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ORIGINAL ARTICLE

Autologous Platelet-Rich Plasma (CuteCell PRP) Safely Boosts *In Vitro* Human Fibroblast Expansion

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Nowadays autologous fibroblast application for skin repair presents an important clinical interest. In most cases, *in vitro* skin cell culture is mandatory. However, cell expansion using xenogeneic or allogenic culture media presents some disadvantages, such as the risk of infection transmission or slow cell expansion. In this study, we investigated an autologous culture system to expand human skin fibroblast cells *in vitro* with the patient's own platelet-rich plasma (PRP). Human dermal fibroblasts were isolated from patients undergoing abdominoplasty, and blood was collected to prepare nonactivated PRP using the CuteCell™ PRP medical device. Cultures were followed up to 7 days using a medium supplemented with either fetal bovine serum (FBS) or PRP. Fibroblasts cultured in medium supplemented with PRP showed dose-dependently significantly higher proliferation rates (up to 7.7 times with 20% of PRP) and initiated a faster migration in the *in vitro* wound healing assay compared with FBS, while chromosomal stability was maintained. At high concentrations, PRP changed fibroblast morphology, inducing cytoskeleton rearrangement and an increase of alpha-smooth muscle actin and vimentin expression. Our findings show that autologous PRP is an efficient and cost-effective supplement for fibroblast culture, and should be considered as a safe alternative to xenogeneic/allogenic blood derivatives for *in vitro* cell expansion.

Keywords: platelet-rich plasma, PRP, fibroblasts, cell proliferation, cell migration, wound healing, cell culture, cell therapy

Impact Statement

Autologous dermal fibroblast graft is an important therapy in skin defect repair, but *in vitro* skin cell culture is mandatory in most cases. However, cell expansion using xenogeneic/allogenic culture media presents some disadvantages, such as the risk of infection transmission. We demonstrated that an autologous culture system with the patient's own platelet-rich plasma is an efficient, cost-effective, and safe supplement for fibroblast culture. As it respects the good manufacturing practices and regulatory agencies standards, it should be considered as a potent alternative and substitute to xenogeneic or allogenic blood derivatives for the validation of future clinical protocols using *in vitro* cell expansion.

Introduction

Regenerative medicine has the potential to heal or replace tissues and organs damaged by age, disease, or trauma, as well as to correct congenital defects. Preclinical and clinical data show promise for the treatment of chronic diseases and acute injuries or even some cancers.[1] In the ''toolbox'' of regenerative medicine, cell therapy aims at delivering an autologous or allogenic cellular component to the patient for the repair or regeneration of the damaged tissue to restore the physiological functions.[2] A wide range of cells can be used in cell therapy, including blood and bone marrow cells,[3] mature and immature solid tissue cells,[4] adult stem cells,[5] and, most controversially, embryonic stem cells.[6]

Skin is a multifunctional and protective barrier in humans, which contains essential stem cell populations and various cellular types that are critical for renewing and maintaining its structural integrity and functions.[2] Therapeutic wound

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Table 1. Advantages and Disadvantages of FBS Versus PRP

	Fetal Bovine Serum	*Platelet-Rich Plasma*
Source	Bovine fetus blood	Autologous blood
Platelet content	Rare intact platelets	High level of intact platelets
Key elements for culture media	Growth factors and hormones Binding and transport proteins Amino acids, vitamins, and trace elements Fatty acids and lipids Protease inhibitors	Growth factors and hormones Binding and transport proteins Amino acids, vitamins, and trace elements Fatty acids and lipids Proteases and antiproteases Chemokines and cytokines Antimicrobial proteins
Advantages	''State-of-the-art'' biological supplement for cell culture ''Off-the-shelf'' availability Unimitated quantity Rich scientific data and publication	Nonmanufactured human product Autologous product: nonimmunogenic, no contamination risk Continuous secretion of growth factors and cytokines for >10 days, allowing to change culture medium every 10 days Promote high cell proliferation without affecting the cell phenotype and genotype Can form a hydrogel when activated and be used for 3D culture or as a cell delivery vehicle
Disadvantages	Industry-manufactured animal product Animal source: risk of xenoimmunization and zoonotic transmission Batch-to-batch variations and variability between suppliers Continuous consummation of growth factors and FBS composition variation over time in culture medium requiring medium changes every 3 days Contains chemically undefined components Low cell proliferation capacity High cost Ethical issue for use of animal products	Donor variations Lack of standardized PRP preparation methods Poor scientific data for cell culture in the literature Requires to be used ''fresh'' after blood harvesting: unavailable as ''off-the-shelf'' Relative contraindications for some patients: coagulation troubles, antiaggregant use (aspirin), …

Karnieli *et al.*[13]; Lang *et al.*[47]; Mannello *et al.*[72]; Astori *et al.*[73]; Atashi *et al.*[16]

healing and the restoration of skin structures and functions depend on many factors, including the availability of progenitor cells, extracellular matrix (ECM) components, growth factors, and cytokines for the angiogenesis and regulation of cell–matrix and cell–cell interactions. Fibroblasts, the major cell type of the dermis, produce the key ECM proteins in the dermis, including laminins, fibronectins, collagens, elastic fibers, noncollagen molecules, and growth factors that regulate cell function, migration, and the cell–matrix and cell–cell interactions in normal skin homeostasis and wound healing.[7] Dermal fibroblasts have already demonstrated clinical potential as therapeutic devices in skin wound healing,[8] tissue regeneration,[9] or as a dermal filler in esthetic and plastic surgery procedures.[10] Some authors even suggest that fibroblasts in the context of regenerative medicine may be used as a more practical and potentially more effective cell therapy than mesenchymal stem cells.[11]

In Europe, autologous fibroblast therapy is considered as an Advanced Therapy Medicinal Product with a Hospital Exemption (ATMP-HE) product. Therefore, the medicinal product does not require a centralized marketing authorization from the Europe Medicines Agency (EMA) as an autologous small-scale nonroutine cell therapy production. However, the product must comply with the requirements for medicinal products, including good manufacturing practice (GMP) requirements for ATMPS. Such therapy must be produced at the request of a physician and should only be used within the Member State where they are produced.[12]

