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# Platelet-Rich Plasma (PRP) Promotes Fetal Mesenchymal Stem/Stromal Cell Migration and Wound Healing Process

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**Abstract** Numerous studies have shown the presence of high levels of growth factors during the process of healing. Growth factors act by binding to the cell surface receptors and contribute to the subsequent activation of signal transduction mechanisms. Wound healing requires a complex of biological and molecular events that includes attraction and proliferation of different type of cells to the wound site, differentiation and angiogenesis. More specifically, migration of various cell types, such as endothelial cells and their precursors, mesenchymal stem/stromal cells (MSCs) or skin fibroblasts (DFs) plays an important role in the healing process. In recent years, the application of platelet rich plasma (PRP) to surgical wounds and skin ulcerations is becoming more frequent, as it is believed to accelerate the healing process. The local enrichment of growth factors at the wound after PRP application causes a stimulation of tissue regeneration. Herein, we studied: (i) the effect of autologous PRP in skin ulcers of patients of different aetiology, (ii) the proteomic profile of PRP, (iii) the migration potential of amniotic fluid MSCs and DFs in the presence of PRP extract in vitro, (iv) the use of the PRP extract as a substitute for serum in cultivating AF-MSCs. Considering its easy access, PRP may provide a valuable tool in multiple therapeutic approaches.

**Keywords** PRP · AF-MSCs · Healing · Cell migration

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## Introduction

Skin ulceration is a common complication of vasculopathies and diabetes. It increases morbidity, reduces mobility and it usually requires multiple visits in order to be treated. Therefore, effective treatment of skin wounds requires a full understanding of the physiological process of healing. The optimal healing of skin ulcers requires a well-orchestrated process, incorporating a complex of biological and molecular events that include attracting cells to the wound site, cell proliferation, differentiation and angiogenesis [32, 48]. Numerous research studies have shown the presence of high levels of growth factors, such as platelet derived growth factor (PDGF), fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF) or epidermal growth factor (EGF), in all phases of healing [32]. Growth factors act by binding to specific receptors on the cell membrane of target cells and contribute to the subsequent activation of signal transduction mechanisms in these cells [2]. Experimental evidence strengthens the hypothesis that administration of growth factors can have beneficial effects in healing by inducing cell migration [36].

However, the healing process is disrupted in many chronic diseases, including diabetes [17]. In recent years, the application of plasma rich in platelets (platelet rich plasma, PRP) has become more frequent and naturally accelerates the healing process [3]. The local enrichment of growth factors in the

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wound area after platelet administration seems to be a reliable method for stimulating tissue regeneration [3]. Clinical studies have shown that the application of PRP in bone grafts results in the acceleration of wound healing and increase bone density [54]. Different protocols and devices for collecting and creating PRP and PPP (platelet poor plasma) are available, although the number of clinical studies that support the contribution of PRP in tissue regeneration is limited [33].

During the process of wound healing, activation of cell migration of various cell types, such as endothelial cells and their precursors, stem cells or skin fibroblasts (Dermal Fibroblasts-DFs), in the wound area has been observed [22, 26, 27, 55]. Stem cells have been used in multiple therapeutic approaches and are considered to be effective in accelerating wound healing in the case of diabetic ulcers and skin regeneration [22, 26–28, 55].

Mesenchymal stem/stromal cells (MSCs) represent a cell type, which according to numerous studies, has an important role in the process of wound healing [8, 9, 20, 22, 26]. MSCs have been isolated from different tissues at various stages of development and in the adult, including amniotic fluid (AF), umbilical cord (UC), umbilical cord blood (UCB), bone marrow (BM) and adipose tissue (AT) [1, 10–12, 16, 21, 31, 37, 38, 42, 43, 49, 50].

BM-MSCs have been used with remarkable results in the treatment of diabetic skin ulcers [22, 26, 27, 55]. The direct mechanism has not been fully understood yet. However, in most cases, MSCs are considered to contribute to the wound healing by: (i) differentiation, (ii) autocrine or paracrine mechanisms, or (iii) immune-regulatory action. Recent studies have shown that the local treatment of diabetic wounds with MSCs has resulted in increased angiogenesis, acting through a paracrine mechanism [23, 30] or by differentiation [28].

Our research group has identified MSCs from human second trimester AF (AF-MSCs), and performed systematic phenotypic and functional characterization [42–44, 60]. In addition, previous in vivo and in vitro experimental approaches have shown that AF-MSCs, are located between the state of embryonic and adult stem cells [5, 6, 11, 21, 42–45, 59, 60]. Particularly interesting is the fact that these cells can differentiate in vitro not only into mesodermal (osteoblasts and adipocytes) but into ectodermal (nerve cells) and endodermal (hepatocytes) lines [11, 42, 43, 51, 52]. Results from our group have shown that AF-MSCs can be utilized as vehicles for anticancer drugs in an in vivo model of bladder cancer [5, 6], can also contribute to tissue repair in acute liver failure [59] or can promote angiogenesis in vivo [41].

AF-MSCs, used in this study, exhibit (i) high proliferation rate, which allows a large number of expanded cells obtained in vitro [42, 43, 60], (ii) ability to induce angiogenesis in vivo [45] and wound healing properties [36, 58]. This paper aims to characterize the PRP phenotype, to evaluate the effect of PRP on ulcers from diabetic patients and to study the effect of PRP on the proliferation rate and the cell migration properties of AF-MSCs and DFs in vitro.

