# Platelet-Rich Plasma Influences Expansion and Paracrine Function of Adipose-Derived Stromal Cells in a Dose-Dependent Fashion

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**Background:** Lipofilling is a treatment modality to restore tissue volume. Both platelet-rich plasma and adipose-derived stromal cells have been reported to augment the efficacy of lipofilling, yet results are not conclusive. The authors hypothesized that the variation reported in literature is caused by a dose-dependent influence of platelet-rich plasma on adipose-derived stromal cells.
**Methods:** Whole blood ($n = 3$) was used to generate platelet-rich plasma, which was diluted with Dulbecco's Modified Eagle Medium to 15%, 5%, and 1.7%, with 15% platelet-poor plasma and 10% fetal calf serum as controls. Pooled adipose-derived stromal cells ($n = 3$) were cultured in these media. Gene expression was assessed, along with angiogenic sprouting of endothelial cells by conditioned medium and platelet-rich plasma.
**Results:** platelet-rich plasma in culture medium affected the expression of genes in a dose-dependent manner. The 15% concentration stimulated proliferation almost eightfold. Mesenchymal markers were unaffected. Interestingly, expression of collagens type 1 and 3 increased at lower concentrations, whereas transforming growth factor-βshowed reduced expression in lower concentrations. Proangiogenic gene expression was unaltered or strongly reduced in a dose-dependent manner. platelet-rich plasma promoted endothelial sprouting and survival in a dose-dependent manner; however, conditioned medium from adipose-derived stromal cells exposed to platelet-rich plasma blocked endothelial sprouting capabilities.
**Conclusion:** The dose-dependent influence of platelet-rich plasma on the therapeutic capacity of adipose-derived stromal cells conditioned medium in vitro warrants caution in clinical trials. (*Plast. Reconstr. Surg.* 137: 554e, 2016.)

Fat grafting, or lipofilling, has become an accepted treatment modality in the construction and reconstruction of tissue volume. Indications vary from acquired[1] to hereditary loss[2] of volume in both aesthetic and reconstructive surgery.

Although technical improvements have increased the overall fat graft take dramatically,[3] the level of fat graft survival is still uncertain and suboptimal.[4] Various methods have been suggested to increase graft take, which include negative-pressure garments,[5] addition of adipose-derived stromal cells to grafts,[6] and addition of platelet-derived growth factors[7] or platelet-rich plasma. The efficacy of these procedures, however, still has to be proven in randomized clinical trials.

Besides the volumetric effect observed after a lipofilling procedure, skin rejuvenation features are observed, such as decreased pore size,

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improved elasticity, and suppression of inflammatory skin conditions and reduction of existing scars.[1,8] The presence of adipose-derived stromal cells in the lipograft is suggested to participate in tissue rejuvenation features and improved would healing.[9] These adipose-derived stromal cells achieve this either by direct support of the lipograft during the first days (facilitating vessel ingrowth), by differentiation into adipocytes, or both.[10]

Platelet-rich plasma, as investigated by Marx et al.,[11] is rich in growth factors that support wound healing in normal physiology. These growth factors, such as platelet-derived growth factor, transforming growth factor-β, and vascular endothelial growth factor, locally influence migration, proliferation, and differentiation[12] of several cell-types including endothelial cells (angiogenesis), fibroblasts, and myofibroblasts (deposition of extracellular matrix). In animal studies, platelet-rich plasma augments graft take,[13–16] most likely because of improved vascularization. Moreover, addition of platelet-rich plasma to fat grafts reduces the occurrence of oil cysts.[17] However, the clinical efficacy of the use of platelet-rich plasma is highly variable,[18–20] which causes doubt about the benefit of platelet-rich plasma to support and improve graft take.[21]

From a pharmacologic point of view, it is to be expected that platelet-rich plasma would act in a dose-dependent fashion in combination with lipofilling. Therefore, the observed clinical variation in the effect of platelet-rich plasma could be attributable to concentration differences. In vitro, fibroblasts and osteoblasts react in a dose-related response to platelet-rich plasma.[22–26]

Studies that explore the influence of platelet-rich plasma on the proliferation and cellular function of adipose-derived stromal cells are limited.[21] Besides platelet-rich plasma, adipose-derived stromal cells appear to be a promising factor to influence graft take and tissue regeneration,[10,27,28] which underlies our in vitro study to investigate the influence of platelet-rich plasma concentration—on the proliferation, phenotype, and function of adipose-derived stromal cells.

## MATERIALS AND METHODS

### Adipose-Derived Stromal Cell Isolation and Culture

Human adipose tissue was collected from the resected abdominal skin flaps from three healthy abdominoplasty patients (body mass index < 30 kg/m$^2$) after informed consent was obtained. This source of adipose-derived stromal cells was approved by of the local ethics committee (anonymized waste material).

The skin flaps were processed using a working protocol for adipose-derived stromal cell isolation.[29] Isolated adipose-derived stromal cells were expanded to passage 3 in Dulbecco's Modified Eagle Medium (Lonza, Walkersville, Md.) supplemented with 10% fetal calf serum (Thermo Scientific, Hempstead, United Kingdom), 1% L-glutamine (Lonza Biowhittaker, Verviers, Belgium), and antibiotics at 37°C. For the experiments, adipose-derived stromal cells of all three donors (passage 3 and higher) were pooled.

### Preparation of Platelet-Rich and Platelet-Poor Plasma

Whole blood was drawn (27 ml) from three healthy volunteers and mixed with 3 ml of citrate (standard anticoagulant citrate dextrose solution) to prevent clotting. Additional blood samples were drawn for platelet, red blood cell, and white blood cell counts. The whole blood citrate mixture was introduced into the Biomet GPS-III device (Biomet, Warsaw, Ind.) following the manufacturer's instructions. Fifteen minutes of centrifugation at 2200 *g* separated the blood into three fractions: erythrocytes, platelet-poor plasma, and platelet-rich plasma. Output volume of the platelet-rich plasma was 3 ml, a ninefold reduction of the input volume. Platelet-poor plasma and platelet-rich plasma were collected separately with a syringe. To prevent inhibition of adipose-derived stromal cell proliferation, residual leukocytes and erythrocytes were removed also (300 *g* for 10 minutes).[30]

### Platelet-Rich Plasma Dilution

The platelet-rich plasma was diluted with Dulbecco's Modified Eagle Medium to final concentrations of 15%, 5%, and 1.7%, whereas platelet-poor plasma was diluted to 15% only. Experiments with endothelial cells, were performed in endothelial cell culture medium (endothelial cell culture medium–RPMI-1640), 5 μg/ml endothelial cell growth factors,[31] bovine brain extract, 5 U/ml heparin (LEO Pharma, Amsertdam, The Netherlands), and 2 mM L-glutamine. Diluted platelet-rich plasma was activated through vigorously shaking (clot formation) (Fig. 1). The clot was resuspended by breaking and pipetting with a pipette tip several times. As a control, 10% or 20% fetal calf serum was used instead of platelet-rich plasma or platelet-poor plasma.

