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GMP-grade platelet lysate enhances proliferation and migration of tenon fibroblasts

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1. ABSTRACT

Tenon's fibroblasts (TFs), widely employed as *in vitro* model for many ophthalmological studies, are routinely cultured with FBS. Platelet Lysate (PL), a hemoderivate enriched with growth factors and cytokines has been largely tested in several clinical applications and as substitute of FBS in culture. Here, we investigate whether PL can exert biological effects on TF populations similarly to other cell types. Results show that PL significantly enhances cell proliferation and migration *vs.* FBS, without influencing cell size/granularity. Upregulation of EGF, VEGF, KDR, MMP2-9, FAK mRNA levels also occurs and phosphorylation of AKT but not of ERK1/2 is significantly enhanced. The inhibition of the PI3kinase/AKT pathway with the specific inhibitor wortmannin, decreases PL-induced cell migration but not proliferation. Condition supernatants containing PL show increased bioavailability of Nitric Oxide and reduced levels of 8-Iso-PGF2-alpha, correlating with cell proliferation and migration. Pro-angiogenic/inflammatory soluble factors (GRO, Angiogenin, EGF, I-309, PARC) are exclusively or greater expressed in media containing PL than FBS. GMP-grade PL preparations positively influence *in vitro* biological effects of TFs representing a suitable and safer alternative to FBS.

2. INTRODUCTION

Tenon's capsule mainly functions as a cavity containing the ocular bulb, which is thus supported

during eye movements (1). Fibroblasts derived from Tenon's capsule represent the main stromal fraction. Currently, they are a versatile experimental *in vitro* model for several ophthalmological studies, ranging from *in vitro* drug testing to novel toxicological screenings, to wound healing repair and to inflammatory and pathological process (2-5). Besides, human TFs have been demonstrated to sustain the *in vitro* expansion of limbal stem cells (6) and even to be reprogrammed to generate induced pluripotent stem cells (7), thus paving the way to replace human feeder layers such as dermal fibroblasts with those derived from specific ocular tissues.

For all the aforementioned reasons, TFs are such a widely accepted and suitable model that the *in vitro* expansion is an unavoidable step. Normally, the *in vitro* culturing of human TFs requires the use of foetal bovine (FBS) or autologous serum derived from patients when available. Although bovine serum is enriched in growth factors, hormones and small peptides beneficial to stimulate cell proliferation (8), recently certain soluble factors released by activated platelets have been now demonstrated to ameliorate cell growth than FBS and plasma (8-10). Furthermore, from a clinical-translational standpoint, the culturing of any cell product with FBS including TFs still represents a threat due to potential risk of zoonosis.

Accordingly, Platelet lysate (PL), a hemocomponent enriched with growth factors and cytokines (11) has been currently employed for several treatments including skin ulcers, orthopaedic and ophthalmological conditions (12-17). For instance, a number of clinical studies have indicated that the topical administration of PL can represent an effective procedure to manage both skin and corneal ulcers due to the numerous growth factors and cytokines released by platelets which can be collected during the manufacturing process (18).

Among all soluble factors present in PL preparations, PDGF, TGF-alpha, VEGF, TGF-beta1 and EGF undoubtedly play a key role in stimulating and improving proliferation and angiogenic processes, as well as fibroblast migration and collagen production (19-21). In addition, these growth factors are able to chemotactically recruit monocytes, macrophages and lymphocytes to the site of injury, improving both tissue regeneration and growth of fibroblast-like cells (20-22).

Interestingly, PL has also been successfully applied for the expansion of human mesenchymal stem cells (11), endothelial (23) and stromal cells including dermal fibroblasts (24), acting as an effective replacement of FBS or human serum in culture.

Although PL is widely employed, its biological significance has still to be elucidated. A proper investigation about the efficacy of PL on a non-dermal origin fibroblastoid population could allow to develop a novel *in vitro* culture model to study the biology of human TFs and the effects of platelet-derived products, which have yet to be defined. In other words, the *in vitro* model reproduced by ocular fibroblasts would also represents the reasonable first step in studying the effects of new blood-derived preparations such as PL.

Recently, we have standardized a technique to isolate and expand human TFs (4) and also shown the efficacy of our patented GMP-grade PL (Pub. No. WO/2013/042095) to sustain cell proliferation and mesodermal transdifferentiation of human adipose tissue derived Mesenchymal stem cells (11).

Here we have investigated whether our patented GMP-grade PL (11) was equally effective on human TF growth and differentiation properties. We firstly explore both biological properties and potential molecular mechanisms of PL on non-dermal tissue specific populations such as human TFs.

We report that PL enhances cell proliferation and migration, in correlation with a significant reduction in oxidative stress levels. Moreover, phosphorylation of AKT but not of ERK1/2 is enhanced in presence of PL compared to FBS. The increase in cell migration but not proliferation can be attenuated by wortmannin, a selective inhibitor of PI3 kinase/AKT pathway. Finally, the cytokine profile shows that a set of soluble factors with pro-inflammatory/angiogenic/proliferative activity (GRO, Angiogenin, EGF, I-309, PARC) are exclusively present in PL preparations compared to FBS.

3. MATERIALS AND METHODS

3.1. Human TF cultures

Human primary TF cultures were obtained as we previously described (4,6). Briefly, explants were mechanically minced and cultured with complete medium (DMEM High glucose/1%PenStep/1%Glutamm ine/1%Non-essential aminoacids/10% FBS or PL). After reaching semi-confluence, fibroblasts were trypsinized (0.0.5%Trypsin/0.0.2%EDTA, Cat. N. X0930-100) and expanded with complete medium. Written informed consents were obtained from patients before surgery. The protocol was conducted in compliance with the Tenets of the Declaration of Helsinki for experiments involving human tissues. All data were de-identified and analysed anonymously. All reagents for complete media but PL were purchased from Biowest, Nuaillé, France. Experiments were performed at passages 3 (early passage) and/or 6 (late passage).

3.2. GMP-grade platelet lysate (PL) preparation

GMP-grade PL (Pub. No. WO/2013/042095) was prepared and virally inactivated according to the protocol that we previously described (11). Briefly, after obtaining informed consent, buffy coats from

Table 1. Primers used in RT-PCR

Target	Forward	Reverse
EGF	GGATAGCCAACAAACACACT	GGCACGTGCAGTAATAGGAT
FGF	CCTGGGGAGAAAGCTAT	GCTTCACGGGTAACAG
VEGF	AGCTGCGCTGATAGACATCC	CTACCTCCACCATGCCAAGT
KDR	TGCCTACCTCACCTGTTTCC	CGGCTCTTTCGCTTACTGTT
MMP2	GGAAAGCCAGGATCCATTTT	ATGCCGCCTTTAACTGGAG
MMP9	TTGGTCCACCTGGTTCAACT	ACGACGTCTTCCAGTACCGA
FAK	CCTGGTCCACTTGATCAGCTA	GCCAAAAGGATTTCTAAACCAG
alpha-SMA	CCAGAGCCATTGTCACACAC	CAGCCAAGCACTGTCAGG
18S	CAATTATTCCCCATGAACG	GGGACTTAATCAACGCAAGC

healthy volunteers were centrifuged and treated with InterSol solution (318 mgNa-citrate 2H2O, 305 mg Na 2-phosphate anhydro, 105 mg Na dihydrogen phosphate 2H2O, 442 mg Na-Acetate 3H2O, 452 mg NaCl, 100 ml H2O, Fenwal Inc. Lake Zurich, Illinois) and subsequently with 20-30% human plasma. Potential pathogens were inactivated by using the Intercept™ Blood System for Platelets (Cerus Corporation, Concord, California USA). Platelet preparation was then collected in a final single bag and stored at -80°C for 24 h before thawing at 37°C for 60 min. This procedure was repeated three times to enrich the pool of growth factors. Concentration and sterility of the preparation was determined by the haematology analyzer ABX Pentra DX 120 (Horiba ABX, Montpellier, France) and BacT/ALERT System (bioMérieux SA, Marcy l'Etoile, France), respectively. Before adding PL in culture, 5 U/ml heparin were added to avoid fibrin gel formation.