## Material and Methods

### Collection of Samples and Preparation Platelet-Rich Plasma (PRP) and Platelet-Poor Plasma (PPP)

Seventeen PRP and seventeen PPP samples were prepared from patients suffering from skin ulceration with their prior written informed consent. The PRP and PPP samples were prepared using a specific concentration filter (Fig. 1a) according to the manufacturer's instructions (GPS® III, Biomet Biologics Inc). For the preparation of PRP and PPP, 54 ml of peripheral blood were aspirated in 60 ml syringe containing 6 ml anticoagulant citrate dextrose solution (ACD-A) (Citra Labs Braintree, MA, USA). The sample was placed in the central slot of the filter unit and centrifuged for 15 min at 3200 rpm. After the centrifugation, the blood sample was separated by gravity into cellular components and PRP and PPP were collected respectively. For the preparation of autologous thrombin, 11 ml of peripheral blood were mixed with 1 ml of ACD-A solution using the appropriate device (Biomet Biologics Inc). Four ml of TPD™ Thrombin Reagent (Clotalyst®, Biomet Biologics Inc) were mixed with 12 ml of the citrated whole blood sample and placed in an incubator at a 25 °C for 25 minutes, then the sample was centrifuged for 5 min at 3200 rpm and the autologous thrombin was collected. For the use of PRP as serum substitute in the cell culture, the PRP was initially deep-frozen in - 80 °C, then thawed and centrifuged in order to remove cell debris before placed in the cell culture medium.

### Patient Treatment

After the relevant consent forms, patients who met the inclusion criteria were categorized into separate study groups. Patients with infected wounds, haematologic disorders, or severe systemic disorders were excluded from the study. Autologous plasma gel was applied to the wound according to clinical protocols and guidelines of the manufacturer (Biomet Biologics Inc). The wound dressings were changed at least 3 times a week and the wounds were treated until the wound closure or the successful completion of the study. The method of preparation and application of autologous plasma clot is as follows: Initially, 20 to 60 ml of peripheral blood was used for PRP preparation using an appropriate filter device (GPS (R) III, Biomet Biologics Inc). The wound was cleaned and the necrotic tissue was debrided. Six millilitre of PRP was mixed with 0.6 l thrombin (also prepared from the same blood sample) in ratio of 10:1. Platelet gel of PRP was placed into the wound site by spraying. Then, a semi- permeable film was used to cover the wound.

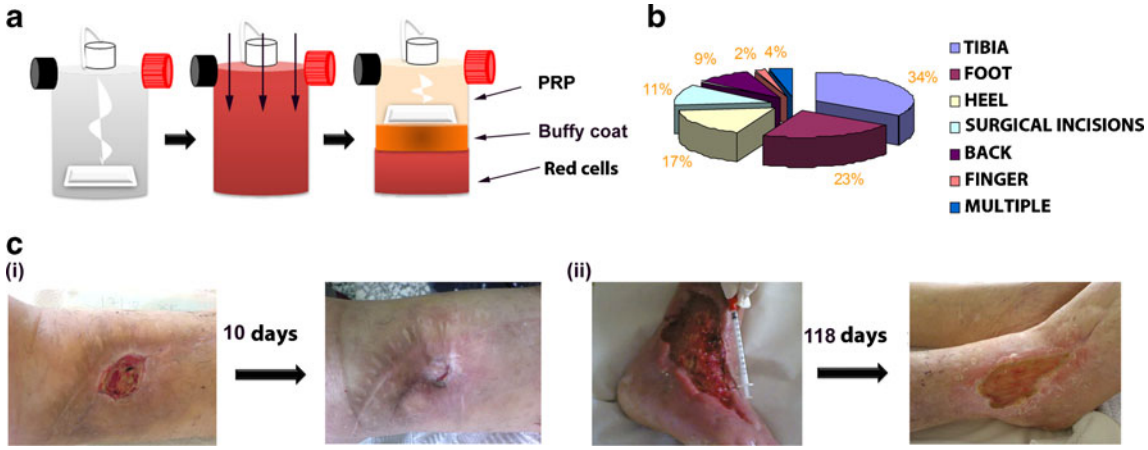



**Fig. 1** Effect of PRP in wound healing **a** Schematic representation of the condensation device for PRP. **b** Ulcer types in patients enrolled in the study. **c** Representative photographs showing the progress of wound healing of diabetic patients after 10 and 118 days of treatment with PRP dressings

## Isolation and Culture of Human AF-MSCs

All protocols involving human subjects were approved by the Ethical Committee of Alexandra Hospital, Athens, Greece and the Bioethics Committee of the School of Medicine of the University of Athens. All samples were collected after informed consent from each individual. The AF-MSCs were isolated from 4 AF samples. Sampling of AF was held during scheduled amniocentesis between the 15th and 19th week of gestation. Using a 22G needle and under ultrasound, 10-15 ml of amniotic fluid was aspirated for each sample. The samples used were derived from the excess volume of amniotic fluid obtained for prenatal diagnosis. The AF-MSCs were cultured according to previously published protocols of our research group [5, 42, 43, 45, 59, 60]. In summary, the AF samples were centrifuged at 1300 rpm for 10 min and the cell pellet was resuspended in Dulbecco's modified Eagle's medium (DMEM, Sigma Aldrich Ltd, Gillingham, Dorset, UK) enriched with 20 % (v/v) fetal bovine serum (FBS, Gibco-BRL, Paisley, Scotland, UK). The samples were incubated at 37 °C and 5 % $CO_2$ for about 15 to 20 days until the first colonies appeared. The spindle shaped (SS) colonies were selected from the initial culture (passage 0) and subcultuted as described previously [42, 60]. The AF-MSCs were used at passage 5-12.

## Culture of Human DFs

Three samples of human hDFs were kindly gifted by Professor Suzanne M. Watt (University of Oxford, UK). The DFs were cultured in medium (DMEM, Sigma Aldrich Ltd) with high glucose and sodium pyruvate supplemented with L-glutamine, penicillin/streptomycin (Gibco-BRL) and 10 % (v/v) FBS (Gibco-BRL). The hDFs were used at passage 5-6.

