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***Title**:*

Plasma rich in growth factors (PRGF-Endoret) stimulates proliferation and migration of primary keratocytes and conjunctival fibroblasts while inhibits and reverts TGF-β1-induced myodifferentiation

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Abstract

Purpose: Plasma rich in growth factors (PRGF-Endoret) technology is an autologous platelet-enriched plasma obtained from patient's own blood, which after activation with calcium chloride allows the release of a pool of biologically active proteins that influence and promote a range of biological process including cell recruitment, growth and differentiation. Since ocular surface wound healing is mediated by different growth factors, we decided to explore the potential of PRGF-Endoret technology in stimulating the biological processes related with fibroblast-induced tissue repair. Furthermore, the anti-fibrotic properties of this technology were also studied.

Methods: Blood from healthy donors was collected, centrifuged and, whole plasma column (WP) and the plasma fraction with the highest platelet concentration (F3) were drawn off avoiding the buffy coat. Primary human cells including keratocytes and conjunctival fibroblasts were used to perform the "in vitro" investigations. The potential of PRGF-Endoret in promoting wound healing was evaluated by means of a proliferation and migration assays. Fibroblast cells were induced to myofibroblast differentiation after the treatment with 2.5 ng/ml of TGF-β1. The capability of WP and F3 to prevent and inhibit TGF-β1-induced differentiation was evaluated.

Results: Results show that this autologous approach enhances significantly proliferation and migration of both keratocytes and conjunctival fibroblasts. In addition, PRGF prevents and inhibits TGF-β1-induced myofibroblast differentiation. No differences were found between WP and F3 plasma fractions.

Conclusion: These results suggest that RGF-Endoret could reduce scarring while stimulate wound healing in ocular surface. F3 or whole plasma column show similar biological effects in keratocytes and conjunctival fibroblast cells.

Introduction

Two tissues compose the ocular surface: the cornea and the conjunctiva. Both provide important functions to the eye including ocular protection, lubrication and refractive power. After an injury, the main layers affected in both tissues are the stratified epithelium, the basement membrane and the stroma. The stroma is one of the most important layers involved in wound healing, and it is composed mainly by fibroblasts. Keratocytes are the specialized corneal fibroblast characterized by their low activity (quiescent) and their distribution through the stroma. Some of their main functions include maintaining the corneal transparency and producing stromal components such as collagen fibers and extracellular matrix. It has been reported that to effectively repair a damage area after an injury, the fibroblasts adjacent to the injury need to proliferate and migrate to repopulate the area.[1,2] These processes are in part mediated by different growth factors such as epithelial growth factor (EGF), fibroblast growth factor (FGF) and transforming growth factor β1 (TGF-β1).[3-6] among others.

In some types of wounds these fibroblasts may develop actin contractile filaments, being differentiated into myofibroblasts.[7,8] During injury repair, myofibroblasts are responsible for wound contraction and extracellular matrix (ECM) deposition and organization. TGF-β has been identified as one of the main inductors of fibroblast differentiation into myofibroblast.[9,10] However, the persistence of myofibroblastic cells after wound healing has been identified as the primary biological episode responsible for the development of scarring tissue.[11,12] The presence of fibrotic tissue at the anterior surface of the eye after an injury or a surgery may induce the opacification of the cornea (corneal haze),[13,14] or may lead to surgical failure.[15,16]

Different approaches to regenerate the ocular surface injury[17,18] and to treat the scar formation have been attempted.[19-21] One interesting alternative is to evaluate the potential of autologous plasma and platelet derived growth factors in stimulating fibroblast-modified wound healing. The technology of plasma rich in growth factors (PRGF®-Endoret®, trademarks for Europe and USA, respectively) consists on the elaboration and use of a platelet enriched plasma obtained from patient's own blood, which after activation with calcium chloride allows the *in-situ* formation of a biodegradable fibrin scaffold and the release of a pool of biologically active proteins that influence and promote a range of biological process including cell recruitment, growth and differentiation.[22,23] Interestingly, some of the proteins secreted from alpha-granules of platelets, including EGF, PDGF and NGF, are necessary to promote wound repair and to maintain ocular surface well preserved.

RGF-Endoret technology has provided significant clinical advances in terms of wound healing and tissue regeneration in different fields including dentistry and

oral implantology,[24,25] orthopedics, sport medicine[26] and ulcer treatment[27] among others. In all these situations, small volumes of plasma rich in growth factors and reduced number of doses are needed to achieve therapeutic efficacy. According to the technique, the fraction with the highest platelet concentration (also known as fraction 3) should be used to promote tissue regeneration as this plasma volume contains the higher amount of proteins. However, this may hamper its use as an autologous eye drop, due to the elevated number of doses necessaries per day and the long period of treatment required to complete healing in several pathologies.