3.3. Flow cytometry analysis

Human TFs (80-90% confluence) were detached by 0.0.5%Trypsin/0.0.2%EDTA, then washed with PBS/2% FBS and resuspended in PBS/FACS Flow as reported elsewhere (4,6,25). Afterwards 1×10^5 cells for each treatment (1, 5 and 10% PL and FBS) were stained with Propidium iodide (10 ng/ml, Sigma) in order to discriminate live/dead cells (25). Samples were acquired by cytofluorimetry (FACSAria II and DiVa Software v6.1.1., B&D, San Jose, USA) and analysed by Flowing Software (v2.5.1., Turku Centre for Biotechnology, Turku, Finland) by evaluating the light scattering properties (forward and side scatter) only on live cells. Identical instrument settings were used for human TFs of each patient cultured in PL or FBS.

3.4. Cell growth

Cell proliferation was evaluated by MTS assay (4,26-27) (CellTiter 96 AQueus One Solution Cell Proliferation Assay, Promega, Milan, Italy) using a colorimetric method according to manufacturer's instructions and seeding 1000 cells/well into 96 well-plates in quintuplicate conditioned for 0, 3 and 7 days with PL or FBS. The optical density signal (492 nm) was normalized to that of day 0 after cell seeding.

3.5. Real time PCR

RNA was extracted using RNeasy MicroKit (Quiagen, Hilden, Germania). C-DNA synthesis was performed from 1 μg RNA by using High Capacity cDNA Reverse Transcription Kit. Total RNA was extracted (Total RNA Purification Kit, Cat. N. 00375, Norgen Bioteck Corp, Thorold, Canada) and reverse-transcribed into complementary DNA (cDNA) using Tetro cDNA Synthesis kit (4,26-27) (Cat. No BIO65043 Bioline, Aurogene, Italy). Transcript levels were assessed by quantitative PCR using Sensimix Sybr Green (Cat. N.QT605-05 SensiMixTM Sybr Kit Bioline, Aurogene, Italy) in a 7900HT Fast (Applied Biosystem, Life Technology, Italy) for a set of gene markers listed in Table 1. qPCR conditions were set as following: 15s at 95°C, followed by 40 cycles of 95° for 15s and 60°C for 1 minute. 18S was used as housekeeping gene.

3.6. Inhibition of the PI3 kinase/AKT pathway

Human TFs were starved for 3 hours in complete medium supplemented with 0.2.% FBS or PL, then trypsinized and seeded for the MTS and migration assays. For cell proliferation and migration tests, wortmannin (Cat. N. 9951, Cell Signaling Technology, Danvers, USA) was added at a concentration of 500nM and 2.5 μM, respectively.

3.7. Migration assay

Semi confluent human TFs were resuspended in serum free medium, seeded at 5×10^4/well in the upper side of 24 well Boyden chamber (8 μm pores, BD Biosciences) and incubated at 37°C for 16 hours. The lower chamber contained DMEM with 10% PL or FBS. Afterwards, the inner side of the insert was wiped with a wet swab, in order to remove the cells and the outer side of the insert gently rinsed with PBS and stained with 0.2.5% Giemsa (Sigma, St. Louis, USA) for 15 min,

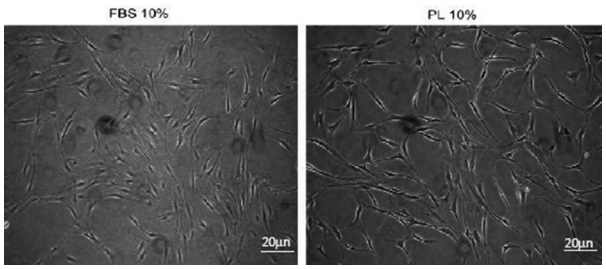

Figure 1. Images from optical microscope. Human TFs at passage 3 showing a more enhanced spindle-shape morphology, when cells are cultured in PL (10%) compared to FBS (10%). PL, platelet lysate.

rinsed again, and then allowed to dry (11,27-28). Migrated cells were counted (ten fields per chamber) under a light microscope. Experiments were repeated three times in duplicate. In order to exclude any potential interference of wortmannin with cell adhesion, we have also seeded in 96 well plates coated with fibronectin (Becton–Dickinson, Bedford, MA) human TFs (1×10^5/well) in culture medium containing FBS or PL with wortmannin. Cells cultured with FBS or PL alone were used as control. After 6 hours of incubation, cells were washed twice with PBS to remove non-adherent cells. The attached cells were analyzed by MTT assay. Data are presented as means ±SD.

3.8. Human TF 8-iso-PGF2-alpha assay and nitric oxide bioavailability

Isoprostanes formation were measured in cell suspension of human TFs treated with PL (%) or FBS (%). The supernatant was stored at -80°C until measurement. Quantification of isoprostanes was performed measuring 8-iso-PGF2-alpha by EIA assay method (Tema Ricerca, Milan, Italy). Intra-assay and inter-assay coefficients of variation were 5.8.% and 5.0.% respectively. A colorimetric assay kit (Tema Ricerca, Milan, Italy) was used to determine the nitric oxide bioavailability, evaluated by metabolites nitrite and nitrate (NOx) in 100 µl of human TF conditioned supernatants maintained under stirring condition 10 min at 37°C. Intra-assay and inter-assay coefficients of variation were 2.9.% and 1.7.%, respectively.

3.9. Western blotting

After a 24 hours of starvation in complete medium containing 0.2.% FBS or PL and stimulation with 10% FBS or PL for 1, 5, 10 minutes, human TFs were washed and lysed for 30 min at +4°C in equal volumes of ice-cold RIPA lysis buffer (10 mM Tris pH 8, 1%Triton, 0,1% SDS, 0,1% Deoxycholate, 140 mM NaCl, 1 mM EDTA) and a cocktail of protease inhibitors (1 mM DTT, 1 mM PMSF, 1 Protease inhibitor tablet/10 mL Sigma, St. Louis, USA). Homogenates were clarified by centrifugation. Supernatants were boiled for 5 min in Laemmli sample buffer. Equivalent protein quantities, as determined by Bradford, were loaded on each gel. Proteins were separated by SDS-PAGE electrophoresis and electrotransferred to polyvinylidene difluoride membranes and incubated with the primary antibody against human phospho-AKT, AKT, phospho-ERK1/2 and ERK (All Cell Signaling Technology, Danvers, USA Cat. N. 9271S, Cat. N. 9272S, Cat. N. 9106S and Cat. N. 4695S, respectively) and a horseradish peroxidase-conjugated mouse or rabbit secondary antibody (1:10000, GE Healthcare Bio-Science, Piscataway, NJ, USA). The membranes were developed by enhanced chemiluminescence kit (ECL, GE Healthcare Bio-Science, Piscataway, NJ, USA).