## Antibodies and Flow Cytometry

PRP ($n$=17) and PPP ($n$=17) samples and AF-MSCs ($n$=4) (after being cultured in medium enriched with PRP or PPP), were examined for the expression of cell surface markers by flow cytometry. Antibodies against surface markers such as: CD90, CD166, CD73, CD105, CD49e, CD29, CD62P, CD62E, CD45, CD34, CD14, CD31, CD50, CD64, CD44, CD1a, CD3, CD13 or CD117 [Becton Dickinson (BD) San Jose, CA, USA] were used, followed by secondary antibodies against mouse immunoglobulins (IgG) conjugated to fluorescein isothiocyanate (FITC) or phycoerythrin (PE) (DAKO Ltd DakoCytomation, Cambridgeshire, UK). The flow cytometric analysis was performed using a Beckman Coulter Cytomics FC 500 cytometer (Beckman Coulter Ltd, Palo Alto, CA).

## Antibody Profiler Arrays for PRP

Three PRP samples were frozen at -80 °C and then thawed to perform cell lysis, followed by centrifugation to remove cell debris at 1200 rpm for 10 min. Protein analysis was performed by using appropriate profiler antibody arrays (Profiler human arrays Catalog # ARY007, R & D Systems Inc, Minneapolis, USA), according to manufacturer's instructions. The quantification of the spots detected was performed using the Quantity One 4.4.1 (Bio Rad Laboratories Inc) software. The values were normalized to the positive controls. Data are presented as mean±SD of three independent experiments.

### Real-Time Quantitative PCR

cDNA samples were mixed with suitable specific primers and polymerase enzyme mixture (PCR master mix) (Applied Biosystems, No. 4312704). TaqMan real-time PCR was performed for the Sox-2, Oct-4 and Nanog genes using the primers and conditions designated by Assays on Demand™ Gene Expression [Applied Biosystems Hs00742896_s1 (oct-4), Hs01053049_s1 9 (Sox-2), Hs02387400_s1 (Nanog), respectively]. Real-time quantitative PCR was performed using an ABI Prism 7000 apparatus (Applied Biosystems) and the analysis was conducted using the ABI Prism 7700 SDS software (Applied Biosystems). The data were analyzed by the relative quantification ($\Delta\Delta Ct$) method as described previously [19, 43]. To determine fold expression differences, the $2^{-\Delta\Delta Ct}$ formula was used. Each assay was performed in duplicate. The data are presented as the mean±SEM for at least three independent experiments. Then, the expression levels of Oct-4, Sox-2 or Nanog in AF-MSCs cultured in medium supplemented with FBS were compared to the expression levels of Oct-4, Sox-2 or Nanog in AF-MSCs cultured in medium enriched with PRP. The statistical analysis was performed using Student's *t*-test.

### Transwell Migration Assay - In Vitro Blocking Experiments

In vitro cell migration assay was performed as described in our previous studies [5, 29, 45, 61]. Briefly, AF-MSCs or DFs at $2\times10^4$/100 ml density in DMEM supplemented with 0.5 % (v/v) FBS, were transferred to an insert of the a transwell plate with pore size of 8 μm (Corning Life Sciences Ltd) (Fig. 4a). Then the AF-MSCs were allowed to migrate for 16 h and DFs for 6 h respectively to DMEM supplemented with: 10 % (v/v) FBS, 10 % (v/v) PRP or 10 % (v/v) PPP. After the appropriate incubation period, the cells that did not migrate were removed from the upper side of the insert with a wet cotton swab. Then, the cells that had migrated were fixed with paraformaldehyde 4 % (wt/v) (Sigma-Aldrich Ltd) on the underside of the membrane and sequentially stained with hematoxylin and eosin (all from Sigma-Aldrich Ltd.) The cell migration was quantitated by measuring the nuclei passing through the pore membrane. Photographs of nuclei were taken from at least 10 fields (20x) for each membrane using an inverted microscope TE300 (Nikon Ltd, London, UK), equipped with a cooled CCD camera and software PCI (Digital Pixel, Brighton, England). All experiments were performed in triplicate. The statistical analysis was performed using Student's *t*-test.

For MMP9 and PDGFR blocking experiments, the MMP9 inhibitor (Santa Cruz Biotechnology, Inc) or the PDGF inhibitor, SU6668 (Calbiochem, Merck Chemicals Ltd, Nottingham, England), was added in DMEM supplemented with 10 % (v/v) PRP, respectively.

### MTS Proliferation Assay

AF-MSCs and DFs were cultured at a density of $10^3$ cells per well in 96-well plate for 5 days in the conditions described below. AF-MSCs were cultured in DMEM supplemented with (i) 20 % (v/v) FBS, (ii) 20 % (v/v) PRP or (iii) 20 % (v/v) PPP. DFs were cultured in DMEM supplemented with (i) 10 % (v/v) FBS, (ii) 10 % (v/v) PRP or (iii) 10 % (v/v) PPP. On the 5$^{th}$ day, the appropriate amount of MTS reagent (Promega Ltd., Madison, WI, USA) was added to each well and the cells were incubated for 3 h, according to the manufacturer's instructions. The absorbance was recorded at 490 nm using microplate reader ELISA (ELX 800; Biotek Inc, USA). The percent increase in proliferation was calculated using the formula: [(ODdayx-ODday0)/ODday0x100)]. All assays were performed in triplicate and the mean value was calculated for each experiment±SEM. The statistical analysis was performed using Student's *t*-test.

## Results

### Healing Progress after the use of PRP Dressings in Patients with Skin Ulcers.

In this study, we reviewed the healing progress in 17 patients who received treatment with PRP dressings for their skin ulcers, followed informed consent, at the Department of General Surgery of 'Amalia Fleming' General Hospital in Athens, Greece. Eleven of these patients were male and 6 female with mean age of 58.2 years (range 38-83). Twelve of the patients were diabetic (70.6 %) whereas, 7 were suffering from chronic renal failure (41 %), with 2 of them needing permanent renal replacement therapy. Five of the patients had history of peripheral vascular disease.