**Proliferation**

Confluent cultures of pooled adipose-derived stromal cells (passage 4) were seeded onto 24-well plates at $4 \times 10^4/cm^2$ (−1 day). After 24 hours (day 0), media were replaced by the platelet-rich plasma–platelet-poor plasma conditions that were generated on day 0 also.

After 4 days, cells were fixed with 2% paraformaldehyde–phospate-buffered saline solution for 20 minutes. After extensive washing, cells were permeabilized with 0.5% Triton X-100–phosphate-buffered saline (Sigma-Aldrich, St. Louis, Mo.). Subsequently, plates were incubated with polyclonal rabbit–anti-human Ki-67 antibody (Monosan, Uden, The Netherlands) diluted 1:250 in phosphate-buffered saline–10% horse serum (First Link Ltd., Birmingham, United Kingdom) for 90 minutes. Control wells received only detection antibody. After washing, the wells were incubated with donkey–anti-rabbit serum conjugated to Alexa Fluor 488 (Life Technologies, Carlsbad, Calif.) diluted 1:300 in 0.5 μg/ml 4′,6-diamidino-2-phenylindole–phosphate-buffered saline and 2% horse serum for 30 minutes followed by a final wash with phosphate-buffered saline.

The wells were scanned using automated immunofluorescent microscopy (TissueFAXS; TissueGnostics, Vienna, Austria) using a Zeiss AxioObserver-Z1 microscope (Carl Zeiss, Oberkochen, Germany) on 10× magnification. Two wells were scanned per condition per platelet-rich plasma donor ($n = 3$). TissueQuest cell analysis software (v4.0.1.0.127; TissueGnostics) determined the total cell-count and percentage of Ki-67–expressing cells.

**Gene Expression by Quantitative Real-Time Polymerase Chain Reaction**

At day −1, confluent cultures of pooled adipose-derived stromal cells (passage 4) were seeded onto six-well plates at $4 \times 10^4/cm^2$. After 24 hours (day 0), media were replaced with medium with 1.7%, 5%, and 15% platelet-rich plasma, respectively, or medium with 15% platelet-poor plasma or 10% fetal calf serum.

After 4 days, cells were lysed using Trizol Reagent (Life Technologies) and total RNA was isolated according to the manufacturer's protocol. Two micrograms of total RNA was reverse transcribed using a First-Strand cDNA synthesis kit (Thermo Scientific, Waltham, Mass.). The cDNA equivalent of 10 ng of RNA was amplified (in triplicate) using the SyberGreen method (Life Technologies) using specific primers (Table 1).

Human beta-actin was used as a reference. Polymerase chain reactions were performed by means of a ViiA-7 real-time polymerase chain reaction system (Life Technologies), with cycle threshold values for individual reactions obtained from the ViiA-7 software. Relative expression was calculated using the delta–cycle threshold method.

**Endothelial Sprouting**

Conditioned medium from pooled adipose-derived stromal cells (passage 4) was prepared

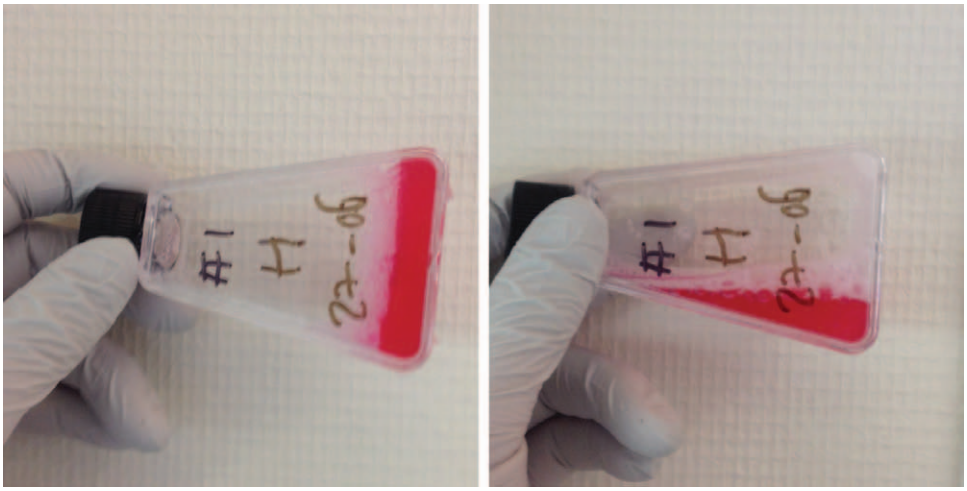

**Fig. 1.** Activation, gelling, and resuspension of platelet-rich plasma. The platelet-rich plasma was diluted with Dulbecco's Modified Eagle Medium to final concentrations of 15%, 5%, and 1.7%, whereas platelet-poor plasma was diluted to 15% only. (*Left*) Platelet-rich plasma was activated separately after dilution, through vigorously shaking the tubes, which resulted in the formation of a clot. (*Right*) Resuspension of the clot by pipetting up and down several times.