Autologous cell therapy aims at decreasing the risk of an adverse immune reaction or infection transmission. In most cases, the cells must be expanded *in vitro* before treatment to obtain a homogeneous cell population and a sufficient number. Recently, the United States Food and Drug Administration (FDA) observed that >80% of investigational new drug applications for cell therapy products used fetal bovine serum (FBS) during the manufacturing process.[13] However, this xenogeneic additive presents a potential risk of infection and immunological reaction, and offers a slow cell proliferation (Table 1). Therefore, the search for a safe and efficient serum substitute is primordial. Some authors have proposed to replace FBS in culture media with autologous human serum for cell expansion, but as cell proliferation was slow, it still required a long culture time (3 weeks) and several cell passaging before transplantation.[14,15]

In a previous study, we demonstrated that autologous platelet-rich plasma (PRP) could be used as a safe, efficient,

and cost-effective culture medium for adipose-derived mesenchymal stem cell (ADSC) proliferation.[16] Media supplied with 20% autologous PRP increased cell proliferation >13-fold without changing the cell phenotype. Currently, PRP obtained from patient's own blood is already used efficiently in the clinical setting for wound healing,[17–22] bone regeneration,[23–27] or skin rejuvenation.[28–31] Platelets are a natural supplier of autologous growth factors, key controllers of cell proliferation, differentiation, and tissue regeneration. Under physiological conditions, platelets secrete in a controlled manner gradually the growth factors and cytokines stored in their α-granules, such as platelet-derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (FGF), transforming growth factor-β (TGF-β), and vascular endothelial growth factor (VEGF).[32–37]

We thus postulate that autologous PRP can serve as a safe and effective biological supplement alternative to current nonautologous products for skin fibroblast expansion.

In this study, we assessed the efficiency of autologous PRP compared with the classical FBS-supplemented culture medium to define an autologous system for normal human dermal fibroblast (NHDF) proliferation. We investigated the optimal PRP concentration, and assessed the biological effects of media supplemented with PRP on migration, adhesion, differentiation, and genomic stability of NHDF.

Materials and Methods

Cell isolation

NHDF was isolated from 10 healthy women undergoing abdominoplasty in our Plastic, Reconstructive and Aesthetic Surgery Department at Geneva University Hospitals (Geneva, Switzerland). The procedure conformed to the principles of the Declaration of Helsinki and was approved by the local institutional ethics committee (protocol #3126). Informed written consent was obtained from all the donors. NHDF was isolated as described elsewhere[38] and cultivated in complete growth medium (Dulbecco's modified Eagle's medium [DMEM]; Life Technologies, Paisley, United Kingdom) supplemented with 10% FBS, 1% HEPES 1 M buffer solution (Life Technologies), 1% nonessential amino acid mixture 100× (Life Technologies), 1% L-glutamine 100× (Life Technologies), 1% penicillin/streptomycin 100× (Life Technologies), and 1% sodium pyruvate 100× (Life Technologies) and stored up to 1 month at 4°C.

For cellular analyses cells were used at passages 1 to 2.

Preparation of human autologous PRP

From the same NHDF donor, 30 mL of human peripheral blood was collected into three specific medical devices (Regen Lab SA, Le Mont-sur Lausanne, Switzerland). The collected blood was centrifuged for 5 min in a standard laboratory centrifuge at 1500 *g* force (corresponding to 3100 rounds per minute [RPM]) at room temperature. Subsequently, the red and white blood cells accumulated at the bottom of the tube under the separator gel, whereas the plasma and platelets remained above the gel layer. Plasma-containing platelets were homogenized by returning the tube five times to obtain 6.0 mL of PRP per tube, which was collected in a polypropylene tube (Becton-Dickinson, Franklin Lakes, NJ) until use. Platelets, red and white blood cells, as well as MPV in whole blood were counted (KX-21N; Sysmex, Lincolnshire, IL) before centrifugation and in the prepared PRP before addition to culture media.

Cell proliferation assay

Cell proliferation was assessed by CellTrace™ Violet (Molecular Probes, ThermoFischer Scientific, Waltham, MA) staining. At passage 2, NHDFs were seeded in 24-well plates at a density of 8×10^3 cells per well. Based on our previous results on the proliferative effect of PRP in ADSCs,[16] we chose to culture cells at different PRP concentrations (1%, 5%, 10%, 20%, 30%, 40%, and 50%) with 2 U/mL heparin (Liquemin 5000; Roche, Basel, Switzerland) and to compare them with classical culture conditions (FBS 10%). Previously we showed that 50% of platelets are still viable and functional in this medium after 10 days. Therefore for ADSCs culture we changed the medium only after 10 days with an efficient proliferation rate.[16] As the proliferation rate of fibroblasts is known to be higher and reach cell confluence faster, we decided to culture NHDF for 7 days without medium change. Quantification of cell proliferation was performed by flow cytometry using the Attune Acoustic Focusing Cytometer (Life Technologies) according to the manufacturer's protocol. To perform cell cycle analysis, we measured the DNA content. In brief, NHDFs were seeded onto 12-well plates at a density of 4×10^5 cells per well, and cultured in 10% FBS or treated with different PRP concentrations (1%, 5%, 10%, 20%, 30%, 40%, and 50%) for 2 days and 7 days. Cells were trypsinized using Tryple X, and then fixed with 70% ethanol. Cells were then permeabilized, and nuclear DNA content was stained with FxCycle PI solution (Molecular Probes) at room temperature. Samples were run using the Attune Acoustic Focusing Cytometer (Thermofisher Scientific). The cell cycle analysis was performed using FlowJo software.