With regards to the etiology, 2 of the patients had dehiscence of surgical wound, 8 had an ulcer of neuropathic origin and 7 had an ischemic or mixed ischemic/neuropathic ulcer. In terms of staging, 7 of the patients had ulceration in necrotic stage while 10 were in exudative stage (Table 1). Each of the patients had an initial assessment where the approximate volume of tissue loss was measured using simple measurement technique. This was expressed in centimeters cubed. Following that, the treatment with PRP dressings was initiated (Fig. 1a). A file was created for each patient, which included the ulcer type (Fig. 1b), the healing progress (Fig. 1c) and the date of transition to granulation and epithelialsation stage. After reaching the epitheliasation stage, the majority of the patients were converted to a different method of wound healing with the use of other types of dressings.

**Table 1** Patient information

| Gender | Number | Percentage (%) |
|---|---|---|
| Male | 11 | 64.7 |
| Female | 6 | 35.3 |
| History | | |
| Diabetes | 12 | 70.6 |
| Chronic renal failure | 7 | 41.2 |
| Peripheral vascular disease | 5 | 29.4 |
| Ulcer Type | | |
| Dehiscence of surgical wound | 2 | 11.8 |
| Ischemic/neuropathic | 7 | 41.2 |
| Neuropathic | 8 | 47.0 |

On average, the volume of tissue loss was 34.06±43.1 $cm^3$ (1-150 $cm^3$) and the duration of treatment with PRP dressings lasted a mean of 75.3±58 (19-196) days. The number of separate sessions required was 10.6±9.6 (3-35). Granulation of the ulcer occurred in a mean of 30.2±34.2 days from the initiation of treatment, while epithelialisation was noted in 60.4±47.8 days (Table 2). The epithelialisation period was found to be quicker than traditional methods of ulcer treatment like negative pressure or advanced moist wound therapies [7].

To summarize, in all cases an accelerated healing process was identified, with increased neovascularisation of the ulcerative area. This occurred frequently even after the first application of the PRP dressing. Interestingly, most of the ulcers were almost healed completely after the $1^{st}$-$6^{th}$ treatment session, while pain in the area was also reduced for the majority of the patients.

## Phenotypic Characterization of PRP Cells

The phenotype of cells found in PRP was determined by flow cytometry using antibodies for a number of surface markers (Fig. 2a). Seventeen PRP samples were tested and found positive for the hematopoietic markers CD45 (leukocyte common antigen), CD14 (LPS-R), for the superfamily members Ig [CD31 (PECAM-1), CD50 (ICAM-3) and CD1a (MHC-glycoprotein)] and other markers, such as CD44 (HCAM-1), CD3 (T3) and CD13 (aminopeptidase N) (Fig. 2a). A more detailed analysis of the PRP profile indicated that CD73 (SH3, SH4), CD105 (endoglin), and CD166 (SB10/ALCAM) were expressed at high levels, and CD90 was expressed at low levels, (Thy-1), while these cells were negative for CD34 and CD117 (c-kit). The integrins, CD29 (β1-integrin) and CD49e (α5-integrin), and the selectins, CD62E (E-selectin) and CD62P (P-selectin) were also expressed in these cells (Fig. 2a).

## The Effect of PRP or PPP on AF-MSC Phenotype

Based on these findings, PRP can enhance the wound healing by up to now unknown mechanisms. In addition, MSCs can be activated and migrate towards the wound site or can be used as cellular grafts in clinical applications for wound healing [6, 9,

**Table 2** Healing data

| Number | Age | Stage | Approximate volume of tissue loss ($cm^3$) | Treatment (days) | Date of transition to granulation stage | Date of transition to epithelialsation stage | Number of separate sessions |
|---|---|---|---|---|---|---|---|
| 1 | 41 | Necrotic | 100 | 98 | 57 | 86 | 11 |
| 2 | 63 | Exudative | 20 | 21 | 7 | 14 | 3 |
| 3 | 45 | Necrotic | 1 | 21 | 7 | 14 | 3 |
| 4 | 83 | Necrotic | 25 | 120 | 99 | 120 | 6 |
| 5 | 73 | Necrotic | 9 | 182 | 124 | 168 | 12 |
| 6 | 78 | Necrotic | 30 | 93 | 15 | 48 | 9 |
| 7 | 60 | Exudative | 4.5 | 36 | 14 | 31 | 5 |
| 8 | 62 | Exudative | 3 | 58 | 16 | 31 | 9 |
| 9 | 35 | Exudative | 8 | 136 | 35 | 115 | 26 |
| 10 | 35 | Exudative | 150 | 26 | 0 | 26 | 3 |
| 11 | 59 | Exudative | 4 | 20 | 14 | 18 | 5 |
| 12 | 64 | Necrotic | 32 | 85 | 23 | 78 | 9 |
| 13 | 78 | Exudative | 4 | 43 | 15 | 36 | 9 |
| 14 | 47 | Exudative | 90 | 105 | 47 | 88 | 27 |
| 15 | 75 | Exudative | 4 | 19 | 12 | 19 | 5 |
| 16 | 37 | Necrotic | 50 | 196 | 21 | 118 | 35 |
| 17 | 54 | Exudative | 15 | 21 | 8 | 16 | 4 |
| AVERAGE | 58.2 | | 34.1 | 75.3 | 30.2 | 60.3 | 10.6 |
| STDEV | | | 43.1 | 58.0 | 34.2 | 47.8 | 9.5 |