**Table 1. Primer Sequences for Quantitative Reverse-Transcriptase Polymerase Chain Reaction**

| | | Sequence | |
|---|---|---|---|
| **Gene Symbol** | **Gen Alias** | **Forward** | **Reverse** |
| *ACTB* | Beta-actin | CCAACCGCGAGAAGATGA | CCAGAGGCGTACAGGGATAG |
| *GAPDH* | G3PD, glyceraldehyde-3-phosphate dehydrogenase | AGCCACATCGCTCAGACAC | GCCCAATACGACCAAATCC |
| *TGFB1* | Transforming growth factor beta 1 | ACTACTACGCCAAGGAGGTCAC | TGCTTGAACTTGTCATAGATTTCG |
| *TGFB2* | Transforming growth factor beta 2 | ATAAATTTCAGCCCAGGTCA | CCAAAAGGGAAGAGATGAAA |
| *TGFB3* | Transforming growth factor beta 3 | ACACTTGGTTAGACGCCTTC | ACCAAATGAACACAGGGTCT |
| *TAGLN* | Transgelin, SM22a | CTGAGGACTATGGGGTCATC | TAGTGCCCATCATTCTTGGT |
| *CNN1* | Calponin-1, basic-calponin, SMCC | CCAACCATACACAGGTGCAG | TCACCTTGTTTCCTTTCGTCTT |
| *PECAM1* | CD31, endoCAM, GPIIA | GCAACACAGTCCAGATAGTCGT | GACCTCAAACTGGGCATCAT |
| *VWF* | von Willebrand factor, factor 8 (F8) | AGTGAGCCTCTCCGTGTATC | TCACCGGACAGCTTGTAGTA |
| *PDGFB* | Platelet-derived growth factor B | CTGCATTTTCCTCTTGTCCT | TTCTGCCCTAGAGAGGAGTG |
| *PDGFRB* | Platelet-derived growth factor receptor beta, CD140b | CCCTTATCATCCTCATCATGC | CCTTCCATCGGATCTCGTAA |
| *ANGPT1* | ANG1, angiopoietin 1 | CTACTGGGCCTCCTCTCATA | TCTCAAATGGAGGAAACCAT |
| *ANGPT2* | ANG2, angiopoietin 2 | CAGTTCTTCAGAAGCAGC | TTCAGCACAGTCTCTGAA |
| *VEGFA* | Vascular endothelial growth factor A, vascular permeability factor | CCTGAAATGAAGGAAGAGGA | AAATAAAATGGCGAATCCAA |
| *FGF1* | Fibroblast growth factor 1, acidic FGF | ACCAAGTGGATTCTGCTTCC | CTTGTGGCGCTTTCAAGACT |
| *FGF2* | Fibroblast growth factor 2, basic FGF | CTGTACCCATACAGCAGCAG | CGCCTAAAGCCATATTCATT |
| *COL1A1* | COL1, collagen type 1 alpha 1 | GGGATTCCCTGGACCTAAAG | GGAACACCTCGCTCTCCA |
| *COL3A1* | COL3, collagen type 3 alpha 1 | CTGGACCCCAGGGTCTTC | CATCTGATCCAGGGTTTCCA |
| *MMP1* | Matrix metalloproteinase-1, collagenase (CLGN) | GCTAACCTTTGATGCTATAACTACGA | TTTGTGCGCATGTAGAATCTG |
| *MMP2* | Matrix metalloproteinase-2, gelatinase A (GLNA) | GTTCCCCTTCTTGTTCAATG | CTTGCCATCCTTCTCAAAGT |
| *MMP9* | Matrix metalloproteinase-9, gelatinase B (GLNB) | GACGATGACGAGTTGTGGT | GAAGATGAAGGGGAAGTGG |
| *HGF* | Scatter factor, hepatocyte growth factor | GTTTCCCAGCTGGTATATGG | GGTCTTTTCAGGAATTGTGC |
| *CSPG4* | Chondroitin sulfate proteoglycan 4, NG2 | GAGAGGCAGCTGAGATCAGAA | TGAGAATACGATGTCTGCAGGT |
| *IGF1* | Insulin-like growth factor 1, somatomedin-C | ACTCGGGCTGTTTGTTTTAC | GTGTGCTTCTTGACGACTTG |
| *IL1B* | Interleukin 1 beta, | GGTTGAGTTTAAGCCAATCCA | TGCTGACCTAGGCTTGATGA |
| *IL6* | Interleukin 6 | AGCTCAATAAGAAGGGGCCTA | TGAGAAACCCTGGCTTAAGTAGA |
| *IL8* | Interleukin 8, granulocyte chemotactic protein 1 (GCP1), CXCL8, TNFAIP1 | CTTTCAGAGACAGCAGAGCA | ACACAGAGCTGCAGAAATCA |
| *TIMP1* | Tissue inhibitor of metalloproteinases 1 | CCAGCGTTATGAGATCAAGA | AGTATCCGCAGACACTCTCC |
| *TIMP2* | Tissue inhibitor of metalloproteinases 2 | GAAGAGCCTGAACCACAGGT | CGGGGAGGAGATGTAGCAC |
| *FN1* | Fibronectin, GFND2 | TCAACTCACAGCTTCTCCAA | TTGATCCCAAACCAAATCTT |
| *LAMA1* | Laminin alpha 1 | ATGGAAAATGGCACACTCTT | AGACTGGGTGTGTGGACTTT |

from 4-day cultures in extracellular matrix with platelet-rich plasma, platelet-poor plasma, or fetal calf serum. This was followed by incubation in the absence of platelet-rich plasma, platelet-poor plasma, or fetal calf serum for 16 hours.

Inner wells of µ-Slide Angiogenesis plates (Ibidi GmbH, Munich, Germany) were coated with 10 µl of Matrigel (BD Biosciences, San Jose, Calif.). Pooled passage 4 human umbilical vein endothelial cells (Lonza Biowhittaker) were suspended (200,000 cells/ml) in the prepared conditioned medium, endothelial cell culture medium–platelet-rich plasma media, and extracellular matrix–platelet-poor plasma medium using the same dilutions as described above. endothelial cell culture medium–20% fetal calf serum and conditioned medium from adipose-derived stromal cells cultured on extracellular matrix–10% fetal calf serum served as a positive control, and serum-free endothelial cell culture medium served as a negative control. In total, 10,000 human umbilical vein endothelial cells were pipetted per well (in triplicate). Light micrographs (2.5× magnification) were evaluated after 6, 16, 24, and 72 hours (72 hours for endothelial cell culture medium–platelet-rich plasma wells only). The number of sprouts, quantified in number of loops and branches and length, was calculated with the Angiogenesis Analyzer (Image J; National Institutes of Health, Bethesda, Md.).[32]

### Statistical Analysis

Data were analyzed by GraphPad Prism (v6.0; GraphPad Software, Inc., La Jolla, Calif.) and presented as means ± SEM. Statistical significance was determined using one-way analysis of variance with Bonferroni comparison post hoc analysis. Differences with $p < 0.05$ were considered significant.