Cell morphology, alpha-smooth muscle actin, and vimentin expression

To study cytoskeletal rearrangements, cells were seeded in black with clear bottom 96-well plates (μClear, Greiner, Kremsmünster, Austria) at a concentration of 10^5 cells/mL in DMEM supplemented with 0.5% FBS for 24 h, and then treated with FBS 10% or 1%, 5%, 10%, 20%, 30%, 40%, and 50% of PRP concentrations for 7 days. NHDFs were fixed with 4% paraformaldehyde for 10 min and permeabilized with 0.1% Triton X-100 for 5 min at room temperature. They were then stained with 50 μL of 5 U/mL phalloidin (Life Technologies), washed twice with PBS, and marked with 50 μL of 1 μg/mL DAPI for 5 min. To assess vimentin expression, a mouse monoclonal antivimentin antibody FITC (V9, eBiosciences; ThermoFischer Scientific) at 2.5 μg/mL for 1 h at room temperature was used. To depict alpha-smooth muscle actin (SMA) expression, a rabbit polyclonal antialpha SMA (Abcam; ab 5694) was used at 2 μg/mL for 1 h at room temperature, followed by a secondary goat antirabbit polyclonal Alexa Fluor 594 (Abcam; ab 150080) at 2 μg/mL for 1 h at room temperature. Cytation 3 cell imaging multimode reader (BioTek) was used to visualize immunofluorescence staining.

To measure alpha-SMA protein expression, NHDFs were seeded into six-well plates at a density of 5×10^5 cells per well, and cultured in 10% FBS or treated at different PRP concentrations (1%, 5%, 10%, 20%, 30%, 40%, and 50%) for 4 days. Cells were detached with Tryple X and then fixed with 2% paraformaldehyde in PBS for 10 min. After washing in PBS, cells were permeabilized with 0.1% Triton X-100 for 5 min. Single-cell suspensions of 10^6/mL were incubated with optimal concentrations of antialpha SMA antibody (Abcam; ab5694) at 2 μg/mL in wash buffer (2% normal goat serum in PBS) for 1 h at room temperature, washed three times, followed by a 1-h incubation with a goat antirabbit IgG H&L (Alexa Fluor®594; Abcam). Flow cytometry was performed on Attune Nxt Flow Cytometer (Life Technologies). Control samples consisted of cells without primary antibody binding.

Metabolic activity assessment by MTT assay

To measure the metabolic activity of cells, we assessed the intracellular reduction of the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan. NHDFs were seeded into 96-well plates at a density of 10^4 cells per well and incubated overnight in DMEM containing 0.5% FBS. The following day, cell cultures were treated for 48 h with a range of PRP concentrations (5%, 10%, and 20%) or FBS 10%. Then 10 μL MTT was added (final concentration 500 μg/mL) for 4 h at 37°C. Pictures (100× magnification) were taken with an inverted microscope (Nikon). The medium was then aspirated, and insoluble formazan crystals (blue granules) were dissolved with 100 μL DMSO per well. The plate was then placed on a shaker for 5 min (150 rpm). Absorbance was read at 595 nm on a microplate reader (Biotek).

Cell adhesion assay

For the evaluation of cell adhesion on laminin and collagen type I, experiments were performed in triplicate on 96-well plates. NHDFs were plated at a density of 1×10^4 cells per well on 96-well plates precoated either with laminin (10 μg/mL) or with collagen type I (50 μg/mL). Cells were allowed to adhere for 30 min, 1 h, or 4 h at 37°C in a medium with 10% PRP. Plates were washed three times with PBS, fixed with 4% paraformaldehyde for 10 min, stained with 0.1% crystal violet, and samples were rinsed with flow water and air-dried; images were taken under an inverted microscope. Samples were dissolved with glacial acetic acid, and the optical density at 570 nm was determined by using an automatic microplate reader.

Wound healing assay

NHDFs were seeded into 96-well plates at a density of 4×10^4 cells per well and grown to confluence for 24 h. A scratch was created in the NHDF monolayer using a 10-μL pipette tip. The wells were washed twice with PBS to remove detached cells, and pictures were taken at the center of each well. NHDFs were then incubated for 8 h in 10% FBS or a range of PRP concentrations (1%, 5%, 10%, 20%, 30%, 40%, and 50%). After incubation, NHDFs were washed with PBS and fixed in 4% paraformaldehyde. One image per well (center position, same as time 0) was obtained on a high-throughput Cytation 3 cell imaging multimode reader (BioTek). Quantification was performed using ImageJ software by measurements of the wounded areas at time 0 and 8 h.

Comparative genomic hybridization array

Genomic stability was determined by comparative genomic hybridization (CGH) array. NHDFs treated with FBS 10% or PRP 10% for 6 days were compared by using CGH. DNA was extracted using the QIAGEN QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Array CGH was performed using the Agilent SurePrint G3 Human CGH Microarray kit 4_180K (design ID 022060) with 43 kb overall median probe spacing (Agilent Technologies). Practical resolution was ~129 kb. DNA extracted from cultured NHDF and control DNA (Promega male DNA, ref G1471) were pooled. Donor DNA and DNA of a sex-matched control (1 μg each) were labeled with Cy3-dUTP and Cy5-dUTP, respectively (Sure Tag labeling kit, Agilent Technologies). Labeled products were purified by Amicon Ultra 30 K filters (Millipore, Burlington, MA). Hybridization was performed according to the protocol provided by Agilent. Donor and control DNA were pooled and hybridized with 2 mg of human Cot-I DNA at 65°C with rotation for 24 h. Arrays were analyzed using an Agilent SureScan Microarray scanner and the Agilent Feature Extraction software (v11.5), and results were presented by Agilent Genomic Workbench (v.7.0).

Statistical analysis

For each cell culture experiment, treatments were performed in triplicate or quadruplicate. Unless otherwise stated, each experiment was repeated three times. Data are expressed as means ± SEM. One-way ANOVA was used for multiple comparisons in experiments with one independent variable. Dunnet's test was used for *post hoc* analysis of the significant ANOVA. A difference in mean values between groups was significant when $p \le 0.05$.

