical applications. *Am J Sports Med* 2009;37:2259-2272.
4. Dai R, Wang Z, Samanipour R, Koo K, Kim K. Adipose-derived stem cells for tissue engineering and regenerative medicine applications [published online February 21, 2016]. *Stem Cells Int*. doi: 10.1155/2016/6737345.
5. Jo CH, Shin JS, Lee YG, et al. Platelet-rich plasma for arthroscopic repair of large to massive rotator cuff tears: A randomized, single-blind, parallel-group trial. *Am J Sports Med* 2013;41:2240-2248.
6. Kosaka M, Nakase J, Hayashi K, Tsuchiya H. Adipose-derived regenerative cells promote tendon-bone healing in a rabbit model. *Arthroscopy* 2016;32:851-859.
7. Manning CN, Schwartz AG, Liu W, et al. Controlled delivery of mesenchymal stem cells and growth factors using a nanofiber scaffold for tendon repair. *Acta Biomater* 2013;9:6905-6914.
8. Oshita T, Tobita M, Tajima S, Mizuno H. Adipose-derived stem cells improve collagenase-induced tendinopathy in a rat model. *Am J Sports Med* 2016;44:1983-1989.
9. Wolf MT, Dearth CL, Sonnenberg SB, Loboa EG, Badylak SF. Naturally derived and synthetic scaffolds for skeletal muscle reconstruction. *Adv Drug Deliv Rev* 2015;84:208-221.
10. Bensaïd W, Triffitt JT, Blanchat C, Oudina K, Sedel L, Petite H. A biodegradable fibrin scaffold for mesenchymal stem cell transplantation. *Biomaterials* 2003;24:2497-2502.
11. Nathan C, Sporn M. Cytokines in context. *J Cell Biol* 1991;113:981-986.
12. Arnoczky SP, Warren RF, Spivak JM. Meniscal repair using an exogenous fibrin clot: An experimental study in dogs. *J Bone Joint Surg Am* 1998;70:1209-1217.

13. Kamimura T, Kimura M. Meniscal repair of degenerative horizontal cleavage tears using fibrin clots: Clinical and arthroscopic outcomes in 10 cases. *Orthop J Sports Med* 2014;2:2325967114555678.
14. Surer L, Yapici C, Guglielmino C, van Eck CF, Irrgang JJ, Fu FH. Fibrin clot prevents bone tunnel enlargement after ACL reconstruction with allograft. *Knee Surg Sports Traumatol Arthrosc* 2017;25:1555-1560.
15. Henning CE, Lynch MA, Yearout KM, Vequist SW, Stallbaumer RJ, Decker KA. Arthroscopic meniscal repair using an exogenous fibrin clot. *Clin Orthop Relat Res* 1990;252:64-72.
16. Zieren J, Paul M, Osei-Agyemang T, Maecker F, Müller JM. Polyurethane-covered dacron mesh versus polytetrafluoroethylene DualMesh for intraperitoneal hernia repair in rats. *Surg Today* 2002;32:884-886.
17. Ra HJ, Ha JK, Jang SH, Lee DW, Kim JG. Arthroscopic inside-out repair of complete radial tears of the meniscus with a fibrin clot. *Knee Surg Sports Traumatol Arthrosc* 2013;21:2126-2130.
18. Hensler D, Illingworth KD, Musahl V, et al. Does fibrin clot really enhance graft healing after double-bundle ACL reconstruction in a caprine model? *Knee Surg Sports Traumatol Arthrosc* 2015;23:669-679.
19. Lee SY, Kwon B, Lee K, Son YH, Chung SG. Therapeutic mechanisms of human adipose-derived mesenchymal stem cells in a rat tendon injury model. *Am J Sports Med* 2017;45:1429-1439.
20. Zhang X, Yong M, Xin F, et al. Runx2-modified adipose-derived stem cells promote tendon graft integration in anterior cruciate ligament reconstruction. *Sci Rep* 2016;6:19073.
21. Chen RR, Mooney DJ. Polymeric growth factor delivery strategies for tissue engineering. *Pharm Res* 2003;20:1103-1112.
22. Strioga M, Viswanathan S, Darinskas A, Slaby O, Michalek J. Same or not the same? Comparison of adipose tissue-derived versus bone marrow-derived mesenchymal stem and stromal cells. *Stem Cells Dev* 2012;21:2724-2752.
23. Kern S, Eichler H, Stoeve J, Klüter H, Bieback K. Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue. *Stem Cells* 2006;24:1294-1301.
24. Matsuda K, Falkenberg KJ, Woods AA, Choi YS, Morrison WA, Dilley RJ. Adipose-derived stem cells promote angiogenesis and tissue formation for in vivo tissue engineering. *Tissue Eng Part A* 2013;19:1327-1335.
25. Tsuji W, Rubin PJ, Marra KG. Adipose-derived stem cells: Implications in tissue regeneration. *World J Stem Cells* 2014;6:312-321.
26. Clevenger TN, Hinman CR, Rubin RKA, et al. Vitronectin-based, biomimetic encapsulating hydrogel scaffolds support adipogenesis of adipose stem cells. *Tissue Eng Part A* 2016;22:597-609.
27. Wasterlain AS, Braun HJ, Dragoo JL. Contents and formulations of platelet-rich plasma. *Operative Tech Orthop* 2012;22:33-42.