## RESULTS

### Platelet-Rich Plasma Donors

A total of 3 ml of platelet-rich plasma was generated per donor, with donor platelet counts all within normal range (251 ± 39.13 cells-platelets/L; range, 180 to 315 cells-platelets/L). Donors' red and white blood cell counts were normal.

### Platelet-Rich Plasma Promotes Cell Proliferation in a Dose-Dependent Fashion

Human adipose-derived stromal cells proliferated irrespective of supplemented platelet-rich plasma, platelet-poor plasma, or fetal calf serum. Adipose-derived stromal cells had a fibroblast-like appearance (Fig. 2, *above*), with no visual signs of apoptosis such as blebbing. Moreover, both 5% and 15% platelet-rich plasma had visibly higher cell numbers after 4 days of culture (Fig. 2, *above*). This dose-dependent influence of platelet-rich plasma on proliferation of adipose-derived stromal cells was corroborated by quantification (Fig. 2, *below*). Total cell numbers were increased in media with 15% platelet-rich plasma ($p < 0.05$), whereas in media with 5% and 15% platelet-rich plasma, more proliferating cells were also present as observed by increased expression of Ki-67 (Fig. 2, *below*). In contrast, proliferation was hampered in media with 1.7% platelet-rich plasma. Proliferation in media with 15% platelet-poor plasma and 10% fetal calf serum was comparable.

### Gene Expression in Adipose-Derived Stromal Cells Is Influenced by Platelet-Rich Plasma in a Dose-Dependent Fashion

The beneficial function of adipose-derived stromal cells relies on the expression of genes that influence angiogenesis, inflammation, and remodeling of the extracellular matrix. The expression of representative genes showed a dose-dependent expression to platelet-rich plasma as compared to controls (platelet-poor plasma and fetal calf serum) (Fig. 3).

#### Mesenchymal Differentiation

All platelet-rich plasma media induced minor changes in *TAGLN* expression compared with 10% fetal calf serum ($p > 0.05$) and 15% platelet-poor plasma ($p > 0.05$). Relative gene expression of *CCN1* was unchanged (Fig. 3, *above*, *left*). In contrast, the pericytic nature of adipose-derived stromal cells was stable, as expression of *PDGFR* and *NG2* was stable (Fig. 3, *above*, *left*).

#### Matrix Remodeling

Expression of *COL1A1* increased with 15%, 5%, and 1.7% platelet-rich plasma compared with 10% fetal calf serum ($p < 0.05$), with a peak at 5% platelet-rich plasma, followed by a decrease at 15% platelet-rich plasma (Fig. 3, *above*, *center*). Matrix metalloproteinases *MMP1* and *MMP2* followed a similar pattern, with the highest expression at 5% and 1.7% platelet-rich plasma but unchanged expression in 10% fetal calf serum (Fig. 3, *above*, *right*). All platelet-rich plasma concentrations increased expression of *MMP1* and *MMP2* compared with 15% platelet-poor plasma ($p < 0.05$). However, the highest expression of *MMP2* was in medium with 10% fetal calf serum compared to medium with platelet-rich plasma ($p < 0.05$). A strong decrease in gene expression of *MMP2* was