3.10. Cytokine profile assay

The simultaneous detection of 80 multiple cytokines/growth factors in media containing PL (DMEM/10%PL) was performed by using the human Cytokine Antibody Array C5 (Ray Biotech, Inc.) according to the manufacturer's instructions. An equal volume of media and protein has been loaded on the arrays. Samples have been normalized on positive controls (Array). Media containing FBS (10%) has been used as reference.

3.11. Statistical analysis

Statistical analysis was performed using the statistical software GraphPad Prism 5 software (San Diego, USA). The independent sample two-tailed t-test with associated 95% confidence intervals was used to compare the single data. For multiple comparisons, the One or Two-way analysis of variance (ANOVA) test and Bonferroni post-hoc test were used. Data are expressed as mean±SD unless specified. A $p<0.0.5$ was considered statistically significant. For oxidative stress experiments, normal distribution of parameters was assessed by Kolmogorov–Smirnov test. Student unpaired t test and Pearson product-moment correlation analysis were used for normally distributed continuous variables. Appropriate nonparametric tests (Mann-Whitney U test and Spearman rank correlation test) were employed for all the other variables.

4. RESULTS

4.1. Cell size and proliferation

In order to assess the effects of PL on cell cultures, we isolated human TFs, by halfing the original biopsy and either treating with PL (10%) or FBS (10%, control). In order to minimize the interpatient variability experiments were performed on the same set of patients. As shown in Figure 1, human TFs cultured with the standard *in vitro* concentration of PL (10%) (11,29) displayed a more enhanced spindle-shape morphology compared to FBS (10%), when observed under inverted-phase microscope. In order to verify this, serum (PL)-starved TF cultures were stimulated with scalar concentrations (1,5,10%) of PL, FBS or DMEM alone (negative control). Three different batches of FBS and PL (lot. 8,9,20) have been tested. Cell size was analysed by flow cytometry with regard to physical characteristics

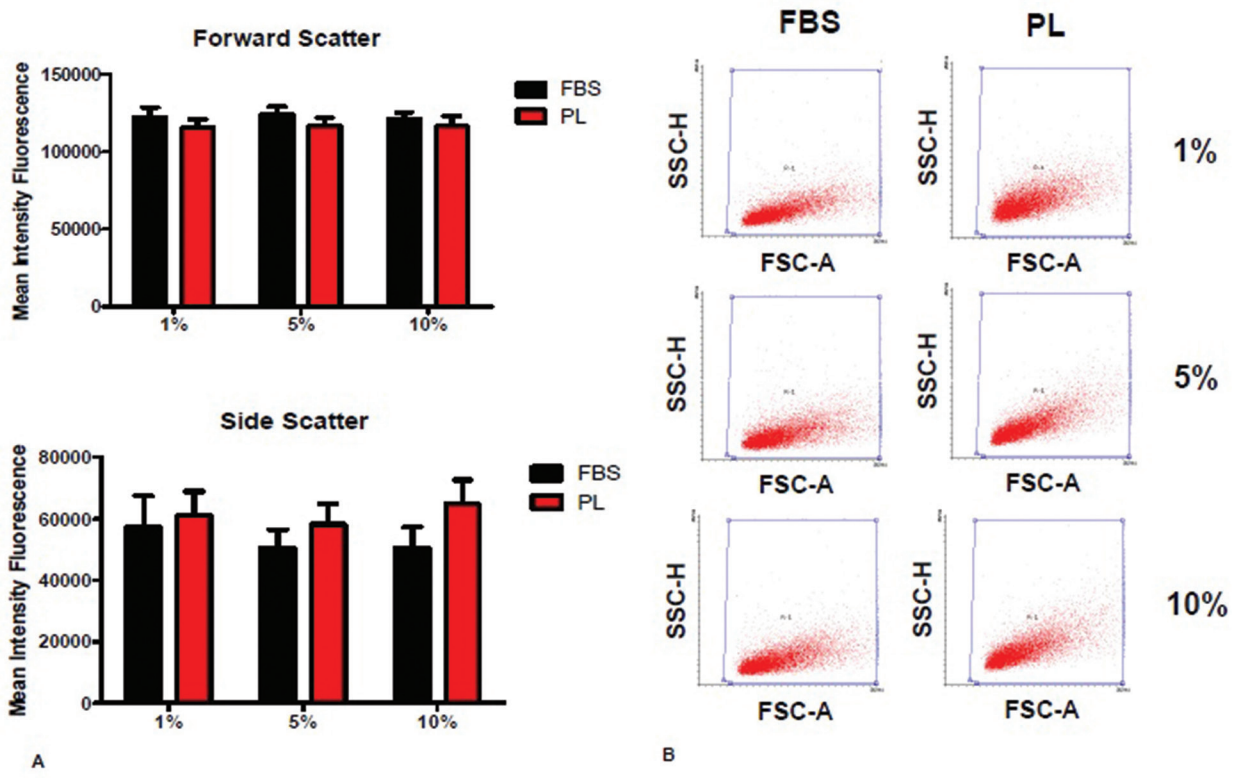

Figure 2. A-B. Flow Cytometry Analysis. (A) Graphs of the Forward and Side scatter Analysis of human TFs at passage 3 displaying that in a concentration range of 1, 5 and 10%, PL is not able to significantly influence physical cell properties such as cell dimension and granularity (p>0.0.5) relative to FBS. (B) Representative cytofluorimetric dot plots showing the Side and the Forward scatter at different concentrations (1, 5, 10%) of FBS and PL used. PL, platelet lysate; SSC, Side scatter; FSC, Forward scatter; H, height; A, Area.