Assuming that function of fibroblasts is critical during ocular surface healing, the purpose of the present study is to assess the potential of PRGF-Endoret technology as an innovative approach for enhancing ocular repair and regeneration. To address these issues, the effects of fraction 3 (F3) or whole plasma (WP) column (with lower amount of proteins than F3) obtained from PRGF-Endoret technology were assessed over primary keratocytes and conjunctival fibroblasts proliferation and migration. In addition, the potential of WP and F3 to inhibit and revert TGF-β1 stimulated myodifferentiation was also evaluated.

MATERIALS AND METHODS

Cells

Primary human cells including keratocytes (termed HK) and conjunctival fibroblasts (termed HConF) (ScienCell Research Laboratories, San Diego, CA), were cultured according to manufacturer's instructions. Briefly, cells were cultured until confluence in Fibroblast medium supplemented with Fibroblast Growth Supplement (Complete FM) (ScienCell Research Laboratories, San Diego, CA) and then were detached with animal origin-free trypsin-like enzyme (TrypLE Select, Gibco-Invitrogen, Grand Island, NY, USA). Cell viability was assessed by trypan blue dye exclusion. Passage 3-6 cells were used in all experiments.

Immunolabelling of cells

The fibroblast-like morphology of cells and the absence of dedifferentiation were confirmed by phase-contrast microscopy and immunolabelling for Collagen Type I (Chemicon – Millipore, Billerica, MA, USA), Fibronectin and Vimentin (Sigma–Aldrich, St Louis, MO,USA). The cells were also tested against typical endothelial cells and hematopoietic progenitor cells markers: CD105 and CD34 (BD Biosciences, San Jose, CA, USA) and against alpha-Smooth muscle actin (Sigma-Aldrich, St Louis, MO, USA) in order to check the spontaneous differentiation to myofibroblasts in culture.

Briefly, 9500 cells/well were plated on 24-well plate with poly-L-lysine-coated glass coverslips (BD BioCoat, BD Biosciences, San Jose, CA, USA). Cells were fixed for 10 minutes in 4% formaldehyde for CD34, CD105 and type I collagen

antigens, in methanol: acetic acid (3:1) for vimentin antigen and in methanol pre-cooled at - 20 ºC for fibronectin and alpha-smooth muscle actin (α-SMA). Cells for type I collagen staining were permeabilized with 1% Triton X-100, in phosphate-buffered saline (PBS) for 10 minutes. After that, cells were blocked with fetal bovine serum (FBS) (10% in PBS) for 30 minutes, and incubated for 1 h with the primary antibodies in the following dilutions: 1:20 for type I collagen, 1:30 for CD34 and CD105; 1:50 for vimentin and 1:800 for fibronectin and α-SMA. Next, cells were incubated with their appropriate secondary antibodies, goat anti-mouse IgG conjugated with Alexa Fluor 488 or goat anti-rabbit IgG conjugated with Alexa Fluor 594 (both from Molecular Probes-Invitrogen, Grand Island, NY, USA). Finally, cells nuclei were stained with Hoechst 33342 (Molecular Probes-Invitrogen, Grand Island, NY, USA), mounted, and visualized under a fluorescence microscope (Leica DM IRB, Leica Microsystems, Wetzlar, Germany).

PRGF-Endoret preparations

Blood from one healthy young male donor was collected after informed consent into 9-ml tubes with 3.8% (wt/v) sodium citrate. The study was performed following the principles of the Declaration of Helsinki. Samples were centrifuged at 580 g for 8 min at room temperature in a PRGF-Endoret System centrifuge (BTI Biotechnology Institute, S.L., Miñano, Álava, Spain). Half of the tubes were used to separate the whole plasma column (WP) over the buffy coat and the other half to take the immediately upper milliliter over the buffy coat called Fraction 3 (F3)—the platelet enriched fraction (see Fig. 1). In both cases, care was taken to avoid the buffy coat containing the leukocytes. Platelets and

leukocytes counts were performed with a hematology analyzer (Micros 60, Horiba ABX, Montpelier, France). Both preparations were incubated with PRGF-Endoret activator (BTI Biotechnology Institute, S.L., Miñano, Álava, Spain) at 37°C in glass tubes for 1 hour. The released supernatants were collected by aspiration after centrifugation at 1000 g for 20 min at 4°C. Finally, plasma obtained from WP and F3 was aliquoted and stored at -80°C until use. Growth factors (TGF-β1, PDGF-AB, VEGF, HGF, EGF, IGF-I and TSP-1) were measured in the supernatants using commercially available Quantikine colorimetric sandwich enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, USA) (Table 1).