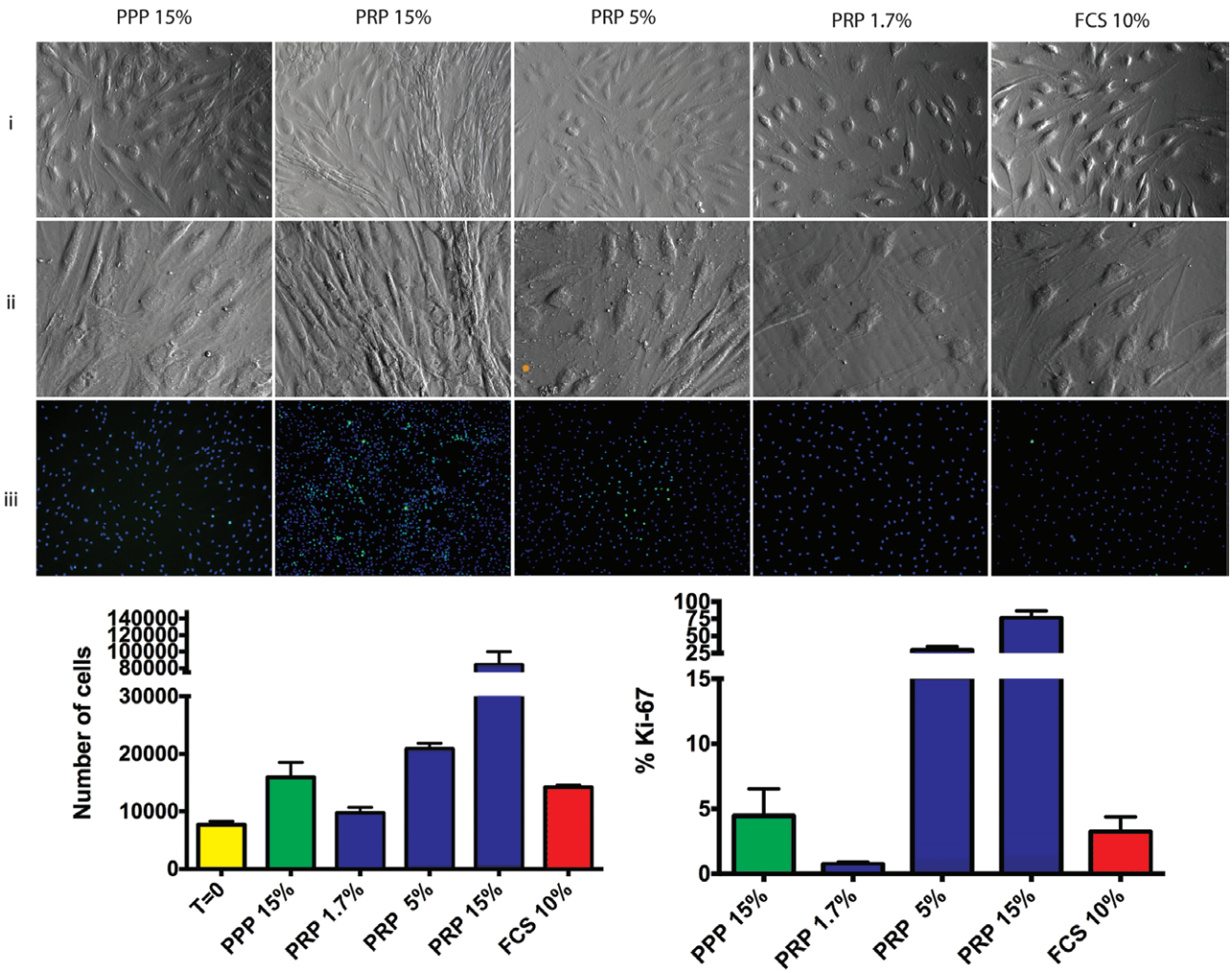


**Fig. 2.** Platelet-rich plasma promotes cell proliferation in a dose-dependent fashion. (*Above* and *second row*) Light micrographs (original magnification, × 10 and × 20, respectively) showing the platelet-rich plasma concentration–dependent increased proliferation of adipose-derived stromal cells after 3 days' culture. (*Third row*) Platelet-rich plasma concentration–dependent increased proliferation [Ki-67 immunofluorescent staining (green), nuclei 4′,6-diamidino-2-phenylindole (blue)] of adipose-derived stromal cells cultured for 4 days. (*Below*) Quantification of actual total numbers of cells per well or fraction of proliferating adipose-derived stromal cells (Ki-67) by automated image analyses (TissueGnostics TissueFAXS) corroborates the qualitative observations. Graphs represent triplicate (with SEM) data from three independent experiments from three platelet-rich plasma donors.

observed with 15% platelet-rich plasma compared to 10% fetal calf serum ($p < 0.05$) and the 5% and 1.7% platelet-rich plasma ($p < 0.05$) (Fig. 3, *above*, *right*). Expression of *COL3A1* was unaltered by platelet-rich plasma compared to controls (fetal calf serum and platelet-poor plasma) (Fig. 3, *above*, *center*).

**Paracrine Factors**

The expression of *TGFB1* increased with increasing platelet-rich plasma concentrations; all concentrations induced a higher expression compared to fetal calf serum ($p > 0.05$) (Fig. 3, *below*, *center*). Similarly, the expression of *FGF1* followed the concentration of platelet-rich plasma, with the highest expression in 15% platelet-rich plasma. Both 15% and 5% platelet-rich plasma increased expression compared to 15% platelet-poor plasma and 10% fetal calf serum ($p < 0.05$) (Fig. 3, *below*, *right*). The expression of *IL1B* decreased in a concentration-dependent manner with increasing platelet-rich plasma concentrations in the medium. In medium with 15% platelet-rich plasma, the expression of *IL1B* was lower compared to media with 10% fetal calf serum and 5% and 1.7% platelet-rich plasma ($p < 0.05$) (Fig. 3, *below*, *center*). The gene expression of *IGF1* followed a similar pattern, with decreased expression at increasing platelet-rich plasma concentrations. At 15%,