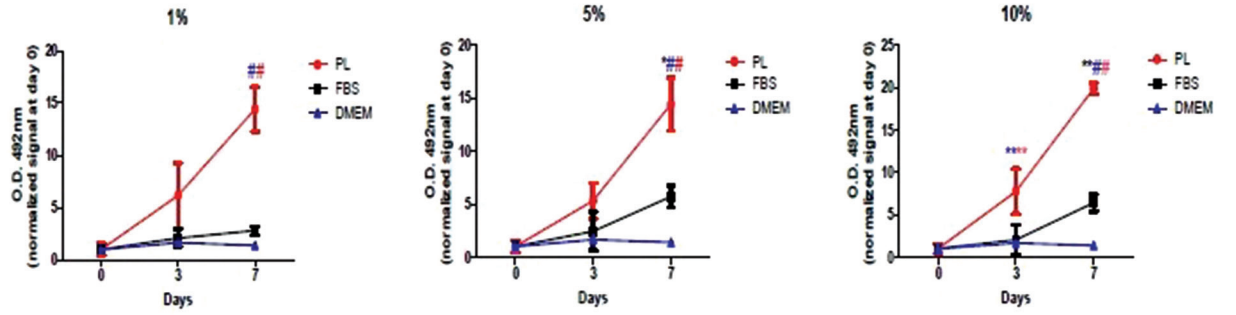

Figure 3. Cell growth. MTS proliferation assay (0, 3, 7 days) showing human TFs at passage 3 cultured with increasing concentration of PL or FBS (1, 5, 10%). PL is able to increase hTF cell proliferation at all concentrations after 7 days of culture compared to FBS. *p<0.0.5, **p<0.0.1. PL, platelet lysate; O.D., optical densities.

such as surface area and cell granularity. Results have shown that after treatment with different concentrations of PL, neither the measurement of the forward (which is proportional to cell-surface area or size) nor of the side scatter (which reflects the cell granularity and complexity) show statistically significant differences compared with FBS (p>0.0.5 at all concentrations, Figure 2A-B). Propidium Iodide staining, used to discriminate live/dead cells within the TF population has shown no statistically significant difference between all samples, indicating high viability in all cultures (Figure 12).

Next, we asked whether PL could influence cell proliferation. By MTS assay we show that after 3 and 7 days of culture with PL (10% at day 3 and 1, 5, 10% at day 7), human TF cell proliferation is statistically

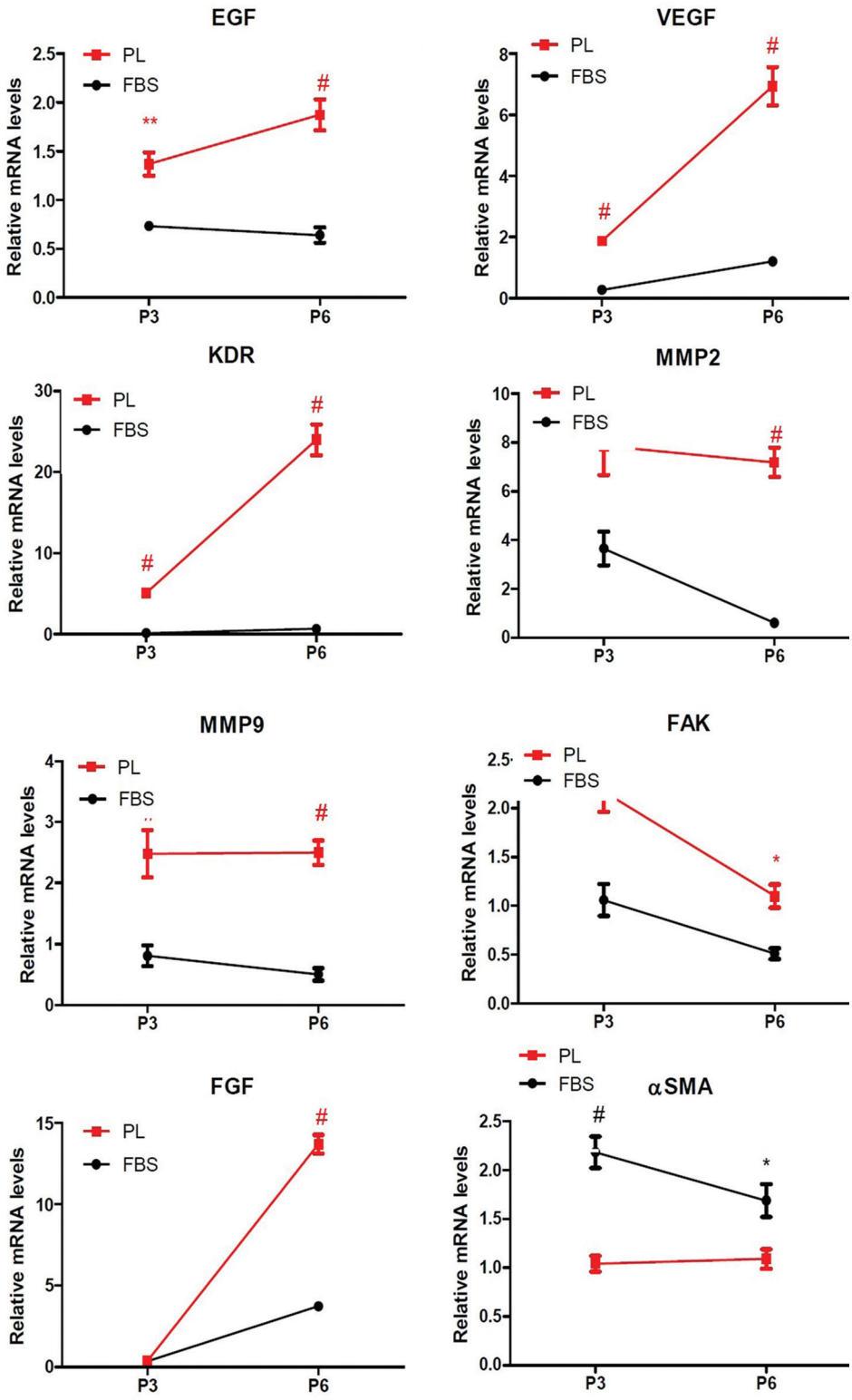

Figure 4. RTPCR Analysis. Phenotypical modifications of human TFs after treatment with PL or FBS (10%) evaluated by RT-PCR. A significant upregulation of EGF, VEGF, KDR, MMP2, MMP9 and FAK mRNA levels is observed when human TFs are cultured with PL *vs.* FBS at both early (P3) and later (P6) passages. FGF mRNA levels are enhanced only in presence of PL and at later passage relative to FBS. Conversely, αSMA mRNA levels are upregulated at the same passages only in presence of FBS but not of PL, where they are maintained unchanged. 18S has been used as housekeeping gene. PL, platelet lysate; P3, passage 3; P6 passage 6. *p<0.0.5, **p<0.0.1, #p<0.0.001.

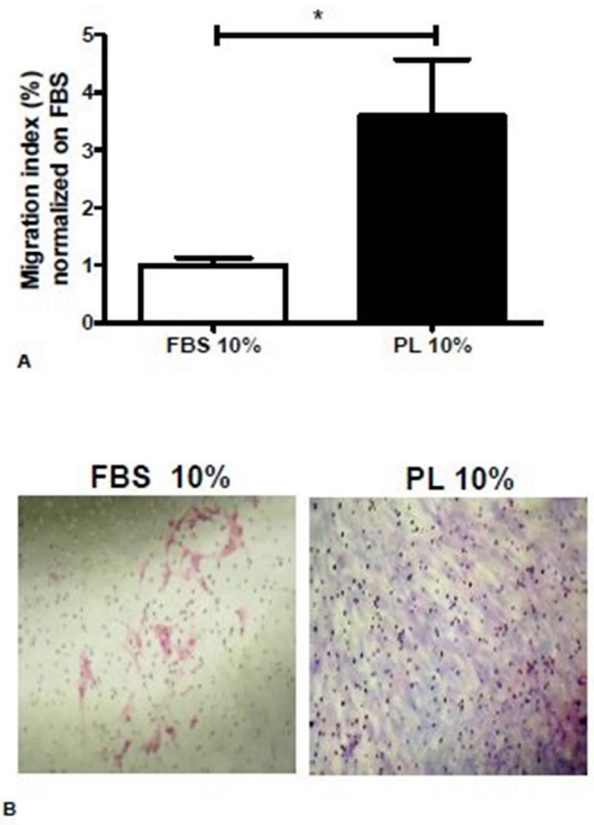

Figure 5. A-B. Cell migration. (A) Graph showing the ability of PL (10%) to statistically increase cell migration at passage 3 compared to FBS. Migration index expressed in percentage (%) and normalized on FBS has been calculated as the following ratio: number of cells migrated/number of cells plated. *p<0.0.5. (B) Representative microscopic fields of the migration assay after Giemsa staining, highlighting a distinct increased number of human TFs migrated at the bottom of the well in presence of platelet lysate. PL, platelet lysate.