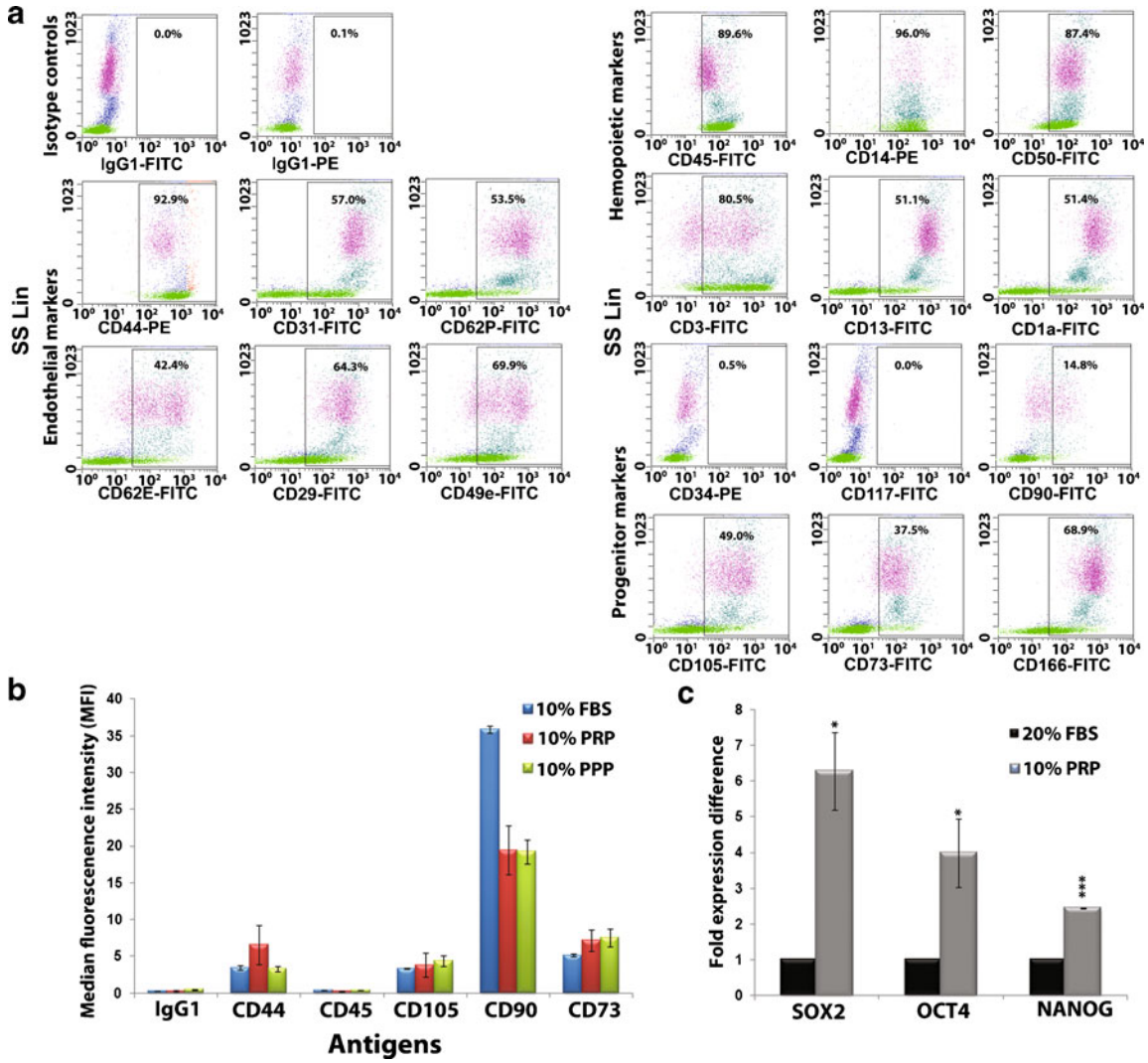



**Fig. 2** Phenotypic analysis of PRP cells and AF-MSCs cultured in the presence of PRP extract as serum substitute. **a** Representative FACS histograms of PRP cells for endothelial and hematopoietic progenitor marker expression. **b** Flow cytometry analysis of AF-MSCs cultured in the presence of PRP or PPP extracts as serum substitute for 10 days, for markers associated with MSC phenotype. The values shown are averages of the mean fluorescence intensity (MFI) ± SD for $n$=3 independent experiments. **c** Analysis of the expression levels of Oct-4, Sox-2 and Nanog in AF-MSCs cultured in the presence of PRP extract as a substitute for serum for 10 days. The values shown are the means ± SD, (*$p$<0.05 and ***$p$<0.001, Student's $t$-test)

24, 45]. We then sought to evaluate in detail the effect of PRP or PPP on the phenotype of AF-MSCs. Cells were cultured for 10 days in medium enriched with 10 % (v/v) PRP or PPP extract. The expression levels of MSC markers were examined by flow cytometry and directly compared to AF-MSCs which were grown in DMEM supplemented with 20 % (v/v) FBS (control cells). It was found that control cells, AF-MSCs cultured in 10 % (v/v) PRP and AF-MSCs cultured in 10 % (v/v) PPP were positive for the MSC-related markers, such as CD90, CD73, CD105 and the surface glycoprotein CD44 (Fig. 2b). Notably, all three cell types did not express the hematopoietic marker CD45. A small decrease in the levels of expression of CD90 in AF-MSCs cultured in 10 % (v/v) PRP or PPP was observed, but this had any effect on the cell phenotype and morphology [42] (Fig. 2b) .

Accumulating evidence from our group and others showed that AF-MSCs express high levels of the pluripotency markers Oct-4, Sox-2 and NANOG [11, 21, 40, 42–44, 51, 60]. Interestingly, AF-MSCs exhibited statistical significant increased expression levels of Oct-4, Sox-2 and NANOG when cultured in medium supplemented with PRP (Fig. 2c).