Proliferation assay

Keratocytes and conjunctival fibroblasts were seeded at a density of 10000 cells per cm^2 on 96 well optical bottom black plates and maintained with serum-free medium for 48 h. Then, culture medium was replaced by serum-free medium supplemented with either: (i) the culture medium alone (FM) with 0.1% FBS as a control of non-stimulation (NS) (ii) 20% (v/v) WP, or (iii) 20% (v/v) F3. The study period was 48 h. Density of cells in culture was estimated using the CYQUANT Cell Proliferation Assay (Invitrogen, Carlsbad, CA, USA). Briefly medium was removed and wells were washed carefully with phosphate buffered saline (PBS). Then microplate was freezed at –80°C for efficient cell lysis in the CyQUANT assay. After thawing the plates at room temperature, samples were incubated with RNase A (1.35 Ku/ml) diluted in cell lysis buffer during 1 hour at room temperature. Then 2x CyQUANT GR dye/cell lysis buffer was added to each sample well, mixed gently and incubated for 5 minutes at room

temperature protected from light. Sample fluorescence was measured using a fluorescence microplate reader (Twinkle LB 970, Berthold Technologies). A DNA standard curve ranging from 7.8 to 1000 ng/ml was included in all fluorescence quantifications. As an index of cell number, calibration curves ranging from 2500 to 90000 cells per cm^2 were established using the Cyquant assay.

Migration assay in response to WP and F3

In order to quantify the migratory potential of conjunctival fibroblasts and keratocytes, they were plated in culture inserts (Ibidi, GmbH, Martinsried, Germany) placed on a 24-well plate at high density and were grown with Complete FM until confluence. After carefully remove the inserts, two separated cell monolayers leaving a cell-free gap of approximately 500 μm thickness were created. The cells were washed with PBS and incubated with the same treatments as in the proliferation assay (0.1% FBS, 20% WP or 20% F3) in quintuplicate for 24 hours. After this period, the different culture mediums were removed and cells were incubated with 1/500 Hoechst 33342 in PBS for 10 min. To quantify the number of migratory cells, phase contrast images of the central part of the septum before treatment and phase contrast and fluorescence photographs after the treatment time were captured with a digital camera coupled to an inverted microscope (Leica DFC300 FX and Leica DM IRB, Leica Microsystems). The gap area and the migratory cells found in this gap after the 24 hours of treatment were measured using the Image J Software (NIH, Bethesda, Maryland, USA). The results were expressed as number of cell migrated per mm^2 of area.

Myofibroblast differentiation

Differentiation of conjunctival fibroblasts and keratocytes to myofibroblasts was induced by 2.5 ng/ml of TGF-β1 (Chemicon-Millipore, Billerica, MA, USA) for 72 hours. Passage 4 cells were plated at a density of 5000 cells per cm^2 in 48-well tissue-culture plates and maintained with serum-free culture medium for 48 h. After this period, the medium was replaced by FM and keratocytes and conjunctival fibroblast were stimulated with either (i) 2.5 ng/ml TGF-β1; (ii) 2.5 ng/ml TGF-β1 + 20% (v/v) WP; or (iii) 2.5 ng/ml TGF-β1+ 20% (v/v) F3 supernatant for 72 hours. In the following experiments, cells were initially pre-treated with 2.5 ng/ml TGF-β1 for 3 days. After this pre-treatment, culture medium was replaced by FM and supplemented with either one of these three treatments: (i) 2.5 ng/ml TGF-β1; (ii) 2.5 ng/ml TGF-β1 + 20% (v/v) WP supernatant; or (iii) 2.5 ng/ml TGF-β1 + 20% (v/v) F3 supernatant for 3 days. In both protocols, a supplement of 0.1% FBS was added to the 2.5 ng/ml TGF-β1 treatment in order to maintain cell viability in the control group. Experiments were performed in quintuplicate.

After incubation time, medium was removed and cells were fixed for 10 minutes in methanol. Cells then were blocked with FBS (10% in PBS) for 30 minutes, and incubated for 1 hour with mouse anti α-SMA antibody at 1:800, followed by incubation with goat anti-mouse IgG conjugated with Alexa Fluor 488 at 1:100 for 1 h. Finally, cell nuclei were counterstained with Hoechst 33342 mounted using an anti-fade solution (SouthernBiotech, Birmingham, AL, USA) and visualized under a fluorescence microscope (Leica DM IRB). Control isotype

was performed by substituting the primary antibodies by 10 % of FBS diluted in PBS.

For myofibroblast cell counting two random 10x microscopic fields were photographed on each well. The digitalized images were analyzed using the Image J software. Hoechst (+) cells were counted to obtain the total cell number. Hoechst (+) and α-SMA (+) cells were counted as myofibroblasts. Cells showing any kind of greenish staining were considered α-SMA positive. Expression of α-SMA was also evaluated on cells treated with 20% (v/v) WP or F3 in order to check the myofibroblast differentiation.