significantly increased compared with FBS and DMEM alone (our negative control) (Figure 3, 1 and 5% day 7 p<0.0.01 vs FBS and DMEM; 10% day 3 p<0.0.1 vs DMEM and FBS; 10% day 7 p<0.0.1 and p<0.0.01 vs DMEM and FBS, respectively). A significant increase in cell proliferation can be also observed after 7 days when TFs are cultured with FBS (5 and 10%) compared to DMEM alone (p<0.0.5 day 3 and p<0.0.1 day 7) as expected.

4.2. Phenotype analysis and migration assay

We then aimed to assess whether the standard *in vitro* concentration of PL (10%) could induce in human Tenon's fibroblasts any variation in the specific mRNA levels of genes known to be involved in basic biological process including wound healing and/or repair/angiogenesis (26,30-32). The genes investigated were: epidermal growth factor (EGF), fibroblast growth factor (FGF) and the vascular endothelial growth factor (VEGF)/kinase insert domain receptor (KDR) axis. We also investigated whether PL could affect cell adhesion, motility and myofibroblast transdifferentiation of human TFs by modulating the transcriptional levels of the metalloproteinase (MMP) 2 and 9, the Focal Adhesion Kinase (FAK) and the alpha smooth muscle actin (alpha-SMA) genes, all of them active in the aforementioned stromal processes (32-34). We observed (Figure 4) a statistically significant upregulation of EGF, VEGF, KDR, MMP2, MMP9 and FAK mRNA levels at both early (passage 3, p=0.0.026, p<0.0.001, p<0.0.001, p=0.0.22, p=0.0.078, p=0.0.057) and later passages (passage 6, p=0.0.004, p=0.0.001, p<0.0.001, p=0.0.004, p=0.0.009, p=0.0.11) of human TFs cultured in media supplemented with PL compared to FBS. FGF mRNA levels increased in presence of PL only at later passage (p<0.0.001).

Whereas, alpha-SMA mRNA levels were upregulated at both passage 3 and 6 of human TFs (p=0.0.007 and p=0.0.38, respectively) only when cultured with FBS compared to PL, which are maintained stable. Afterwards, in order to explore the influence of PL on the migratory activity of human TFs, cells were subjected to Transwell migration assay. Results show that PL significantly increased cell migration compared with FBS (p=0.0.1, Figure 5A-B).

4.3. Intracellular pathway activation

In order to evaluate if intracellular signalling nodes along the MAPK pathway were activated in presence of PL, human TFs were stimulated for 1, 5 and 10 minutes and the protein levels of phosphorylated-AKT and ERK1/2 (23,35) detected by Western Blot. Cells stimulated with PL showed a striking increase in AKT phosphorylation after 1, 5 and 10 minutes of stimulation (Figure 6A-B, p<0.0.5, p<0.0.01 and p<0.0.01, respectively) compared to FBS. This last also had some effect on AKT phosphorylation by enhancing the expression over time (p<0.0.5 and p<0.0.1 at 5 and 10 minutes *vs.* starved samples, respectively). Both PL and FBS were equally effective to enhance phosphorylation of ERK1/2 with maximum effect after 5 minutes of stimulation (p<0.0.5 PL and p<0.0.1 FBS *vs.* starved samples, Figure 6C-D).

Afterwards, in order to clarify the role of the AKT pathway in mediating the effects of PL on human TFs, we examined cell proliferation and migration after treatment with wortmannin, a specific inhibitor of the PI3 kinase/AKT pathway. We show that wortmannin had no effect on human TF proliferation rate in presence of FBS or PL (Figure 7, p>0.0.5), without exerting cytotoxic effect at the concentration used, whereas cell migration was significantly decreased in presence of both agents (p=0.0.043 and p=0.0.42 FBS and PL, Figure 8). A potential interference of wortmannin with human TF adhesion has been ruled out by MTT assay, showing the inability of the specific inhibitor to affect TF adhesion onto a fibronectin substrate either in presence of FBS or PL (Figure 13).

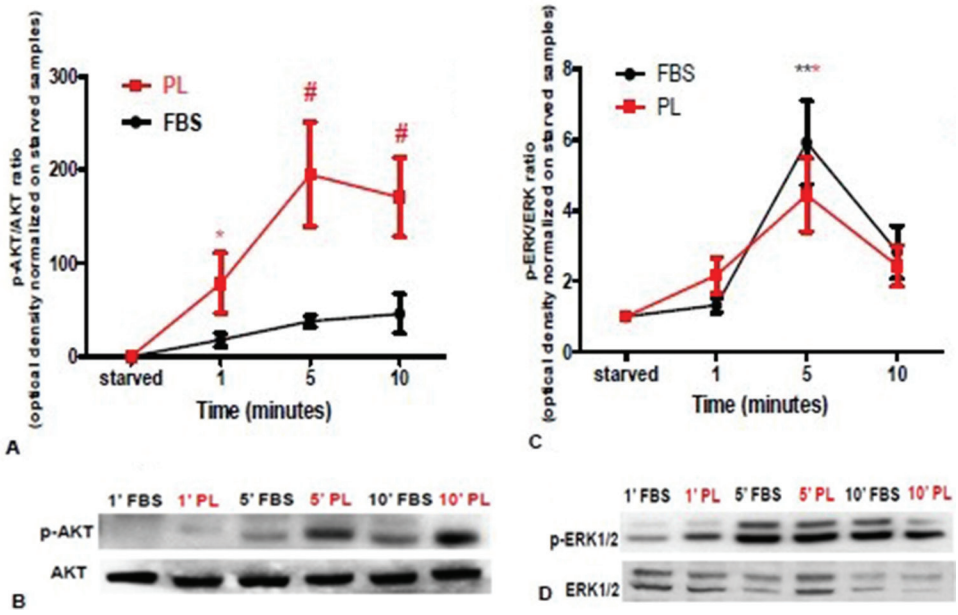

Figure 6. A-D. Analysis of the signalling pathways. (A) The graph shows that the stimulation with PL (10%; 1, 5 and 10 minutes) is significantly able to enhance the AKT phosphorylation over time compared to FBS (10%). (B) Both PL and FBS treatment induce a significant and equal increase in phosphorylation of ERK1/2 vs. starved samples. (C) Representative images of the Western Blot assay displaying phospho-AKT and AKT total and (D) phospho-ERK1/2 and ERK total at the time course of stimulation with PL and FBS. The phospho-AKT/AKT and phospho ERK1/2-ERK1/2 ratio have been normalized on the starved samples. PL, platelet lysate. *p<0.0.5, **p<0.0.1, #p<0.0.01