Results

Blood cell and platelet counting in whole blood and PRP

After centrifugation, platelets formed a thin pellet over the separator gel below the plasma. The platelets were put back in suspension in the plasma (mean volume 6 mL per tube), and this suspension was used as PRP. The final mean platelet volume (MPV) of PRP was $2.5\times10^5 \pm 1.21$ platelets/μL. This concentration was 1.53 times more than the whole blood before centrifugation ($1.64\times10^5 \pm 0.64$ platelets/μL). The platelet recovery rate in PRP from the whole blood was 96%. MPV was comparable between whole blood and PRP (8.7 fL ±0.87 in whole blood vs. 8.2 fL ±0.82 in PRP). The mean white blood cell concentration was significantly lower in PRP compared with whole blood ($0.75\times10^3 \pm 0.7$ cells/μL vs. $6.75\times10^3 \pm 2.88$ cells/μL, respectively; $p<0.05$). The mean red blood cell concentration was also significantly reduced ($0.03\times10^6 \pm 0.001$ cells/μL vs. $4.17\times10^6 \pm 0.49$ cells/μL, respectively; $p<0.05$) ($n=10$) (Fig. 1A).

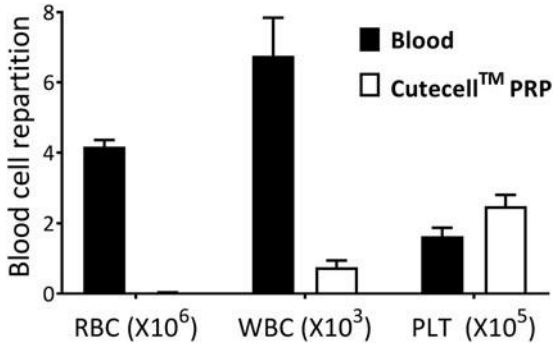

FIG. 1. Blood and PRP cellular counts. Analysis of the number of platelets (PLT $\times 10^5$), *white* blood cells (WBC $\times 10^3$), and *red* blood cells (RBC $\times 10^6$) in whole blood compared with PRP prepared with the CuteCell™ PRP device. $N = 10$ patients. PRP, platelet-rich plasma.

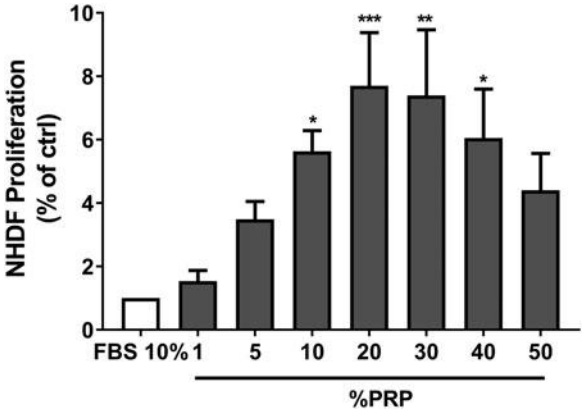

FIG. 3. Assessment of PRP proliferative effect by flow cytometry using CellTrace *Violet* (vital dye). Proliferative effect of increasing PRP concentrations in comparison (1–50%) with FBS 10% ($n = 10$ different patients) on NHDF for 7 days without medium change in a complete autologous system (cells and PRP from the same patient). $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. FBS, fetal bovine serum.

PRP promotes a dose-dependent increase of the proliferation together with cell cycle modifications

After 7 days of culture without changing the media, cultures supplemented with different PRP concentrations showed a higher viable NHDF number compared with FBS-containing media (Fig. 3). This proliferative effect of PRP followed a dose-dependent bell-shaped curve. The optimal culture condition was PRP 20% where the NHDFs number was 7.7-fold higher than FBS 10% ($n = 10$; $p < 0.001$).

It was observed that the treated samples with PRP exhibited significant cell cycle modifications compared with the control FBS-treated cells after 48 h. The mean percentage of cells in the ''G0/G1'' phase changed from 74.5% in the FBS 10% group to 62% in the 20% PRP group; in the ''S'' phase from 9.5% in the FBS 10% group to 13% in the PRP 20% group; in the ''G2/M'' phase from 14% in the FBS 10% group to 23% in the PRP 20% group (Fig. 4A, B). After 7 days of treatment, cells were confluent, DNA synthesis and mitosis were stopped, and cells entered the G0/G1 phase in a quiescent status with media supplemented with PRP (Fig. 4C).

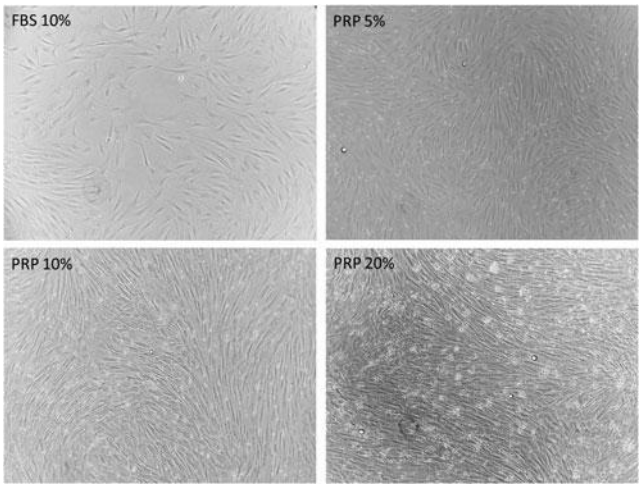

FIG. 2. Bright-field optical photography of NHDF in the presence of FBS 10% or PRP (5–20%) after 7 days of culture. Magnification 10×. Pictures are representative of one donor. NHDF, normal human dermal fibroblast.

PRP treatment increases cell metabolic activity

Under microscopic examination of the FBS-treated cultures (Fig. 5A), formazan granules were found in intracellular organelles. However, when the cells were treated with PRP (5–20%) for 48 h, needle-like crystals appeared on the surface of the cells, representing exocytosed MTT formazan (Fig. 5A). Moreover, when we quantified the amount of solubilized formazan by optical densitometry, we evidenced an increase in PRP-treated cells, directly reflecting an increase in cell metabolic activity (Fig. 5B) peaking at 3.12-fold in PRP 20%-treated cells compared with FBS 10%-treated cells after 48 h of treatment.

Assessment of PRP effects on cell shape, cytoskeleton, vimentin, and alpha-SMA expression

NHDF cultured in classical FBS-supplemented culture medium showed a regular flattened cell shape while NHDF treated with PRP (10–50%) were spindle shaped, a morphology that is closer to 3D matrix cultures or *in vivo* setting (Fig. 2).