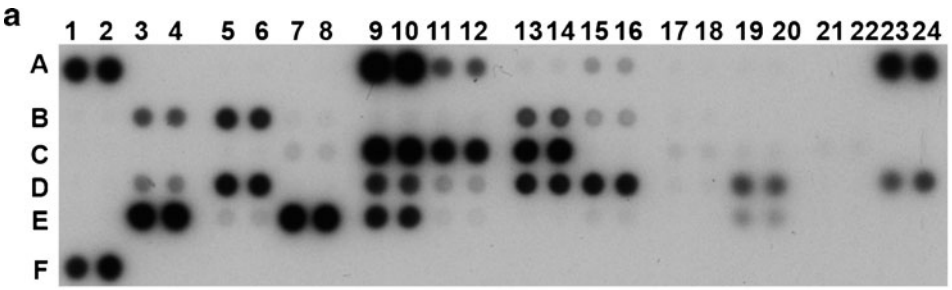
## Proteomic Profile of the PRP Cell Extract

For the identification and quantification of proteins contained in the cell extract derived from PRP, proteomic analysis was conducted using antibody profiler arrays. Thirty three factors

were detected, of which the most interesting were categorized according to their known functions (Fig. 3). Thus, factors that regulate angiogenesis such as, PDGF-AA, PDGF-AB/BB, angiopoietin-1, angiopoietin-2, VEGF and EG-VEGF, and others such as endostatin, endothelin-1 and IGFBP-1, -2, -3 were identified. Molecules such as uPA, thrombospondin-1, FGF-1, EGF, HGF, angiostatin, related to tissue repair were detected at low levels. In contrast, factors associated with cell migration such as TIMP-1, TIMP-4, MMP8 and MMP9 were detected in high levels (Fig. 3a and b).

## The Role of PRP or PPP in Cell Migration of AF-MSCs and DFs

During the process of wound healing, cell migration of various cell types, such as endothelial cells and their precursors,




| | 1,2 | 3,4 | 5,6 | 7,8 | 9,10 | 11,12 | 13,14 | 15,16 | 17,18 | 19,20 | 21,22 | 23,24 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | POS | | Activin A | ADAMTS-1 | Angigenin | Angiopoietin-1 | Angiopoietin-2 | Angiostatin/ Plasminogen | Amphire-gulin | Artemin | | POS |
| B | Coagulation factor III | CXCL16 | DPPIV | EGF | EG-VEGF | Endoglin | Endostatin/ Collagen XVIII | Endothelin-1 | FGF acidic | FGF basic | FGF-4 | FGF-7 |
| C | GDNF | GM-CSF | HB-EGF | HGF | IGFBP-1 | IGFBP-2 | IGFBP-3 | IL-1β | IL-8 | LAP (TGF-β1) | Leptin | MCP-1α |
| D | MIP-1α | MMP-8 | MMP-9 | NRG1-β1 | Pentra-xin-3 | PD-ECGF | PDGF-AA | PDGF-AB/ PDGF-BB | Persephin | Platelet factor 4 | PIGF | Prolactin |
| E | Serpin B5 | Serpin E1 | Serpin F1 | TIMP-1 | TIMP-4 | Thrombo-spondin-1 | Thrombo-spondin-2 | uPA | Vassohibin | VEGF | VEGF-C | |
| F | POS | | | | | | | | | | | NEG |

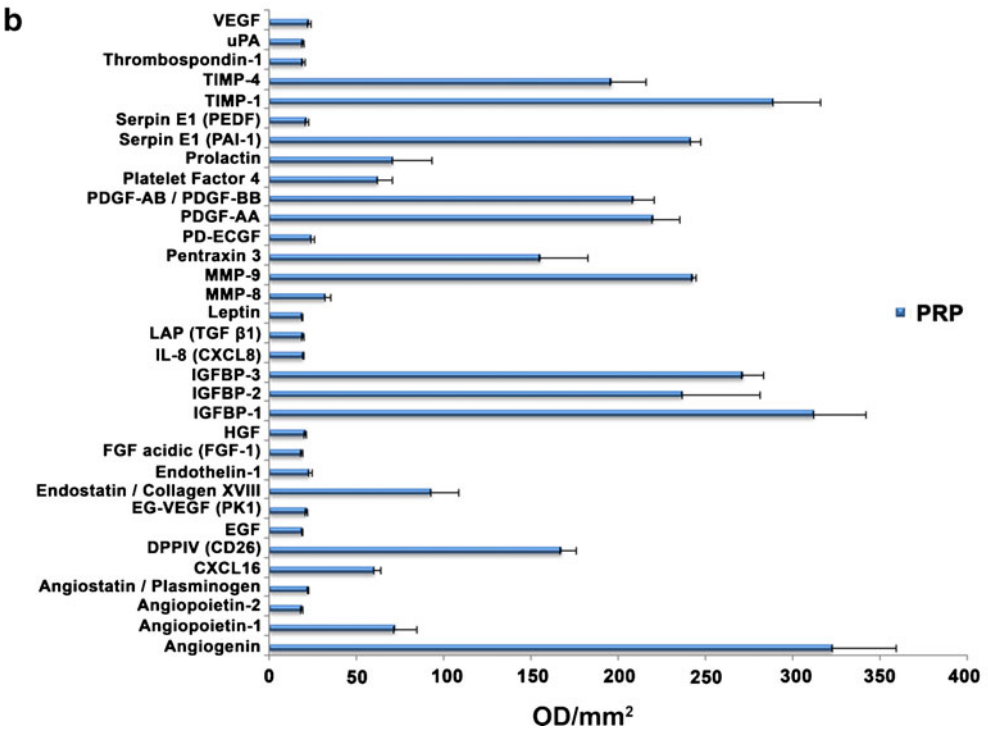



**Fig. 3** Analysis of growth factors contained in PRP using antibody profiler arrays. **a** Representative image of the antibody profiler array for PRP. Each spot corresponds to a growth factor. The table summarizes the names of the molecules within the array. **b** Relative expression of the growth factors detected in PRP. The values are normalized according to the positive controls in the array. The values represent average ± SD