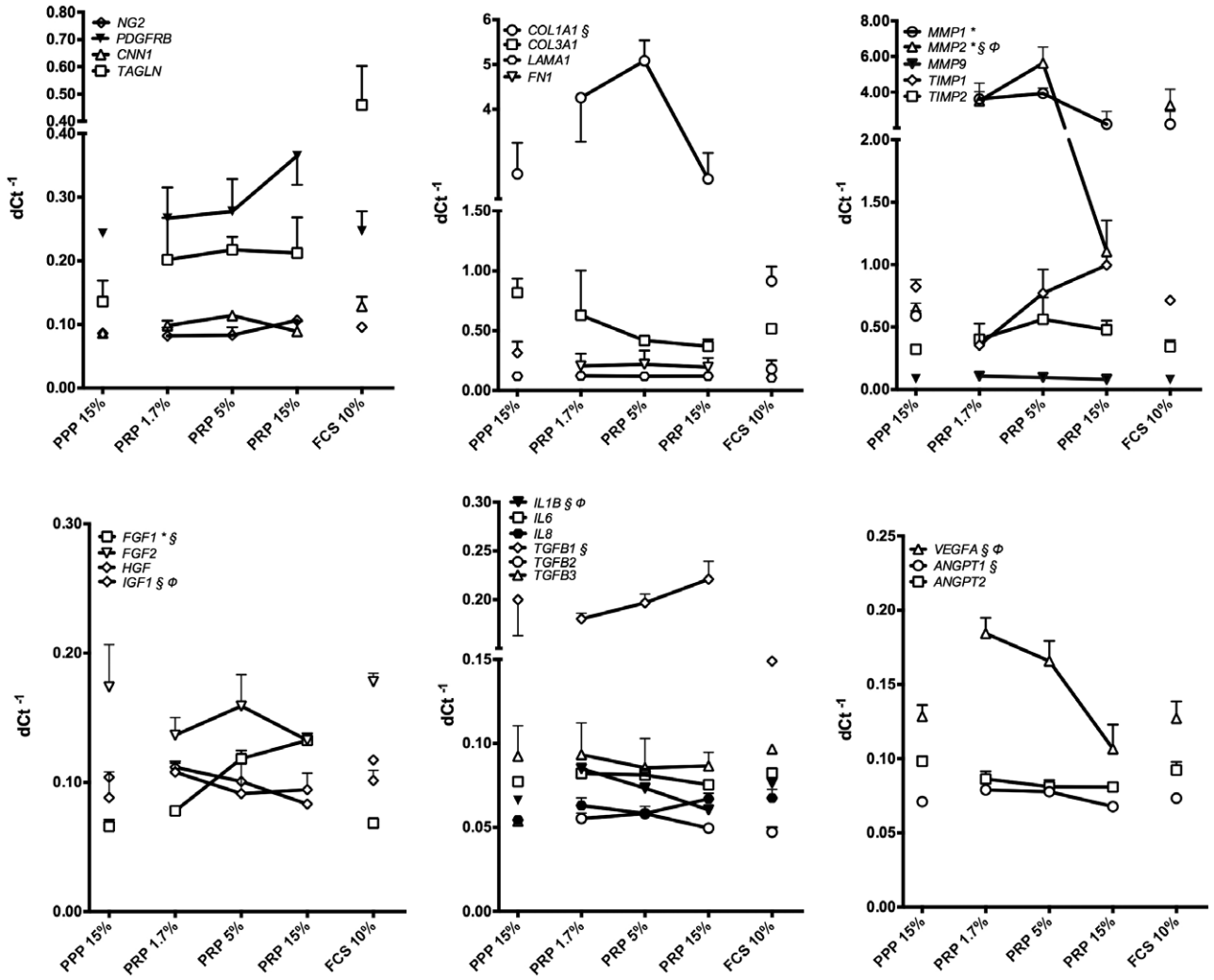


**Fig. 3.** Platelet-rich plasma influences gene expression in adipose-derived stromal cells in a dose-dependent fashion. Quantitative real-time polymerase chain reaction analysis of gene transcript levels normalized to *ACTB* expression. None of the serum- or plasma-derived medium additives caused differentiation to smooth muscle–like cells (*above*, *left*, *TAGLN*, *CCN1*) nor altered the pericytic nature of adipose-derived stromal cells (*above*, *left*, *PDGFRB*, *NG2*). Of all four extracellular matrix genes, *COL1A1*, *COL3A1*, *LAMA1*, *FN1*, and *COL1A1* were down-regulated in platelet-rich plasma media compared with fetal calf serum controls (*above*, *center*). In contrast, of the extracellular matrix remodeling genes, *MMP1*, *MMP2*, *MMP9*, *TIMP1*, *TIMP*2, and *MMP2* (a gelatinase) were up-regulated in high-concentration platelet-rich plasma medium compared with lower concentrations and to platelet-poor plasma and fetal calf serum controls (*above*, *right*). *MMP1* was up-regulated compared with both platelet-poor plasma controls (*above*, *right*). Of the promitotic growth factor genes *FGF1*, *FGF2*, *HGF*, and *IGF1*, *FGF1* was up-regulated compared with both controls, whereas *IGF1* was up-regulated compared with fetal calf serum controls and showed a platelet-rich plasma dose-dependent increased expression also (*below*, *left*). Inflammatory genes (*IL1B*, *IL6*, *IL8*, *TGFB1*, *TGFB2*, and *TGFB3*) were not regulated, except for *TGFB*, which was up-regulated compared with fetal calf serum controls, whereas *IL1B* was down-regulated in a dose-dependent fashion compared with fetal calf serum controls (*below*, *center*). The proangiogenic genes *VEGFA* and *ANGPT2* were both regulated compared with fetal calf serum controls, whereas *VEGFA* showed a platelet-rich plasma dose-dependent down-regulation also (*below*, *right*). Graphs represent triplicate (with SEM) data from three independent experiments from three platelet-rich plasma donors. *PPP*, platelet-poor plasma; *PRP*, platelet-rich plasma; *FCS*, fetal calf serum. Significant changes in expression ($p < 0.05$): *one or more platelet-rich plasma concentration(s) compared to 15% platelet-poor plasma; §one or more platelet-rich plasma concentration(s) compared to 10% fetal calf serum; Φin platelet-rich plasma concentrations.

platelet-rich plasma affected *IGF1* expression most compared with 10% fetal calf serum ($p < 0.05$) (Fig. 3, *below*, *left*).

**Angiogenesis**

The expression of *VEGFA*, as a marker for proangiogenic capacity of adipose-derived stromal

cells, showed a reciprocal relation with increasing concentrations of platelet-rich plasma in the medium (Fig. 3, *below*, *right*), whereas only 1.7% platelet-rich plasma in medium caused a significant decrease of the expression of *VEGFA* ($p < 0.05$). *ANGPT1* expression was also affected; 15% platelet-rich plasma induced a down-regulation compared to 10% fetal calf serum ($p > 0.05$) and 5% and 1.7% platelet-rich plasma ($p < 0.05$). *ANGPT2* showed a small decrease in the platelet-rich plasma conditions compared with 15% platelet-poor plasma and 10% fetal calf serum ($p > 0.05$) (Fig. 3, *below*, *center*).

### Dose-Dependent Influence of Platelet-Rich Plasma on Adipose-Derived Stromal Cell–Induced Endothelial Sprouting

The addition of 1.7%, 5%, or 15% platelet-rich plasma to endothelial culture medium instead of 20% fetal calf serum resulted in the ready formation of an endothelial sprouting network in all concentrations at 6 hours that lasted for at least 24 hours. (**See Figure, Supplemental Digital Content 1,** which shows sprouting networks of human umbilical vein endothelial cells on Matrigel with platelet-rich plasma as serum component. Light micrographs of individual wells of μ-Slide Angiogenesis plates coated with Matrigel were taken at 2.5× original magnification, ***http://links.lww.com/PRS/B607***.) The number of loops, branches, and branch lengths peaked at 15% platelet-rich plasma after 6 hours ($p > 0.05$) (Fig. 4, *above*, *left*). Moreover, networks that had formed in endothelial cell culture medium–15% platelet-rich plasma media remained intact for 72 hours, whereas in all other conditions, the networks had collapsed ($p < 0.05$). Much to our surprise, conditioned medium of adipose-derived stromal cells that were cultured in the presence of 5% or 15% platelet-rich plasma strongly inhibited sprouting of human umbilical vein endothelial cells in vitro as observed by the absence of loops or branches (Fig. 4, *above*, *left*). (**See Figure, Supplemental Digital Content 2,** which shows sprouting networks of human umbilical vein endothelial cells on Matrigel with conditioned medium derived from adipose-derived stromal cells cultured in media with 1.7%, 5%, or 15% platelet-rich plasma. Light micrographs of individual wells of μ-Slide Angiogenesis plates coated with Matrigel were taken at 2.5× original magnification, ***http://links.lww.com/PRS/B608***.) Conditioned medium from adipose-derived stromal cell cultured medium with 1.7% platelet-rich plasma, however, induced network formation with comparable number of loops and branches to control media (conditioned medium 15% platelet-poor plasma, and conditioned medium 10% fetal calf serum). Endothelial cell culture medium-0% fetal calf serum resulted in lowest number of loops and branches ($p > 0.05$).