We sought to investigate whether the morphological change occurring at 7 days of PRP treatment (Fig. 6A) was related to a phenotypical change. We first demonstrated prominent F-actin reorganization from cortical actin localization (FBS 10%) into thick cell-spanning filaments (PRP 20%) (Fig. 6B). We further assessed the changes in alpha-SMA expression upon PRP treatment by flow cytometry and immunofluorescent analysis (Fig. 7A). Alpha-SMA expression was significantly increased with a high PRP concentration (40–50%), while FBS- and PRP 5–10%-treated cells showed a basal perinuclear staining (Fig. 7B).

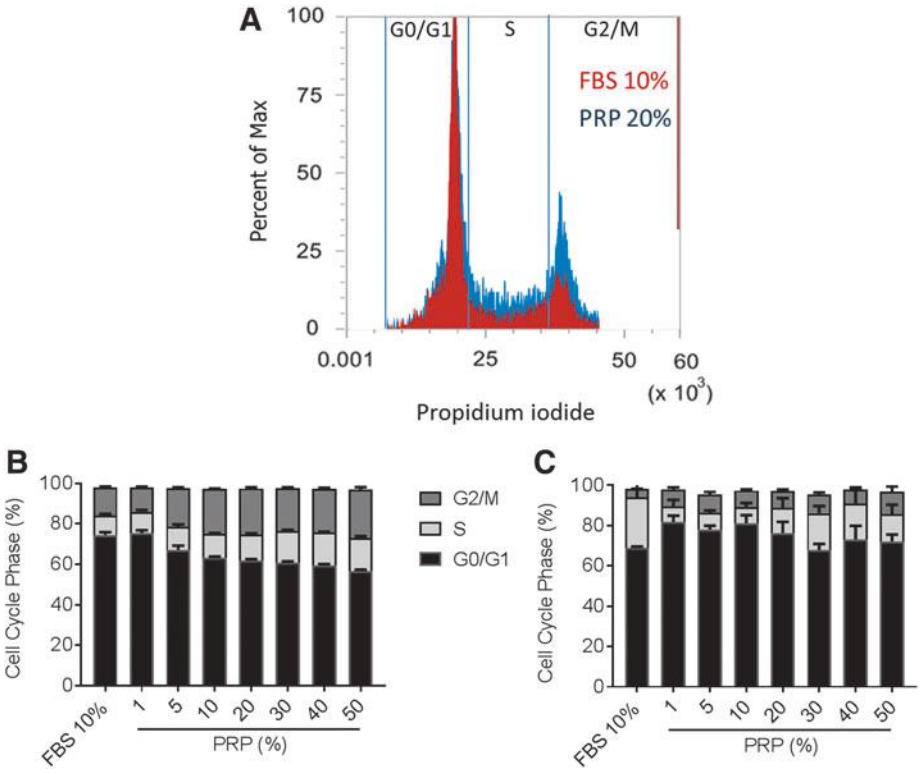

FIG. 4. PRP-dependent cell cycle modulation in NHDFs. **(A)** Descriptive cell cycle data of NHDFs after 48 h of incubation of treatment with PRP 50%. The histogram designates the increases in cell number in ''G2/M'' phase arrest and decreases in cell number in the ''G1'' phase. The graphical representation of cell numbers in the G1/G0, S, and G2/M phases after 48 h of treatment **(B)** and 7 days of treatment **(C)**.