MSCs or DFs, seems to be having an important role. In the present study, we examined the possible induction of cell migration due to PRP, using in vitro cell migration assay (Fig. 4). For this purpose, AF-MSCs or DFs were transferred to the upper side of a transwell insert and exposed to culture medium supplemented with 10 % (v/v) PRP, 10 % (v/v) PPP or 10 % (v/v) FBS. Statistically significant increase in migration of AF-MSCs towards to medium enriched with 10 % (v/v) PRP (196 %± 29.85 %, $p<0.001$, Student's *t*-test) compared to medium supplemented with 10 % (v/v) FBS was observed. AF-MSCs migrated to a lesser extent towards to medium enriched with 10 % (v/v) PPP (152 %±17.28 %, $p<0.05$, Student's *t*-test) [Fig. 4b (i)]. Accordingly, medium enriched with 10 % (v/v) PRP induced mobility of DFs (239 %±1.94 %, $p<0.01$, Student's *t*-test) compared to medium supplemented with 10 % FBS. The presence of PPP had no positive effect on the migration properties of these cells [Fig. 4b (ii)].

The proteomic analysis of PRP (Fig. 3) showed high levels of expression of factors associated with the cell migration such as MMP-9 [30, 47] and PDGF [58]. To investigate whether the increased motility of AF-MSCs and DFs towards to PRP was related to the high concentration of these factors, MMP9 and PDGF inhibitors were used prior to the in vitro cell migration assay. The results showed a statistically significant decrease in cell mobility of AF-MSCs to medium enriched with 10 % (v/v) PRP in the presence of MMP9 inhibitor (concentrations of 5 μM and 15 μM resulted in 42.37 %± 4.7 and 63.27 %±7.2 motility reduction, respectively) [Fig. 4c (i)]. Similarly, the presence of the PDGF inhibitor in the medium supplemented with 10 % (v/v) PRP led to decrease of AF-MSCs migration at 40.11 %±6.4 (5 μM) and 76.83 %± 7.2 (15 μM). Similar results were observed when studying the migration of DFs in the presence of MMP9 inhibitor resulting in 85.69 %±1,8 (5 μM) and 92.9 %±7.1 (15 μM) motility reduction and of PDGF inhibitor (concentration of 5 μM and 15 μM) resulting in 78.64 %±4.17 and 93.39 %±4.27, respectively [Fig. 4c (ii)].

These observations suggested that PRP is rich in growth factors that may regulate the migration of MSCs and fibroblasts in vitro through a combined effect of different components. Similar mechanisms are likely to promote wound healing in vivo.

### The Effect of PRP and PPP on the Proliferation Rate of the AF-MSCs and DFs

To gain further insight into the effect of the PRP and PPP extracts on the proliferation of AF-MSCs and DFs, MTS assay was performed. Thus, the effect of PRP extract as a substitute for FBS in AF-MSCs was investigated.

Cells were cultured for 5 days in serum-free medium supplemented with of 2-20 % (v/v) PRP or 5-10 % (v/v) PPP, respectively. The proliferation rate was estimated on the 5$^{th}$ day and compared to that of control cells (cultured in DMEM supplemented with 10 % or 20 % (v/v) FBS). As shown in Fig. 4d (i), all groups of PRP treated AF-MSCs exhibited a statistically significant increase in their proliferation rate compared to the control cells. PRP was found to be an effective alternative to FBS. More specifically, at a concentration of 5 % (v/v) PPP caused a statistically significant increase in the proliferation rate of AF-MSCs compared to the control cells [Fig. 4d (ii)]. Similarly, DFs in medium enriched with 10 % (v/v) PRP exhibited increased proliferation rate [Fig. 4d (iii)]. Moreover, in case of DFs, PPP at a concentration of 10 %, had a similar effect on their proliferation rate to FBS [10 % (v/v)] [Fig. 4d (ii)].

## Discussion

PRP according to several studies represents a rich source of growth factors [30, 47] which can be used in clinical practice for wound healing [58]. Interestingly, our studies showed accelerated healing of ulcers after treatment with PRP dressings and faster neovascularisation of the affected area. Similar results have been published from other studies non- randomized, prospective and retrospective. In more detail, the use of platelets seems to achieve a quicker healing compared to conventional methods [3, 25, 34, 35, 46].

The beneficial effects of PRP seem to occur in many types of wounds and ulcers and in different parts of the body, including surgical wounds [14, 35, 46, 53]. While the results of this method are clearly evidence based, the exact mechanism underlying the wound healing process, constituted the main part of our study.

The healing process comprises the attraction of various types of cells at the wound site. It has also been observed that the application of PRP at the wound enhances the secretion of local growth factors which increase cell migration and proliferation of the target cells [39]. In this study, we defined the proteomic profile of PRP and detected the expression of growth factors and molecules related to angiogenesis and cell migration, such as PDGF- AA, PDGF-AB/BB, MMP-9, angiopoietin-1, angiopoietin-2, VEGF and EG-VEGF, endostatin, angiogenin, endothelin-1 and IGFBP-1, -2, -3.