Inhibition of myofibroblastic differentiation

Fibroblasts were seeded at a density of 5000 cell per cm^2 in a 48-well plate and were pre-treated with 2.5 ng/ml of TGF-β1 plus 0.1% FBS as differentiation medium. After 72 hours, one part of the cells were fixed and stained for α-SMA and Hoechst 33342 as a positive control of differentiation. The remaining cells were then treated with FM plus (i) 0.1% (v/v) FBS + 2.5 ng/ml TGF-β1, (ii) 20% (v/v) PRGF + 2.5 ng/ml TGF-β1 or (iii) 20% (v/v) F3 + 2.5 ng/ml TGF-β1 for 3 days. All samples were performed in quintuplicate. The immunolabelling for α-SMA and Hoechst and cell counting were performed as previously described.

Statistical analysis

Means and their respective 95% confidence intervals were calculated for each of the treatments, proceeding to review the potential differences between

treatments for each experimental process (proliferation, migration, and protective and reversible effect of WP and F3). Differences between treatments were considered to be significant in cases where the boundaries of the respective 95% did not overlap.

RESULTS

The human primary conjunctival fibroblasts (HConF) and keratocytes (HK) showed the typical spindle-shaped aspect in culture and did not spontaneously differentiate into myofibroblasts as confirmed by the absence of α-SMA expression (data not shown). Cells were positive for all the fibroblast markers (Collagen Type I, vimentin and fibronectin) and negative for markers of hematopoietic and endothelial cells (data not shown).

Platelet enrichment of the PRGF-Endoret preparations were 2.6-fold for WP (481 x 10^6 platelets/ml) and 3.6-fold for F3 (663 × 10^6 platelets/ml) over the baseline concentration in whole blood. None of the preparations contained detectable levels of leucocytes. Table 1 shows platelet and leukocyte concentration for each sample (WP and F3) and the levels of some of the most important growth factors.

Cell proliferation

Proliferation of conjunctival fibroblasts and keratocytes significantly increased after treatment with both PRGF-Endoret preparations (20%WP or 20%F3) as it is shown in Figure 2. In fact, conjunctival fibroblasts proliferated 3.1-fold and 3.4-fold after treatment with WP and F3 respectively. On the other hand, keratocytes showed a significant increase over basal conditions of 2.8-fold with WP and 2.7-fold with F3. No significant differences were found between the autologous treatments.

Migration assay

WP and F3 stimulated significantly the migratory capacity of both HConF and HK. In particular, migration of HConF increased 1.8-fold and 1.7-fold over the non-stimulatory situation for WP and F3 respectively (Fig. 3A) whereas migration of keratocytes increased 2.3-fold for WP and 1.8-fold for F3 (Fig. 3B). The number of migrating cells was significantly higher with plasma preparations than with NS cells. No statistical differences were found between PRGF treatments. Fig. 3C shows phase contrast images of HConF and HK cells after a 24-hour period of migration and highlights the potent stimulatory effect of WP and F3 over the treated cells.

Protective effect of plasma preparations

The effects of plasma preparations (WP and F3) on the prevention of the TGF-β1-stimulated myofibroblastic differentiation were evaluated. Cells treated with either 20% WP or 20% F3 alone were not differentiated into myofibroblasts (data not shown). HConF and HK cells showed a spontaneous differentiation to myofibroblasts in a percentage of 16% ± 2% and 14% ± 8% respectively after 72-hour-culture with 0.1% of FBS. After 3 days of stimulation with 2.5 ng/ml of TGF-β1, conjunctival fibroblasts and keratocytes showed a 61% ± 32% and a 48% ± 23% of α-SMA positive cells respectively (Fig. 4A and 4B). The immunofluorescence for α-SMA exhibited that after culturing the cells 3 days either with 20% of WP + 2.5 ng/ml TGF-β1 or with 20% of F3 + 2.5 ng/ml TGF-β1, the percentage of positive HConF cells decreased drastically to 0.2% ± 0.1% and 0.1% ± 0.3% respectively. This decrease was statistically significant

for both types of fibroblasts respecting to the spontaneous transformation in culture and also with respect to the condition of TGF-β1 alone. Figure 4C shows the protective role of WP and F3 against the effect of TGF-β1 over HConF and HK cells. No significant differences were found between the responses induced by WP or F3 on fibroblasts transformation to myofibroblasts.

Inhibition of myofibroblastic phenotype

The effects of both plasma fractions (WP and F3) on the inhibition of the TGF-β1-stimulated myofibroblasts differentiation were evaluated. After pre-treating the cells during 3 days with 2.5 ng/ml of TGF-β1, a 60% ± 25% of α-SMA positive HConF cells and a 77% ± 7% of α-SMA positive HK were found (Fig. 5A and 5B). Then cells were treated for another 3 days with either TGF-β1, a combination of TGF-β1 + 20% WP or a combination of TGF-β1 + 20% F3. Interestingly, the percentage of myofibroblasts in the HConF population was reduced significantly to 7% ± 2% when WP was added and to 11% ± 3% when F3 was added (Fig. 5A). In the case of HK cells, the number of α-SMA positive cells decreased drastically to 8% ± 2% and to 12% ± 2% when WP and F3 were added respectively (Fig. 5B). Therefore, the use of PRGF-Endoret treatments reduced significantly (with a 95% of confidence interval) the number of myofibroblasts. As counterpoint, cells treated with 0.1% FBS + 2.5 ng/ml TGF-β1 for 3 days maintained the myofibroblastic phenotype. In fact, an 86% ± 8% of the HConF cells and a 71% ± 7% of the HK cells were positive for α-SMA.