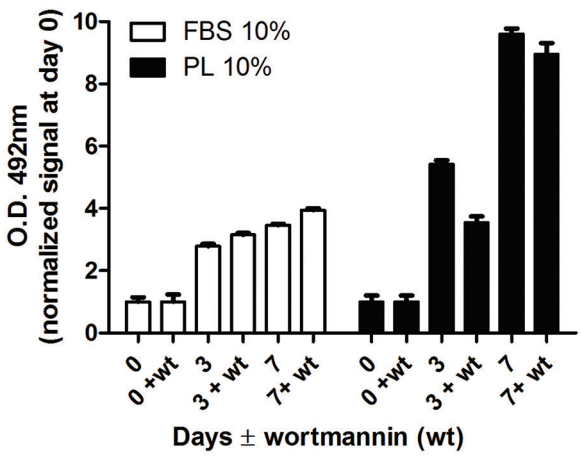

Figure 7. Effect of the inhibition of the PI3K/AKT pathway on cell proliferation. MTS assay (0, 3, 7 days) of human TFs after treatment with FBS or PL and in presence or absence of wortmannin, showing no influence of the specific inhibitor of the PI3 kinase/AKT pathway on hTF cell proliferation either in presence of PL and FBS. O.D., optical densities; PL, platelet lysate; wt, wortmannin.

4.4. Oxidative stress analysis

Given the involvement of oxidative stress mediated by platelets on proliferation, differentiation and pro-fibrotic transformation of fibroblasts (36-38) and the ability of both PL and ROS species to exert regenerative effects (39-42), we asked whether PL could influence oxidative stress levels. To this purpose, we analysed the production of 8-iso-prostaglandin-F(2alpha) (8-iso-PGF2-alpha-III), a marker of oxidative stress (43) and that of nitric oxide (NO) in conditioned supernatants of human TFs. We show that conditioned media derived from human TFs cultured in presence of PL exhibited higher levels of NO, according to the production of nitrite/nitrate (p=0.0.02, Figure 9A) and significantly lower level of 8-iso-PGF2-alpha (p=0.0.01, Figure 9B), compared to those obtained in presence of FBS. Moreover, univariate correlation analysis demonstrated that both proliferation and migration were correlated positively with NOx formation (Rs=0.6.89; p<0.0.1, Figure 10A; Rs=0.7.25; p<0.0.01, Figure 10C) and negatively with 8-iso-PGF2-alpha (Rs=-0.4.84; p<0.0.5, Figure 10B; Rs=-0.4.76; p<0.0.5, Figure 10D) of cells treated with PL. No correlation was found with FBS treatment (data not shown).

4.5. Detection of cytokines and growth factors in PL

Finally, in order to investigate whether soluble factors within PL may contribute to its biological effects, we have screened the medium containing PL (DMEM/10% PL) regarding its content of 80 pro-inflammatory growth factors and cytokines, known to be involved in cell activation/proliferation, chemotaxis, angiogenesis and wound healing. Medium with FBS (DMEM/10% FBS) has been used as comparison. Results show that pro-inflammatory/angiogenic factors such as GRO, Angiogenin, EGF, I-309 and PARC are

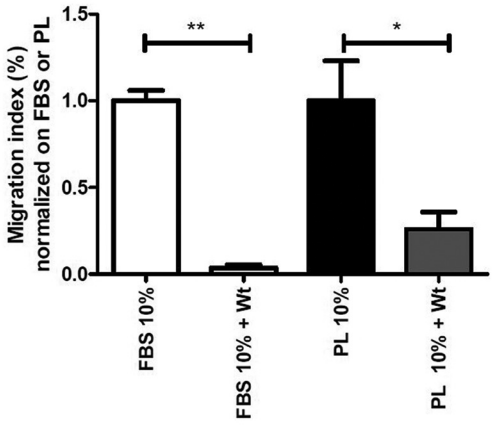

Figure 8. Effect of the inhibition of the PI3K/AKT pathway on cell migration. Migration assay of human TFs after treatment FBS or PL in presence of wortmannin, displaying a statistically significant reduction in TF cell migration when cultured with PL or FBS. *p<0.0.5, **p<0.0.1. PL, platelet lysate; wt, wortmannin.

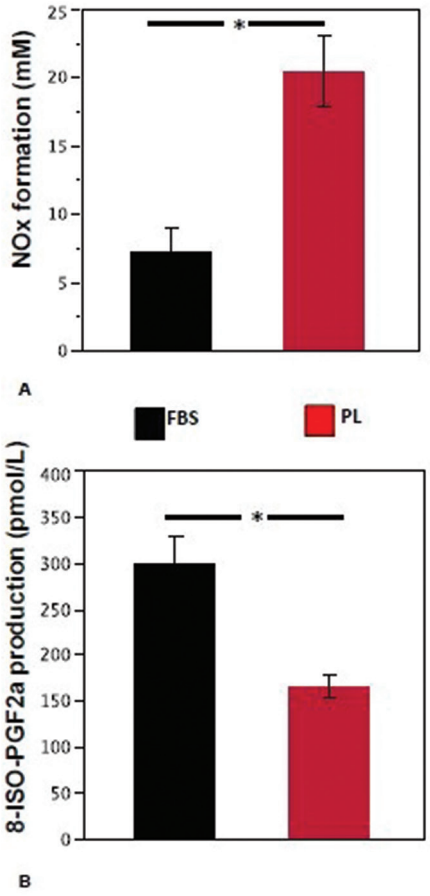

Figure 9. A-B. Analysis of the oxidative stress levels. (A) Conditioned media derived from human TFs at passage 3 cultured in presence of PL showing a significant higher production of nitrate/nitrite (Nox formation μM) but (B) significant lower levels of isoprostanes (8-iso-PGF2-alpha-III, pmol/L) respect to FBS. PL, platelet lysate.

exclusively expressed in DMEM/10%PL and absent in DMEM/10%FBS, whereas TGF-beta2, Plgf, IL-16, osteopontin, NAP-2, Light, LIF and osteoprotegerin are higher expressed in media supplemented with PL than with FBS (Figure 11 A-B).

5. DISCUSSION

PL has been established for a broad range of clinical applications (11), including ophthalmological diseases (44) thanks to its ability to induce tissue regeneration in a paracrine fashion (45-46). Despite this, PL is usually manufactured “in-house”, thus lacking any standardization of soluble factors during its preparation. In addition, although it can be obtained from autologous sources, risk of potential contamination can still occur during the clinical procedure. These limitations can be overcome by a GMP-grade PL formulation, manufactured to strict specific standard procedures in accordance to safety requirements (virally inactivated) (11,47).