## DISCUSSION

Our results show that in vitro adipose-derived stromal cells respond to platelet-rich plasma in a dose-dependent way: platelet-rich plasma caused a dose-dependent increase of the proliferation rate of adipose-derived stromal cells, which coincided with increased expression of Ki-67. Moreover, adipose-derived stromal cells showed a dose-dependent decrease of several paracrine genes, which is relevant to tissue repair or the take of lipografts. This was corroborated by the nearly abolished capacity of adipose-derived stromal cells to support angiogenic sprouting of endothelial cells in vitro after treatment with high concentrations of platelet-rich plasma which, to our knowledge, has not yet been demonstrated.

Platelet-rich plasma is used as an additive in various clinical indications,[21,33] including lipografts. It is generally accepted that the growth factors present in platelet-rich plasma, presumed to be in high concentration also, stimulate wound healing,[34,35] tissue remodeling, and revascularization[21] and improve lipograft take.[15,17,19] Clinical evaluation studies on the use of platelet-rich plasma, however, report a large variation of results,[18–20] which might be attributable to interindividual variations in the composition of platelet-rich plasma–lipograft mixtures. Different concentrations of platelet-rich plasma have been shown to induce varying effects on fibroblasts, osteoblasts, and endothelial cells.[24,25,36–38] With adipose-derived stromal cells playing a key role in fat graft survival and tissue rejuvenation,[28,39] the effect of platelet-rich plasma in a possible dose-dependent response on adipose-derived stromal cells seems to be crucial. A maximum platelet-rich plasma concentration of 15% was chosen because this is the highest clinically feasible concentration available when using disposable platelet-rich plasma kits, already requiring 4.5 ml of platelet-rich plasma in a 25.5-ml lipograft.

Our results demonstrated that platelet-rich plasma is a powerful dose-dependent mitogen for adipose-derived stromal cells, with two- to fivefold more cells at high platelet-rich plasma concentrations compared to "normal" fetal calf serum after 4 days' culture. This finding fits the results of various

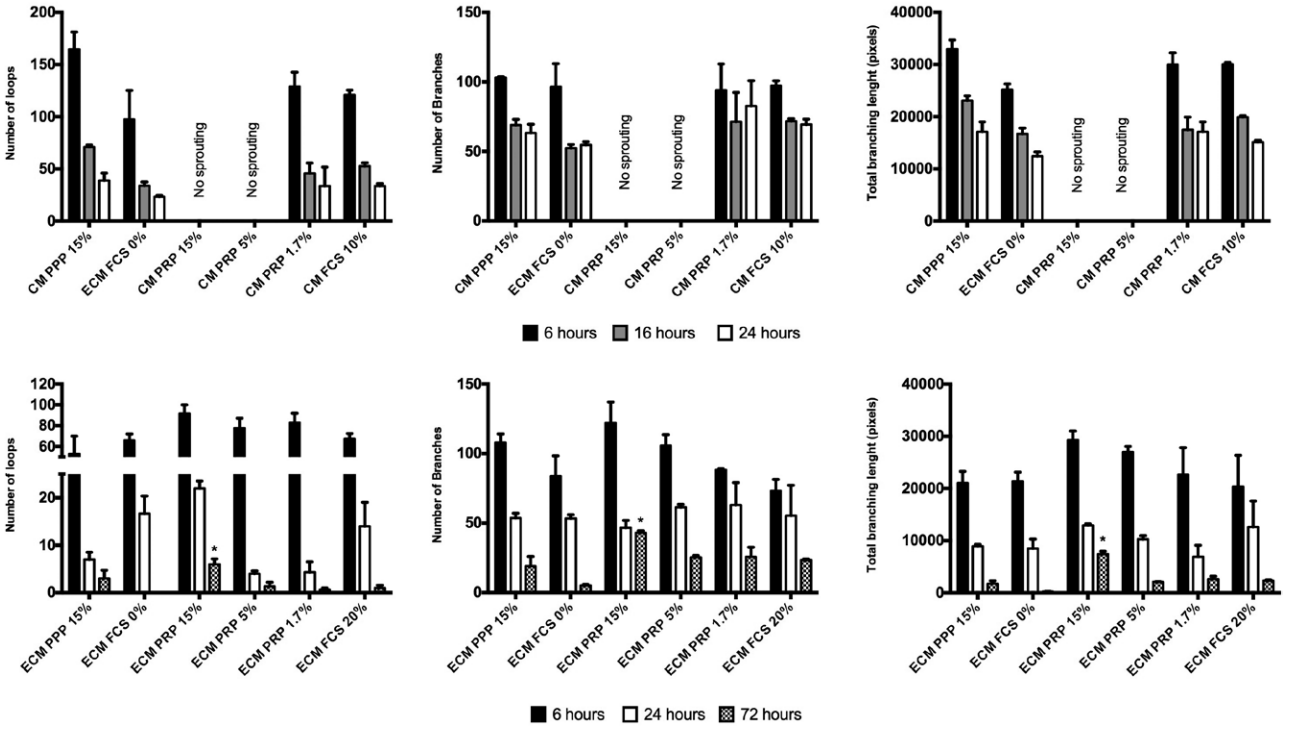


**Fig. 4.** In vitro sprouting of human umbilical vein endothelial cells is affected by platelet-rich plasma alone (*above*) and by platelet-rich plasma–conditioned media (*below*) from adipose-derived stromal cells (see **Figure, Supplemental Digital Content 1**, ***http://links.lww.com/PRS/B607***, and **Figure, Supplemental Digital Content 2, *http://links.lww.com/PRS/B608*** ). (*Above*) Sprouting networks of human umbilical vein endothelial cells on Matrigel were readily (6 hours) formed and remained present at 24 hours, whereas these networks remained stable for 72 hours only in medium with 15% platelet-rich plasma (see **Figure, Supplemental Digital Content 1**, ***http://links.lww.com/PRS/B607***, and **Figure, Supplemental Digital Content 2, *http://links.lww.com/PRS/B608***). (*Below*) Conditioned medium derived from adipose-derived stromal cells cultured in media with 5% or 15% platelet-rich plasma abolished the formation of endothelial sprouting networks compared with controls (see **Figure, Supplemental Digital Content 2, *http://links.lww.com/PRS/B608***). In general, the presence or absence of plasma or serum constituents did not cause differences in the number of loops, branches, and total branching length. The number of sprouts, quantified in number of loops and branches and their length, was calculated by the Angiogenesis Analyzer plug-in for Image J. Significant ($p < 0.05$): *one or more platelet-rich plasma concentration(s) compared to 15% platelet-poor plasma, 10% fetal calf serum. Graphs represent triplicate (with SEM) data from three independent experiments from three platelet-rich plasma donors. *CM*, conditioned medium; ECM, endothelial culture medium; PPP, platelet-poor plasma; *FCS*, fetal calf serum; *PRP*, platelet-rich plasma.