The immunofluorescence detection of α-SMA revealed that both WP and F3 inhibited the differentiation of the different populations of fibroblasts into myofibroblasts (Fig. 5C).

Discussion

Several groups have focused their studies in understanding the different processes related with the wound healing of the ocular surface. There is a general agreement that one of the main events involved in wound healing is cell proliferation as well as the migration of these cells to the damage area.[1,28,29] There are also reports showing that different growth factors and cytokines and an intricate network of signals are implicated in the modulation of wound healing.[30]

Plasma Rich in Growth Factors (PRGF-Endoret) is an autologous platelet rich plasma technology by which it is possible to obtain different growth factor-enriched formulations that can be used in the treatment of several disorders.[31] The effects of PRGF-Endoret on tissue regeneration have been deeply demonstrated in different areas of the medicine, such as dentistry, oral implantology, orthopedics, sports medicine and the treatment of skin disorders.[32]

In this study, we report for the first time the effects of PRGF-Endoret on the proliferation, migration and differentiation of human keratocytes and conjunctival fibroblasts *in vitro*. Results demonstrate that the different plasma formulations evaluated (WP and F3) enhance proliferation and migration of both types of fibroblast populations while significantly protect and inhibit TGF-β1-induced myofibroblast differentiation. Interestingly, there are not significant differences between the effects induced by the platelet enriched plasma fraction (F3) and the whole plasma fraction (WP). Although preliminary, these results may help to understand the potential of the autologous formulations in corneal wound healing.

One important concern in ocular wound healing is scar formation. TGF-β has been identified as one of the most potent inductors of fibroblast differentiation into α-SMA-expressing myofibroblasts.[9,22] According to Masur et al.,[33] a spontaneous fibroblast differentiation to myofibroblasts is observed when cells are cultured at low cell density or at low concentration of FBS. In our study, a spontaneous differentiation of 16% for HConF cells and 14% for HK cells was detected. No additional differentiation was observed after adding the PRGF formulations (WP and F3).

Our current findings confirm that PRGF-Endoret technology protects TGF-β1-induced α-SMA-expression of keratocytes and conjunctival fibroblasts. In fact, when cells were co-cultured either with 20% WP or F3 and TGF-β1 simultaneously, α-SMA-expression was under 0.3%. In addition, both WP and F3 significantly inhibited myofibroblast differentiation even when a previous 3-day culture with TGF-β1 had been carried out.

Although further research is needed to clarify the molecular events that regulate PRGF-Endoret biological activity, it seems reasonable that some of the proteins present in both WP and F3 may have played key roles in cell proliferation, migration and differentiation. Some growth factors present in PRGF-Endoret preparations have been described as key regulators of corneal wound healing. For example, epidermal growth factor (EGF), hepatocyte growth factor (HGF) and keratocyte growth factor (KGF) stimulate epithelial cell proliferation and migration, while in the case of stroma, these processes are mediated by transforming growth factor (TGF), platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF).[34-38]

Some studies have discussed the mechanisms by which the myofibroblastic phenotype disappears from corneal tissue or cultured cells. Interestingly, it has been observed that FGF-1 and -2, some proteins present in PRGF-Endoret formulations, promote the fibroblast phenotype and reverse the myofibroblast phenotype.[11] Some other studies suggest that myofibroblast apoptosis may be one of the initial mechanisms of myofibroblast disappearance; although myofibroblasts transdifferentiation to keratocytes or corneal fibroblasts should also be considered.[39]

Another important conclusion from this study is that WP exerts similar *in vitro* biological effects that F3. It has to be assumed that until now, F3, that is, the plasma fraction with the highest platelet concentration, has been widely used in several medical areas[28-31] including the treatment of ocular diseases[40-43]. The data presented herein may modify the current clinical protocols as WP shows similar biological effects than F3 but represents an improvement in the yield of 400%. In fact, while only one milliliter of F3 can be obtained from 9 mL of blood, almost 4 mL of WP can be obtained from the same blood volume.

In summary, the different formulations of Plasma Rich in Growth Factors (PRGF-Endoret) enhance proliferation and migration of keratocytes and conjunctival fibroblasts while protect and inhibit TGF-β1-induced myofibroblast differentiation. Although further studies are needed to determine the exact mechanisms underlying the effects of this autologous technology, results from this study suggest that the different PRGF-Endoret formulations (WP and F3) could improve the wound healing in ocular surface.