Using in vitro experimental approaches, we further studied the effect of PRP on the proliferation and migration properties of AF-MSCs and DFs. Notably, the presence of PRP in the AF-MSC medium substituting FBS, resulted in a significant induction of the migration ability and the proliferation rate of AF-MSCs and DFs. Different concentrations of PRP ranging

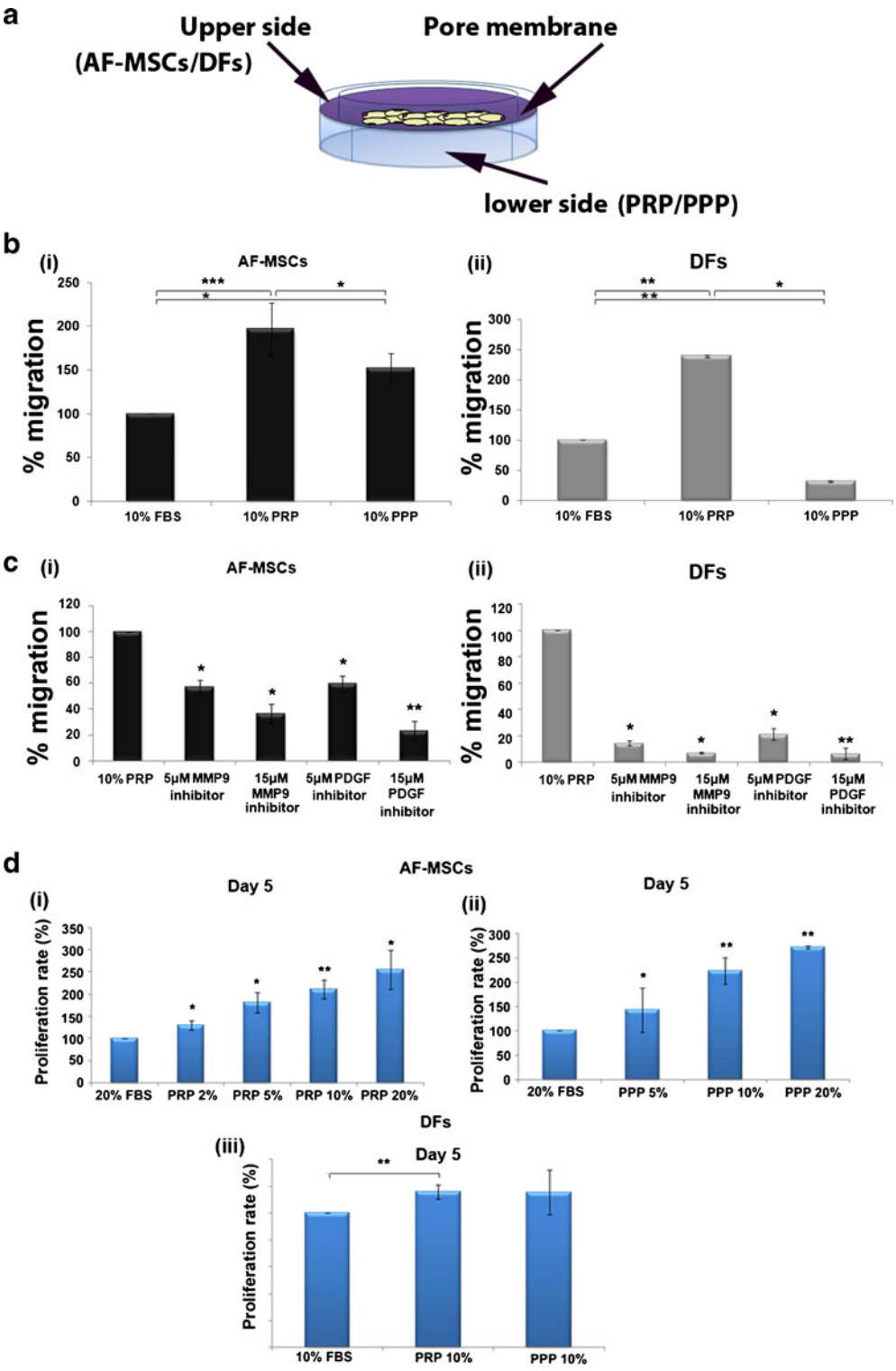



**Fig. 4** Role of PRP on cell migration of AF-MSCs and DFs in vitro **a** Schematic representation of a transwell insert for cell migration in vitro **b** Histograms showing the migration of (i) AF-MSCs and (ii) DFs to 10 % (v/v) PRP and 10 % (v/v) PPP, respectively. **c** Histograms showing the migration of (i) AF-MSCs and (ii) DFs to 10 % (v/v) PRP and 10 % (v/v) PRP in the presence of different concentrations (5 μM-15 μM) of MMP9 and PDGF inhibitors. **d** Assessment of proliferation rate (i, ii) for AF-MSCs and (iii) DFs in the presence of PRP and PPP. The values represent average ± SD,(*$p$<0.05, **$p$ <0.01 and ***$p$ <0.001 Student's $t$-test)

from 2 % to 20 % can exhibited positive effects on proliferation and migration of these cells and can be further used to test the potency of PRP. The highly expressed factors such as PDGF and MMP9 in PRP seem to act positively in inducing migration of these cells. The role of MMP-9 [15, 56] and PDGF [4] on the migration of various cell types is described in a range of recent studies, confirming in this way our findings. During the healing process, cells can be attracted to the wound site through the bloodstream, mainly because of local secretion of growth factors and endogenous signals and thus contribute to a rapid return of the wound [17, 48]. Recent studies [18, 57] along with the experimental data presented in this

paper indicated that PRP can be a powerful tool to attract cell populations, such as MSCs or DFs at the wound area. Recently, our group also showed that the AF-MSCs and DFs in the presence of endothelial precursors cells actively contribute to angiogenesis in vitro and in vivo, a process that plays a central role in the healing process [45]. Furthermore, PRP can be an alternative substitute to bovine serum for growing MSCs [13]. PRP extract as an autologous preparation, may exhibit an advantage over the bovine serum for growing MSCs.

In summary, our data suggest that PRP may act as a regulator of cell migration and wound healing. The effect of PRP may also open the way in order to investigate new therapeutic applications in clinical practice.

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**Authorship Contribution** M.G. Roubelakis: Conception and design, experimental procedures, data analysis, data approval and manuscript writing.

O. Trohatou: Experimental procedures, data analysis and manuscript reviewing

A. Roubelakis: PRP samples provision, patient treatment and surveillance, data analysis and manuscript reviewing.

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Georgios Papazoglou: Patient treatment and surveillance

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N.P. Anagnou: Financial support and manuscript reviewing

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