other studies on platelet-rich plasma–induced proliferation of adipose-derived stromal cells,[40–42] fibroblasts,[24,43] and human umbilical vein endothelial cells.[13,36] In the study by Kølle et al.,[39] increasing numbers of adipose-derived stromal cells in a lipograft had a positive effect on graft take, although a consensus of its effect has not yet been confirmed by others. Positive effects on graft survival could be explained by local support of the adipose-derived stromal cells on surrounding cells or by differentiation[44] of adipose-derived stromal cells into adipocytes. Adipose-derived stromal cells cultured in media with platelet-rich plasma maintain their capability to differentiate into adipocytes.[45–47] Differentiation of adipose-derived stromal cells into adipocytes could significantly contribute to end graft volume, as suggested by Kølle et al.

In addition, we determined that expression of genes related to adipose-derived stromal cell function were altered by exposure to different platelet-rich plasma concentrations compared with control media with 15% platelet-poor plasma and 10% fetal calf serum. These changes found in relative gene expression corroborate data of Amable et al.,[48] who explored the effects of human platelet lysate on several stem cell types.

Expression of mesenchymal markers was not influenced by platelet-rich plasma, which indicates that in platelet-rich plasma, adipose-derived stromal cells do not acquire myofibroblast features. This is relevant because myofibroblasts are related to fibrotic tissue processes. However, rather large changes were observed in expression of adipose-derived stromal cell function–related

genes. Platelet-rich plasma significantly increased expression of genes encoding *COL1A1, MMP1*, and *MMP2*. The increases of these factors indicate that platelet-rich plasma increases the capacity of adipose-derived stromal cells to facilitate tissue remodeling. Moreover, *TGFB1*, *FGF1*, and *IGF1* showed a strong dose-dependent up-regulation, whereas *IL1B* and *VEGFA* showed a down-regulation. The up-regulation of the antiinflammatory *TGFB1* and simultaneous down-regulation of the proinflammatory *IL1B* would be beneficial in graft take and wound healing, where adverse inflammation could cause graft damage and apoptosis. The up-regulation of both strongly mitotic and antiapoptotic growth factors *FGF1* and *IGF1* would translate to improved graft take through stimulation of tissue integration and suppression of apoptosis. Together, these changes in expression of genes encoding paracrine factors indicate that adipose-derived stromal cells switch from a highly proangiogenic phenotype with modest matrix remodeling capacities, to a phenotype that is not in support of angiogenesis, whereas tissue remodeling is enhanced in addition to proliferation and survival of tissue cells. This, however, remains a topic of future research.

Changes in gene expression were confirmed by changes in the effects of conditioned media: adipose-derived stromal cells exposed to higher platelet-rich plasma concentrations seem to lose proangiogenic properties, as endothelial network formation was blocked by their conditioned medium. Possible explanations can be found in the up-regulation of *TGFB1* combined with decreased expression of *VEGFA*. Although both *TGFB1* and *VEGFA* are associated with angiogenesis by influencing endothelial cells,[49,50] changes in their relative availability modify the overall effect and can induce endothelial apoptosis,[51] thus blocking network formation.

In contrast to conditioned medium derived from adipose-derived stromal cells cultured in higher platelet-rich plasma concentrations, direct addition of platelet-rich plasma did not negatively influence endothelial network formation. Increasing platelet-rich plasma concentrations correlated with the formation of more loops and branches, which survived longer compared with control conditions. Platelet-rich plasma effects on endothelial cells are most likely induced by readily available *VEGFA*, *ANG1-Tie2* signaling, and activation of the ERK and phosphatidylinositol-3-kinase–Akt pathways.[52,53] The study of Kakudo et al.[13] reported similar positive findings on endothelial network formation, both in vitro and in vivo.

Platelet-rich plasma, or platelet lysates, have several advantages over the use of animal-derived serums in cell cultures.[40] Risk of contamination with animal pathogens and proteins is absent, which allows for use in human cell therapy. Although platelet-rich plasma allows for rapid cell expansion in vitro, adipose-derived stromal cell gene expression and correlated secretome production changes compared with fetal calf serum.[45,48] The reported therapeutic properties of adipose-derived stromal cells cultured on fetal calf serum[10,21,54] might inadvertently be lost by cultivation on high platelet lysate or high platelet-rich plasma concentrations; therefore, further study of the altered properties is warranted.

Results of this study demonstrate that adipose-derived stromal cells respond to platelet-rich plasma in a dose-dependent way, and emphasize the need for further research and optimization in the use of platelet-rich plasma as an additive to lipofilling, adipose-derived stromal cell–enriched lipofilling, or adipose-derived stromal cell–based therapies. Dose-dependent effects on proliferation and adipose-derived stromal cell function were found, most likely explaining the varying clinical results that have been observed thus far. However, it remains unclear whether and how a higher number of adipose-derived stromal cells with altered function induced by platelet-rich plasma may and will contribute to graft survival. Also, in vivo interaction between endothelial cells, adipose-derived stromal cells, and platelet-rich plasma, and the resulting effect on angiogenesis, requires further study. Furthermore, adipose-derived stromal cells cultured on platelet-rich plasma differ from fetal calf serum–cultured adipose-derived stromal cells, losing proangiogenic properties. This effect should be taken into account by future studies on adipose-derived stromal cell–based cell therapies using platelet-rich plasma or platelet lysates as serum.

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