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Figure legends:

Figure 1. Scheme of the different plasma fractions obtained with the PRGF-Endoret technology. F1: fraction 1; F2: fraction 2; F3: fraction 3; WP: Whole Plasma column containing F1, F2 and F3. In all the different plasma preparations, care was taken to avoid the buffy coat containing leukocytes.

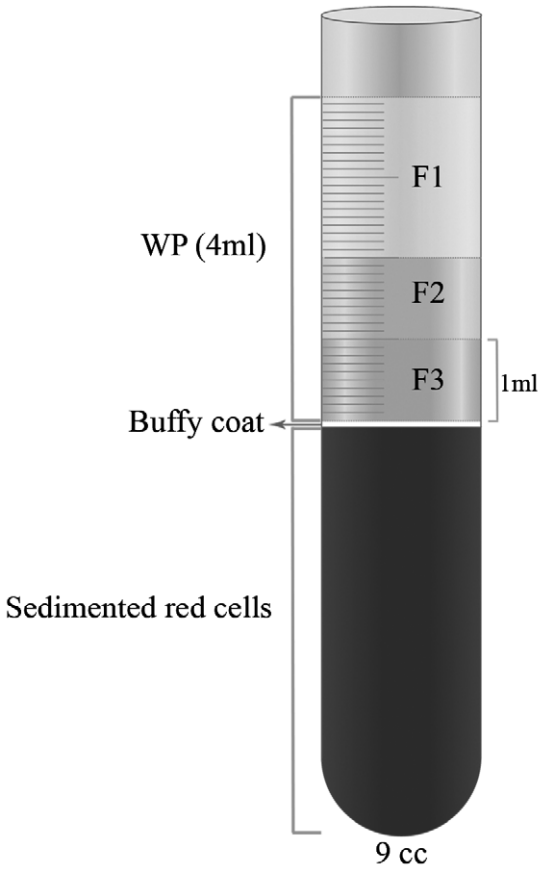

Figure 2. (A) Proliferation of HConF cells after culturing with 0.1% FBS (N.S.), 20% WP or 20% F3 for 2 days. (B) Proliferation of Keratocyte (HK) cells after culturing with 0.1% FBS (N.S.), 20% WP or 20% F3 for 2 days. WP and F3 significantly increased proliferation of both cells compared with non-stimulatory conditions (*, 95% confidence interval). No statistical differences were found between WP and F3.

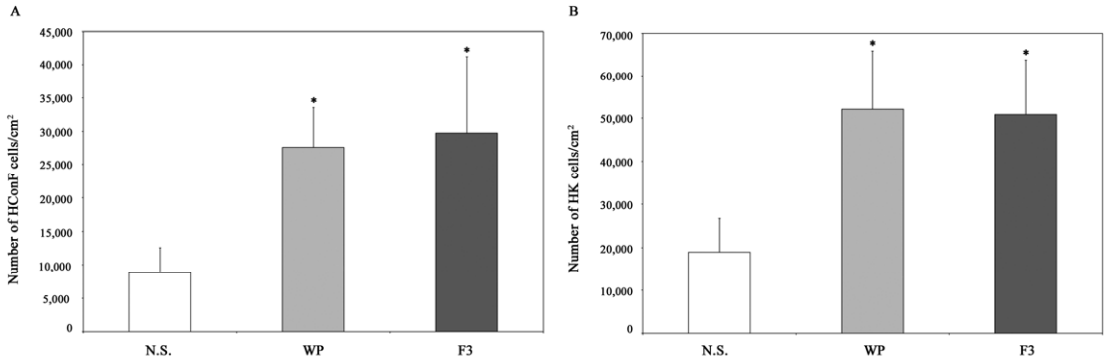

Figure 3. (A) Migration rate of HConF cells after culturing with 0.1% FBS (N.S.), 20% WP or 20% F3 for 24 hours. (B) Migration rate of Keratocyte (HK) cells after culturing with 0.1% FBS (N.S.), 20% WP or 20% F3 for 24 hours. WP and F3 significantly increased migration of both cells compared with non-stimulatory conditions (*, 95% confidence interval). No statistical differences were found between WP and F3. (C) Phase contrast photomicrographs illustrating the migration rate of HConF and HK cells. Scale bar: 300 μm