Given the presence of numerous soluble factors detected in our PL preparations (11,47), we have attempted to establish whether the PL normally employed *in vitro* for culturing Mesenchymal stem cells or dermal fibroblasts (11,45,47) could also allow the culturing of human TFs, a widely employed *in vitro* model of stromal cells for ophthalmological studies. As for Mesenchymal stem cells (11,47), the GMP-grade PL produced in our laboratory enhances human TF cell proliferation. However it does so in a concentration-independent manner, without provoking morphological changes in cell dimension and/or granularity as observed for Mesenchymal stem cells (29) and preserving human TF viability. Also the migratory activity of human TFs is improved in presence of PL. These results confirm the non-specific cell type effect of PL, in agreement with other reports describing similar chemotactic responses in a variety of different cell populations ranging from the myoblast cell line C2C12 to keratinocytes and retinal glial and endothelial cells (48-50).

In addition, our results show that PL compared to FBS can preserve or enhance upregulation of genes such as EGF, MMP2 and 9, VEGF and FGF involved in regeneration or migration of epithelial tissue. Also we did not observe any variation in the expression levels of FAK and alpha-SMA, a myofibroblastic marker, despite the presence in the PL of TGF-beta1 (11-12,47) a fibrotic agent known for inducing myofibroblast activation by acting on both FAK and alpha-SMA (50). For instance, corneal fibroblasts transit to a pro-fibrotic state only when an epithelial injury occurs and the wound healing process starts to switch from the inflammatory to a proliferative phase. Since our system *in vitro* has been designed in absence of injury and immune response, it is conceivable that both the transition to a fibrotic state and the induction of myofibroblast activation cannot be

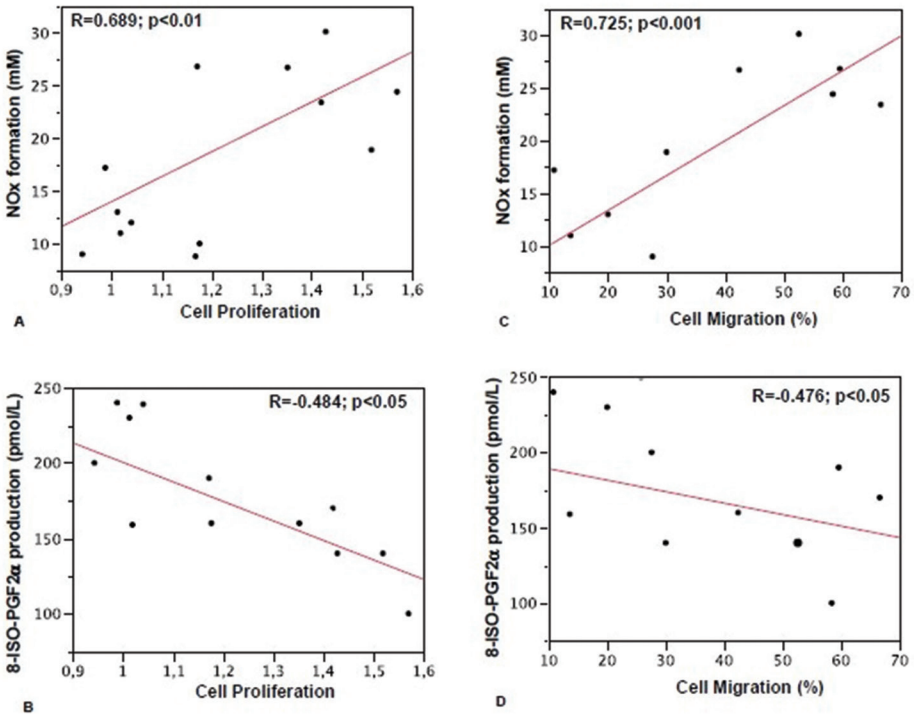

Figure 10. A-D. Statistical correlations between oxidative stress levels and TF proliferation and migration in presence of PL. (A) The production of nitrite/nitrate (NOx formation) positively correlates ($p<0.0.1$) with cell proliferation effect induced by the use of PL in human TFs, whereas (B) 8-iso-PGF2alpha III production inversely correlates ($p<0.0.5$). (C) Cell migration phenomenon displays even a more striking positive correlation with the production of nitrite/nitrate (NOx formation, $p<0.0.01$) and in presence of PL (10%). (D) Differently, an inverse linear correlation with 8-iso-PGF2-alpha-III production can be found ($p<0.0.5$). PL, platelet lysate.

observed (18). Alternatively, in presence of PL human, TFs could be restricted to a pre-fibrotic state and prone to be committed towards the myofibroblast lineage only after injury occurs.

So far, mechanisms by which PL exerts *in vitro* its beneficial effects on human TFs have been largely unexplored. Our data highlight that although multiple modulators of the inflammatory response are either present in the media supplemented with PL and FBS, interestingly, specific factors such as GRO, Angiogenin, EGF, I-309 and PARC are exclusively expressed in media containing PL and absent in that with FBS. The above mentioned chemokines/growth factors play a major role in cell differentiation, proliferation, angiogenesis and wound healing (51-53), converging on the activation of main signalling pathways in several biological systems including induction of NO Synthesis, ERK1/2 and AKT (54-57). Nevertheless, our data show that phosphorylation of ERK1/2 is not increased by treatment with PL compared to FBS, whereas the phospho-AKT signal is promptly activated. This is in line with previous studies reporting that the expression of ERK1/2 is unaffected or not essential in skin fibroblasts as well as HUVEC cells after stimulation with PL (23,45). Therefore, the ability of TFs to further proliferate but not to migrate after treatment with PL or FBS and inhibition of the PI3kinase/AKT pathway by wortmannin, suggests an exclusive role not PL-specific for AKT in the migratory event. Other molecular factors are likely to be involved, for instance p38, essential for PL-induced dermal fibroblast wound healing but not for wound closure (24).

A potentially specific property *in vitro* of PL could be the ability to decrease the oxidative stress levels, resulting in the enhancement of both cell proliferation and migration. Redox reactions, which consist of a fine balance between reductive and oxidative processes, underlie several mechanisms including tissue regeneration (42), lesion repair and wound healing (37). For instance, the management of corneal ulcers in association with antioxidants has been reported where the administration of vitamin C improves the restoration of the stromal matrix following an alkali burn, thus initiating the epithelial regeneration (58). Our study shows for the first time that after *in vitro* treatment with PL, human TF-derived supernatants contain higher levels of nitrate/nitrite compounds but decreased levels of isoprostanes (unique products of arachidonic acid oxidation) compared to cells cultured in presence of FBS alone. These novel results indicate a greater bioavailability in our conditions of