28. Longo UG, Campi S, Romeo G, Spiezia F, Maffulli N, Denaro V. Biological strategies to enhance healing of the vascular area of the meniscus. *Stem Cells Int* 2012;2012:528359.
29. Proctor C. Rotator cuff repair augmented with endogenous fibrin clot. *Arthrosc Tech* 2012;1:e79-e82.
30. Bellon JM, Rodriguez M, Garcia-Honduvilla N, Pascual G, Buján J. Partially absorbable meshes for hernia repair offer advantages over nonabsorbable meshes. *Am J Surg* 2007;194:68-74.
31. Rodriguez IA, Kalaf EAG, Bowlin GL, Sell SA. Platelet-rich plasma in bone regeneration: Engineering the delivery for improved clinical efficacy. *BioMed Res Int* 2014;2014:392398.
32. Shupe PG, Warth RJ, Gao X, et al. Whole blood fibrin clot preserves the viability and bioactivity of human muscle-derived stem cells. ORS Annual Meeting 2017. Paper no. 278.
33. Martino MM, Briquez PS, Ranga A, Lutolf MP, Hubbell JA. Heparin-binding domain of fibrin(ogen) binds growth factors and promotes tissue repair when incorporated within a synthetic matrix. *Proc Natl Acad Sci USA* 2013;110:4563-4568.
34. Mittermayr R, Morton T, Hofmann M, Helgerson S, Van Griensven M, Redl H. Sustained (rh)VEGF 165 release from a sprayed fibrin biomatrix induces angiogenesis, up-regulation of endogenous VEGF-R2, and reduces ischemic flap necrosis. *Wound Repair Regen* 2008;16:542-550.
35. Sahni A, Altland OD, Francis CW. FGF-2 but not FGF-1 binds fibrin and supports prolonged endothelial cell growth. *J Thromb Haemost* 2003;1:1304-1310.
36. Hutton DL, Grayson WL. Hypoxia inhibits de novo vascular assembly of adipose-derived stromal/stem cell populations, but promotes growth of preformed vessels. *Tissue Eng Part A* 2016;22:161-169.
37. Rohringer S, Hofbauer P, Schneider KH, et al. Mechanisms of vasculogenesis in 3D fibrin matrices mediated by the interaction of adipose-derived stem cells and endothelial cells. *Angiogenesis* 2014;17:921-933.
38. Josh F, Tobita M, Tanaka R, et al. Concentration of PDGF-AB, BB and TGF-beta1 as valuable human serum parameters in adipose-derived stem cell proliferation. *J Nippon Med Sch* 2013;80:140-147.
39. Harrison S, Vavken P, Kevy S, Jacobson M, Zurakowski D, Murray MM. Platelet activation by collagen provides sustained release of anabolic cytokines. *Am J Sports Med* 2011;39:729-734.
40. Kano MR, Morishita Y, Iwata C, et al. VEGF-A and FGF-2 synergistically promote neoangiogenesis through enhancement of endogenous PDGF-B-PDGFR signaling. *J Cell Sci* 2005;118:3759-3768.
41. Chung SW, Bae SM, Lee M, et al. LHT7, a chemically modified heparin, inhibits multiple stages of angiogenesis by blocking VEGF, FGF2 and PDGF-B signaling pathways. *Biomaterials* 2015;37:271-278.
42. Fredriksson L, Li H, Eriksson U. The PDGF family: Four gene products form five dimeric isoforms. *Cytokine Growth Factor Rev* 2004;15:197-204.
43. Kokai LE, Marra K, Rubin JP. Adipose stem cells: Biology and clinical applications for tissue repair and regeneration. *Transl Res* 2014;163:399-408.
44. Arkudas A, Pryymachuk G, Hoereth T, et al. Dose-finding study of fibrin gel-immobilized vascular endothelial growth factor 165 and basic fibroblast growth factor in the arteriovenous loop rat model. *Tissue Eng Part A* 2009;15:2501-2511.

45. Nissen NN, Polverini PJ, Koch AE, Volin MV, Gamelli RL, DiPietro LA. Vascular endothelial growth factor mediates angiogenic activity during the proliferative phase of wound healing. *Am J Pathol* 1998;152:1445-1452.
46. Rophael JA, Craft RO, Palmer JA, et al. Angiogenic growth factor synergism in a murine tissue engineering model of angiogenesis and adipogenesis. *Am J Pathol* 2007;171:2048-2057.
47. Ley CD, Olsen MWB, Lund EL, Kristjansen PEG. Angiogenic synergy of BFGF and VEGF is antagonized by angiopoietin-2 in a modified in vivo matrigel assay. *Microvasc Res* 2004;68:161-168.
48. Wong C, Inman E, Spaethe R, Helgerson S. Fibrin-based biomaterials to deliver human growth factors. *Thromb Haemost* 2003;89:573.
49. Seghezzi G, Patel S, Ren CJ, et al. Fibroblast growth factor-2 (FGF-2) induces vascular endothelial growth factor (VEGF) expression in the endothelial cells of forming capillaries: An autocrine mechanism contributing to angiogenesis. *J Cell Biol* 1998;141:1659-1673.
50. Bikfalvi A, Klein S, Pintucci G, et al. Differential modulation of cell phenotype by different molecular weight forms of basic fibroblast growth factor: Possible intracellular signaling by the high molecular weight forms. *J Cell Biol* 1995;129:233-243.