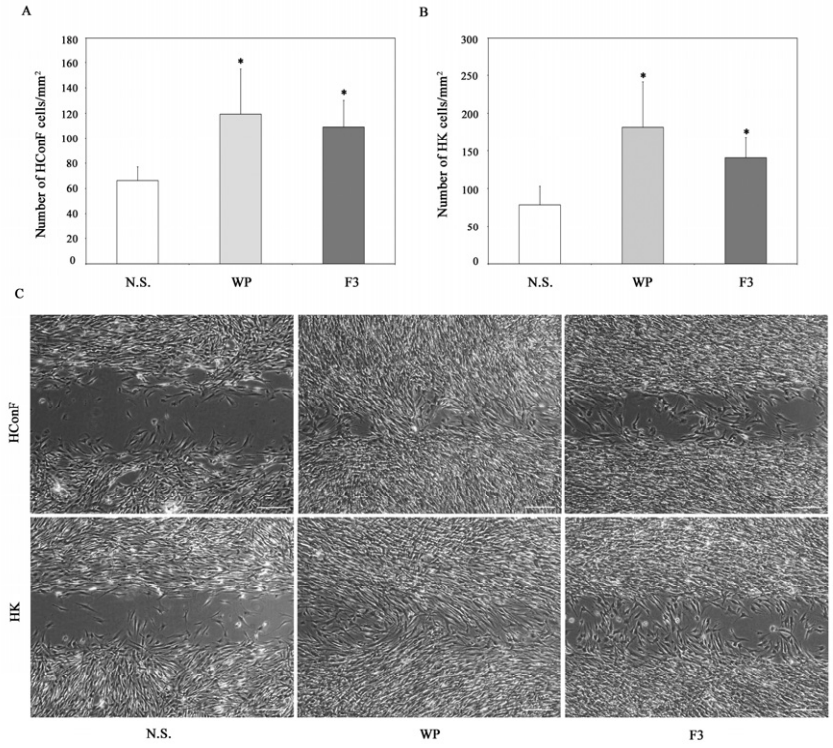

Figure 4. When cells were treated simultaneously with TGF-β1 and WP or TGF-β1 and F3, (A) Conjunctival fibroblasts (HConF) and (B) Keratocytes (HK) number of α-SMA positive cells were significantly lower compared to the TGF-β1 treatment group (95% confidence interval). There is also a significant difference between spontaneous myo-transformation and the number of α-SMA positive cells after treatment with 2.5 ng/ml TGF-β1 + 0.1% SBF or + 20% WP or + 20% F3. (C) Immunofluorescence for detection of α-SMA protein in HConF and HK cultured cells. α-SMA (+) and Hoechst (+) cells are considered as myofibroblasts. Scale bar: 200 μm.