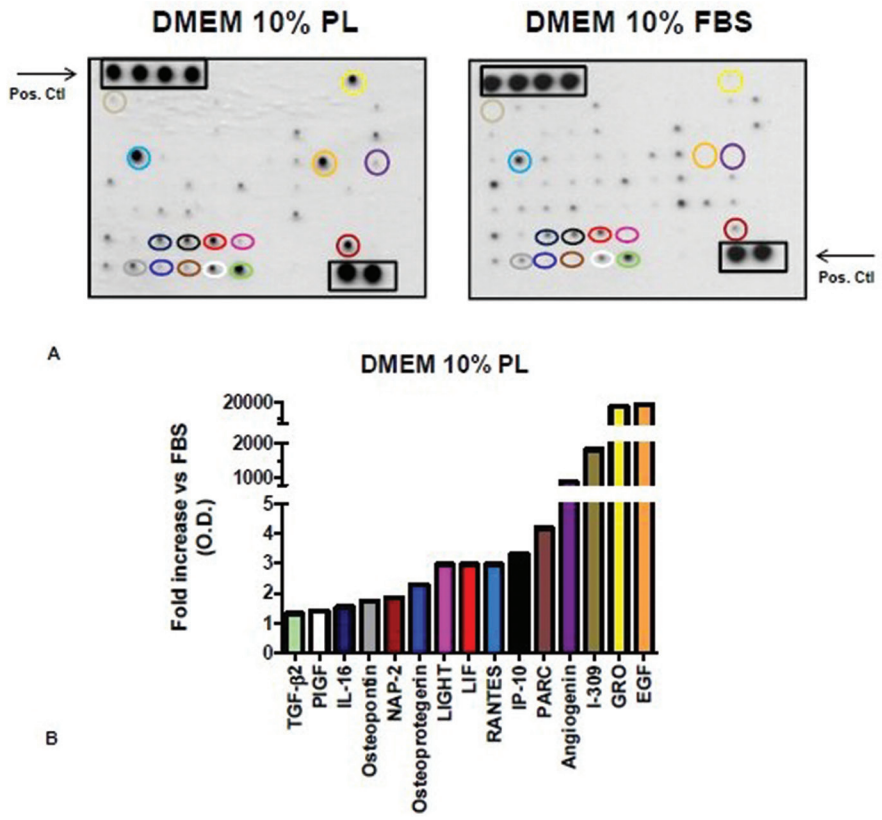

Figure 11. A-B. Cytokines/Growth factors profile. (A) Representative images of the Cytokine Antibody Arrays showing the multiple expression of 80 pro-inflammatory/angiogenic cytokines/growth factors in DMEM 10% PL and FBS. The coloured circles corresponding to the coloured columns in the graph below. (B) The graph displays how some angiogenic factors (GRO, Angiogenin, EGF, I-309, PARC) are exclusively expressed in PL and absent in FBS. Other soluble factors (TGF-beta2, Plgf, IL-16, osteopontin, NAP-2, Light, LIF, osteoprotegerin) are found greater expressed in PL than in FBS. O.D., optical density. Data are normalized on positive controls of the Arrays (black squares) and on FBS.

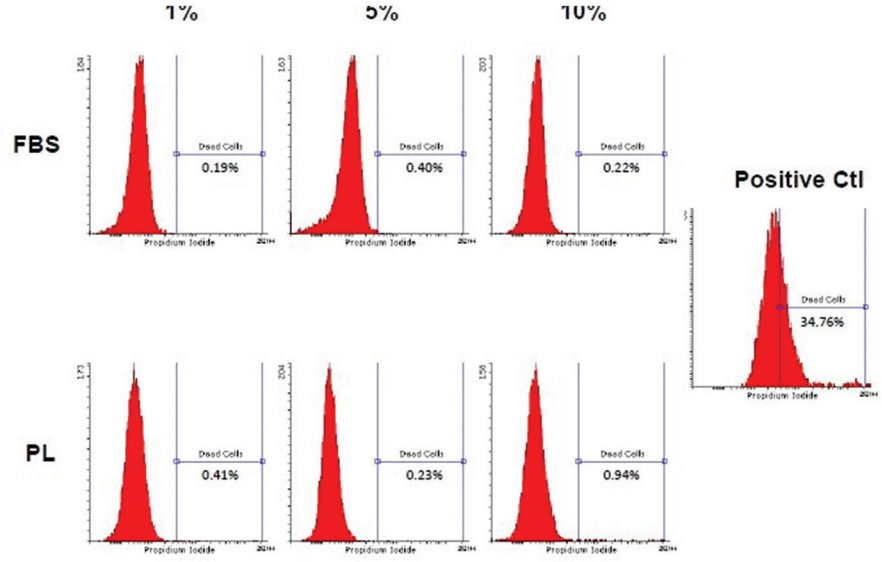

Figure 12. Representative cytofluorimetric histograms showing the Propidium Iodide staining at different concentrations (1, 5, 10%) of FBS and PL used. PL, platelet lysate; PI, Propidium Iodide. Positive Ctl, Positive Control.

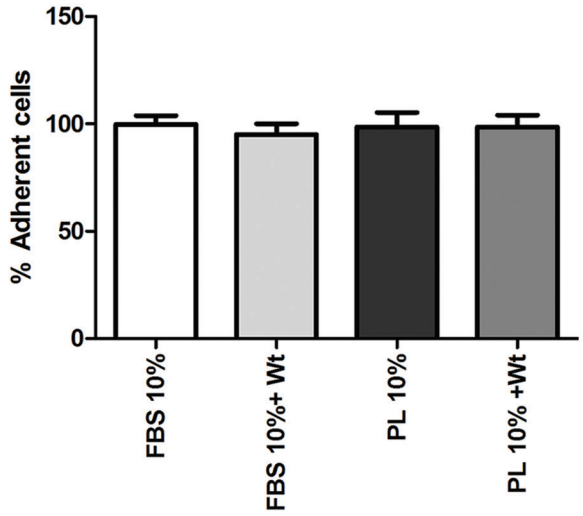

Figure 13. Cell adhesion assay. The graph shows that wortmannin has no effect on cell adhesion either in presence of FBS or PL. Data are presented as means ±SD.

nitric oxide production, that it is involved in enhancing neoangiogenetic processes and in corneal epithelial regeneration (59). Moreover, results indicate also a decrease in isoprostanes production, which is known to exacerbate tissue injury (60), and that may help to reduce the amount of free radical generation and oxidative damage (61-64).

All together our data suggest that PL can be employed as substitute of FBS during human TFs *in vitro* expansion without altering the biological cell properties. The option to employ PL preparations designed for different clinically conditions even for the *in vitro* culturing of TFs could broaden the current therapeutic PL-based strategies. For instance, the preconditioning of TFs with PL could reveal an interesting novel tool for limbal stem cell expansion as well an alternative to the FBS to avoid risk of zoonosis for cell banking purposes. Alternatively, the culturing of TFs with PL could reveal a useful *in vitro* model to investigate the effects of pro-inflammatory cytokines in driving angiogenic and fibrotic process in several ophthalmological diseases.

Finally, a deeper understanding of the molecular basis of the novel biological properties shown *in vitro* by the PL such as that of reducing the level of oxidative stress, could provide new insights for improving its biochemical formulation and biological efficacy.

Further studies are required *in vitro* and *in vivo* to investigate the mechanisms of reduction of oxidative stress and to fully assess the efficacy of the GMP-grade PL prepared in our laboratory also in comparison with different PL preparations and/or blood products.

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Abbreviations: PL: Platelet lysate; FBS: foetal bovine serum; TFs: Tenon's fibroblasts; NO: nitric oxide; EGF: epidermal growth factor; FGF: fibroblast growth factor; VEGF: vascular endothelial growth factor; KDR: kinase insert domain receptor; MMP2 and 9: metalloproteinase MMP 2 and 9; FAK: Focal Adhesion Kinase; alpha-SMA: alpha smooth muscle actin

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