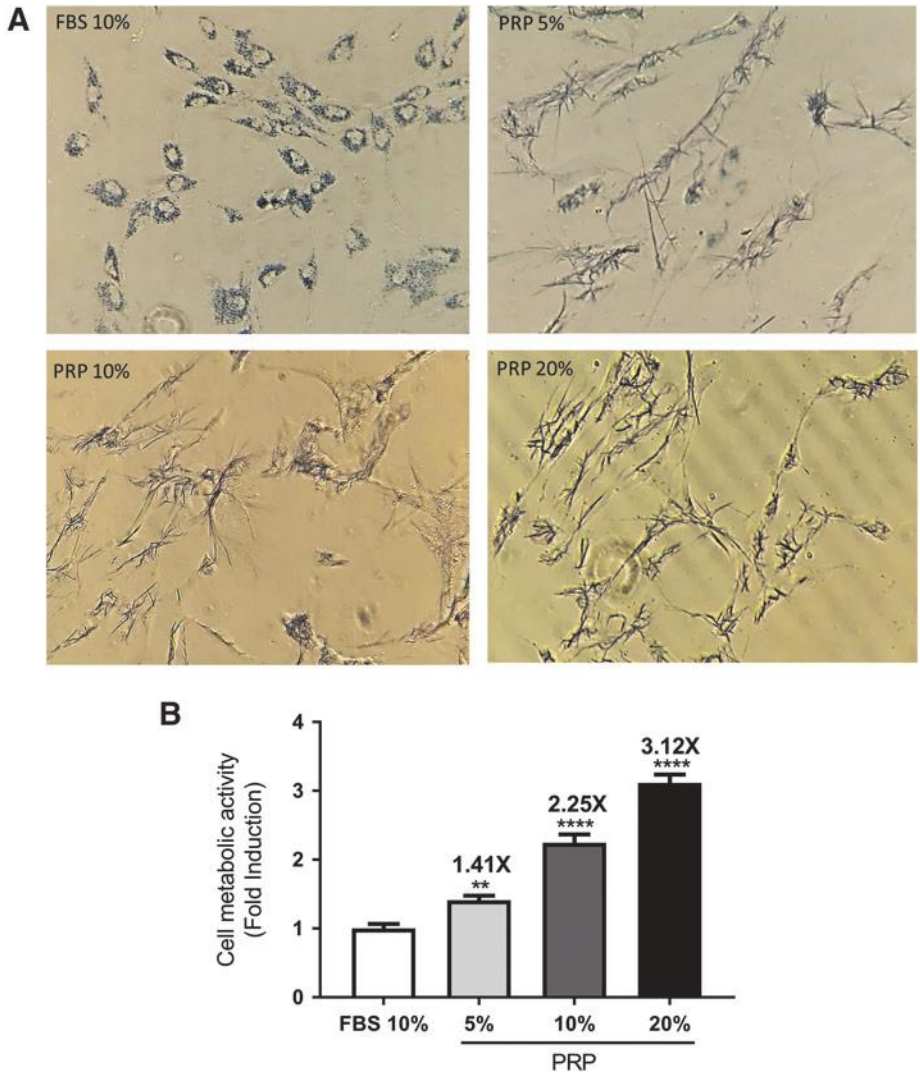

FIG. 5. **(A)** Representative bright-field microscopy images depicting the cellular localization of the MTT formazan in NHDF incubated for 48 h in different culture media. FBS-treated NHDF shows intracytoplasmic dark granules while PRP-treated NHDF shows extruded formazan crystals. Quantification of the solubilized formazan (absorbance measurements 570 nm) **(B)**. Data are expressed as means ± SD. $^{**}p < 0.01$, $^{****}p < 0.0001$.

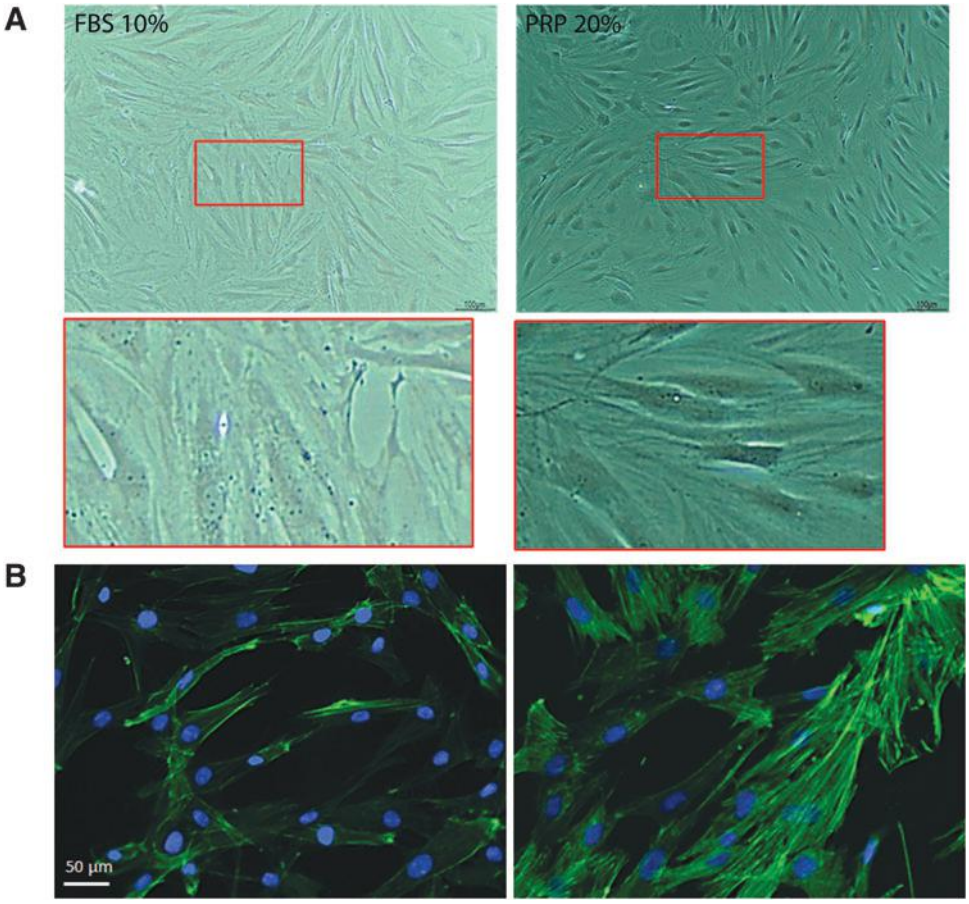

FIG. 6. Long-term PRP treatment (7 days) promotes NHDF cell shape modification, closely mimicking fibroblast to myofibroblast differentiation **(A)**. F-actin cytoskeletal reorganization was assessed. Prominent bundles of actin microfilaments appeared along the cytoplasm in PRP-treated cells **(B)**.

Immunofluorescent analysis showed an increase in vimentin staining in the presence of PRP 20%, but it was completely abolished at high PRP concentration (PRP 50%).

PRP treatment affects cell adhesion on ECM and promotes fibroblast collective migration

To further characterize the biological effects of PRP on NHDF biology, we evaluated the effect of PRP treatment on cell adhesion on laminin and collagen type I. As shown in Figure 8A, PRP decreased the overall attachment of NHDFs to laminin 4 h after seeding. This effect occurred already after 15 min, with a 21% decrease in overall cell adhesion. The same results were obtained for NHDF attachment to the collagen I matrix (Fig. 8B) (41% of total cell adhesion after 15 min).

To study the migratory properties of NHDFs exposed to PRP, we performed an *in vitro* scratch assay (Fig. 9). Eight hours of 20% PRP treatment induced a 10% increase in the number of migrating cells from the scratch margin into the scratch zone compared with cell cultures with FBS. This migration front was a collective cell migration. Conversely, NHDFs exposed to FBS 10% showed features of isolated cell migration.

PRP does not modify cell genomic stability

NHDFs at passage 2 were cultured for 4 days with media supplemented with FBS 10% or PRP 10% to document genetic stability during proliferation. Array CGH analysis of cells treated with the two different culture media did not show imbalanced chromosomal rearrangements. The increased proliferation rate in response to PRP treatment did not provoke genomic instability. To exemplify the data, the benign homozygous deletion depicted on chromosome 4 (region q13.2) was superimposable in NHDF cultivated in FBS 10% or PRP 10%, as well as the benign heterozygous deletion on chr3 in q29 region (Fig. 10).