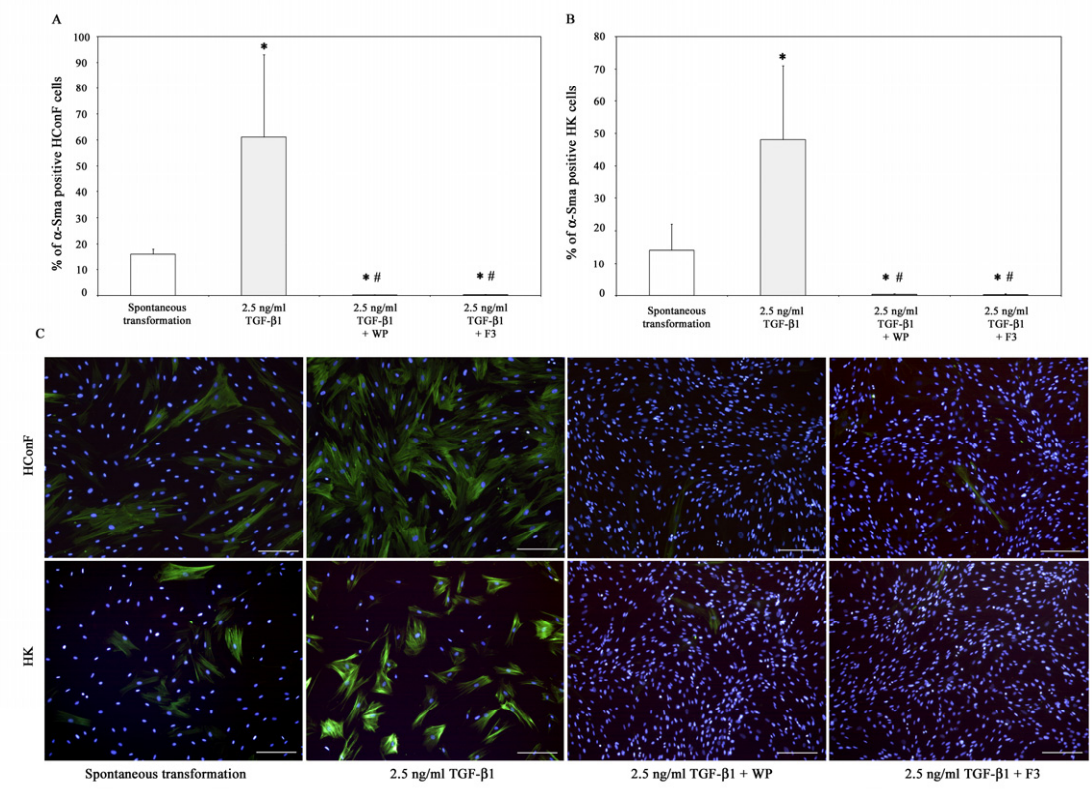

Figure 5. Capacity of reversion of PRGF-Endoret technology over the myofibroblastic phenotype. HConF and HK cells were treated for 3 days with 2.5 ng/ml TGF-β1 as a previous stimulation to get a population of myofibroblasts. Then they were cultured with 2.5 ng/ml TGF-β1 simultaneously with 20% WP of 20% F3 for another 3 days to prove the capacity of WP and F3 to dedifferentiate the cells. There is no statistical difference between the response induced by WP and F3 but there is respecting to starting point (2.5 ng/ml). (*, 95% confidence interval). (C) Immunofluorescence of α-SMA showing positive cells before and after treatment with plasma preparations. Myofibroblasts are α-SMA (+) and Hoechst (+) cells. Scale bar: 200 μm

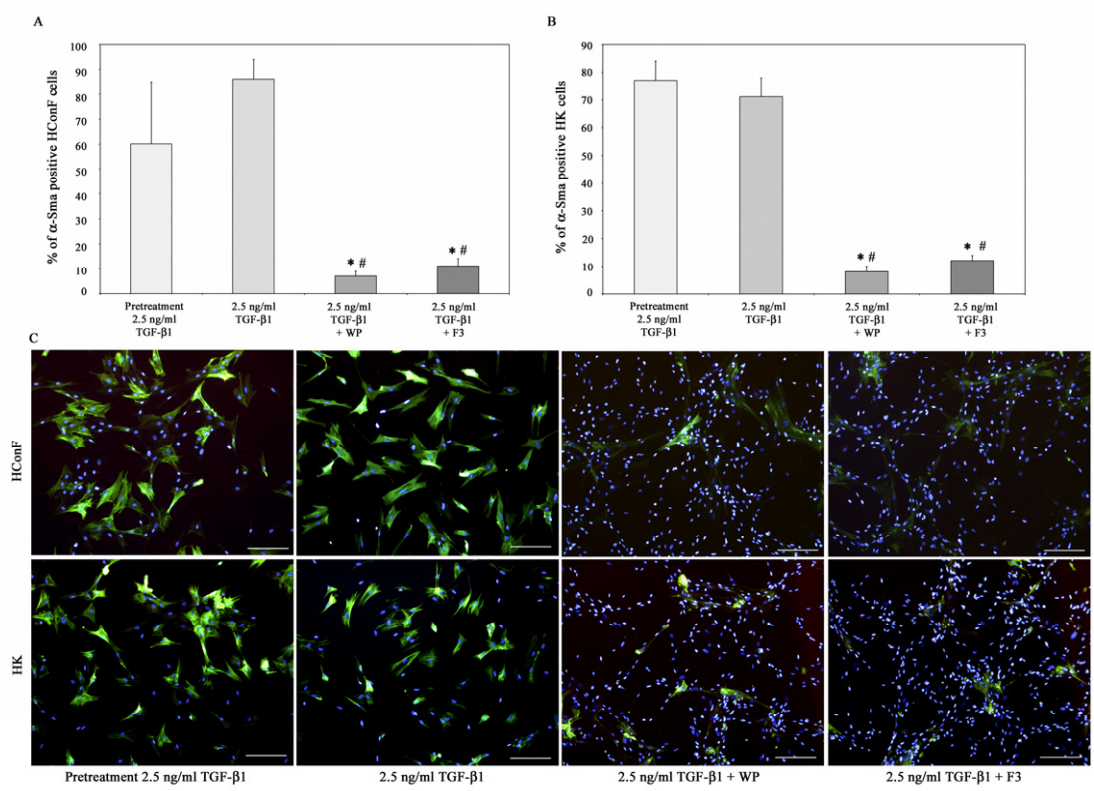

Table 1. Platelet and leukocyte concentration for each sample (WP and F3) and the levels of some of the most important growth factors.

TABLE 1 Platelet and leukocyte count and concentrations of several growth factors in the two different plasma preparations (WP and F3) of the blood´s donor.

			Growth factor levels						
Plasma preparation	Leukocyte count (x 10^6/ml)	Platelet count (x 10^6/ml)	TGF-β1 (ng/ml)	PDGF-AB (ng/ml)	IGF-I (ng/ml)	VEGF (pg/ml)	HGF (pg/ml)	EGF (pg/ml)	TSP-1 (μg/ml)
WP	>0,2	481	63	19	83	568	400	508	29
F3	>0,3	663	81	30	86	791	491	779	50

WP, Whole plasma column (enriched in platelets 2,6-fold over peripheral blood); F3, Fraction 3 (enriched in platelets 3,6-fold over peripheral blood). Peripheral blood contained 182 x 10^6 platelets/ml.