Discussion

Autologous fibroblast treatments have a potential development in a range of esthetic and plastic surgery procedures.[39] This clinical interest was developed after animal studies where it was shown that fibroblasts isolated from mature skin remained alive *in vivo* 5 months after intradermal injection and improved dermal thickness.[40] In 2011, LAVIV (Azficel-T, Fibrocell technologies, Exton, PA), the first personalized autologous fibroblast cell therapy, has been approved by the FDA for enhancement of nasolabial folds in adults.[41] The main drawback of this technology is the cost and the extended time of the manufacturing process[42] (11–22 weeks) before injection, as well as the xenogeneic source of growth factors supplied by FBS to expand the cells *in vitro*.

Providing safe and regulated cell therapy products to patients implies adherence to GMP, and these guidelines

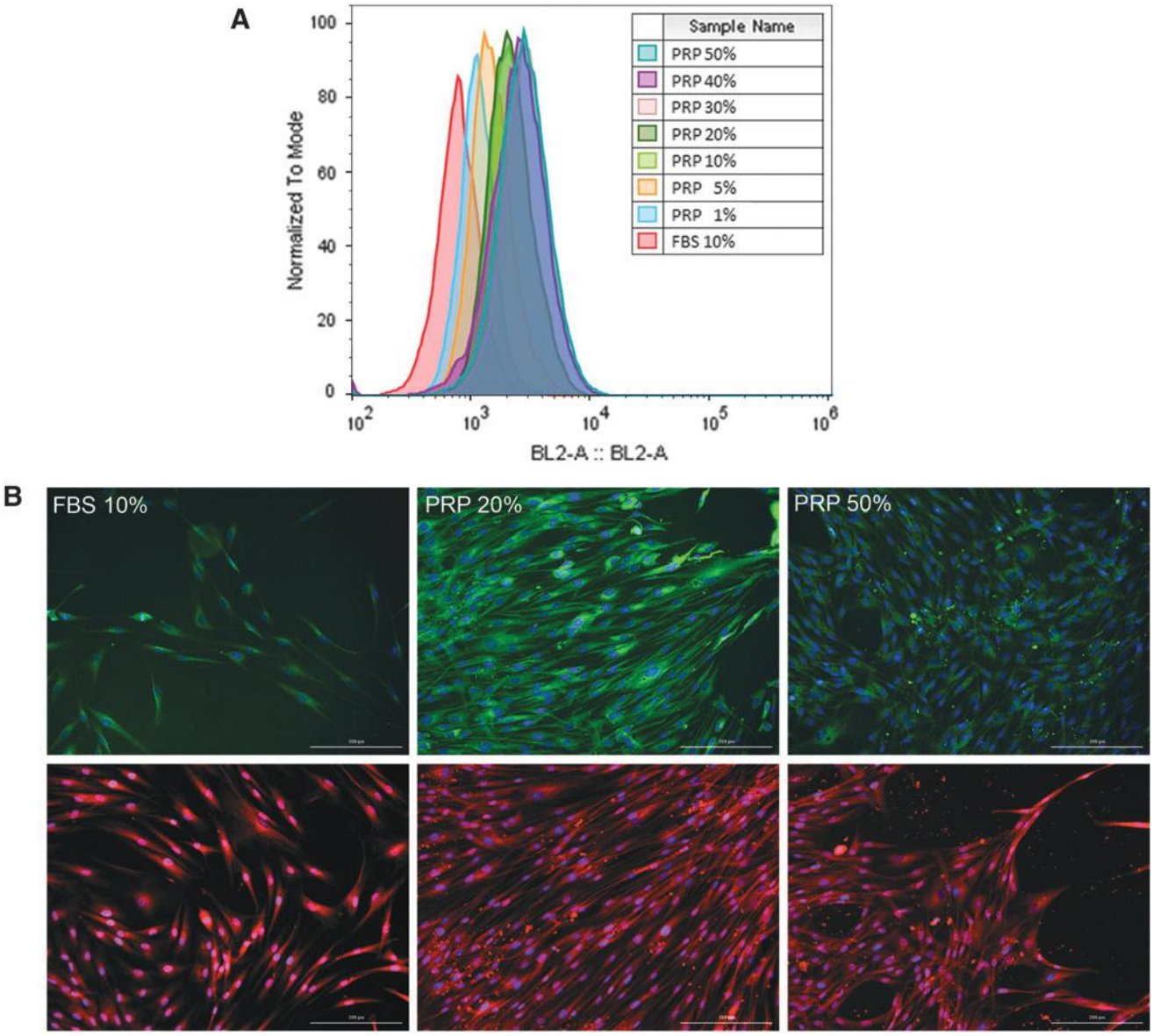

FIG. 7. **(A)**. Flow cytometry histogram overlays of alpha-SMA positive cells of PRP-stimulated NHDF for 4 days compared with FBS 10%. **(B)** Vimentin (*upper line*) and alpha-SMA (*lower line*) immunofluorescence on NHDF after 7 days of culture in the presence of FBS 10% or PRP (20–50%) in the culture media. Nuclei were counterstained with DAPI.

should be applied throughout the all process of tissue harvesting, cell isolation and expansion.[43] Consequences on molecular level on cells that have been long term cultured in bovine serum have not yet been explored deeply. It cannot be excluded that the high failure rate in preclinical research might, at least partly, result from the fact that human cells are confronted with the serum of a foreign species.[44]

For these reasons, attempts have been made to subtract any factors of nonhuman origin in cell therapies (e.g., FBS, FCS).[13] The last decade, human platelet lysate (PL) has been established as an alternative to FBS in cell culture.[45] Despite the promising results, their cell proliferation efficiency is not yet enough optimal (e.g., slow cell proliferation), and their biological activity varies greatly among the protocols.[46] Recently autologous PRP was recognized as an autologous biological supplement to stimulate stem cells proliferation more safely and effectively than FBS.[16,47]

Our current study demonstrated that the culture media containing nonactivated PRP prepared with CuteCell™-PRP tubes present a potent dose-dependent proliferation effect on NHDF. Media supplemented with 20% of nonactivated autologous PRP offered the highest NHDF proliferation rate (7.7 times more than FBS culture medium). The stimulatory effect of PRP on NHDF proliferation was evidenced at the DNA level by assessing the cell cycle phases. After 48h media containing 20% PRP increased significantly cell numbers in S and G2/M phases. This effect was not seen anymore after 7 days where most of the cells were in G0/G1 phase, because they reached confluency.

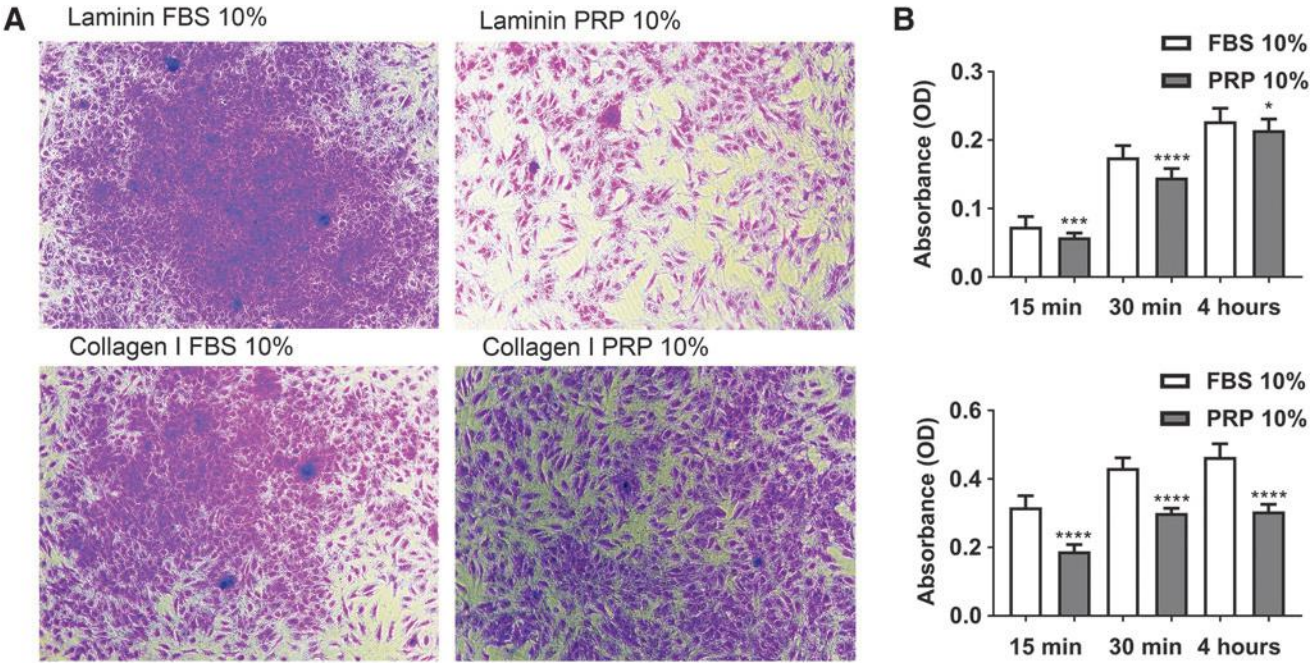

FIG. 8. Short-term PRP effect of NHDF adhesion to laminin and collagen I. NHDFs were stimulated with FBS 10% or PRP 10% for 15 min, 30 min, or 4 h. Unattached cells were removed, and adherent cells were fixed with methanol and stained with 0.1% crystal violet **(A)**. Cells were then solubilized, and the released dye was quantified using a microplate reader (optical density at 590 nm) **(B)**. Bars indicate standard errors. Number of replicates: between 10 and 20 wells per experimental condition. $*p < 0.05$, $***p < 0.001$, $****p < 0.0001$.

Some other studies also aimed to substitute FBS with PRP for the *in vitro* expansion of fibroblast originating from dermis or gingival tissue.[48–52] But most of these experimental settings, using activated PRP, showed limited cell proliferation efficiency. Kakudo *et al.* concluded that human dermal fibroblast proliferation could be enhanced 2.5× after 7 days of culture with 5% activated PRP, while 20% activated PRP did not promote proliferation.[48] Another study with NHDF showed only a 1.5× enhancement of proliferation by adding 5% activated PRP after 5 days.[49] Cho *et al.*[53] also demonstrated a dose-dependent increase in proliferation that is maintained after 5 days in media containing 2% of activated PRP (4.6-fold PLT baseline number). Recently, nonactivated PRP prepared from a pool of 49 patients was shown to have a mild proliferative effect after 7 days of culture (1.3-fold).[52] However, in this study, Noh *et al.* did not replace the FBS by PRP. They added an unknown concentration of PRP to culture media containing FBS, without substituting completely the FBS by PRP.

In most of these studies, FBS substitution was achieved by different concentrations of activated PRP (activation by thrombin or calcium). This artificial activation provokes, as performed in other studies, an immediate and important release of platelet growth factors from 15 min up to 24 h only.[54] Therefore, we believe that platelet activation is undesirable for applications as cell culture where the slow release of growth factors from gradual platelet degranulation is required.

MPV is considered as a potential marker of platelet activity: larger platelets that contain more dense granules are enzymatically and metabolically more active than their smaller counterparts.[55] In our study, MPV of platelets in PRP was comparable with those in the whole blood (8.2 fL vs. 8.7 fL, respectively). As the PRP preparation did not change the MVP, it implies that processing did not cause platelet activation. In a previous study of adipose-derived stem cell expansion with a medium containing nonactivated PRP, we demonstrated that PDGF-AB and FGF concentrations peaked at day 5 and remained stable for >10 days, while TGF-β1 and VEGF were continuously secreted from day 0 to 10.[56] This was in direct correlation with 50% platelet viability after 10 days of culture with no medium change.[16]

Therefore, when nonactivated PRP is added to culture medium, it could be changed every 7–10 days, instead of 3 days when culture medium is supplemented with active PRP.[57] Medium change during the coculture is also affecting the kinetic of the growth factor release. We thus chose to expand the cells for 7 days in culture without interfering with the medium composition. It was recently shown that simple cell manipulation can influence proliferation rate and expression of inflammatory genes, as demonstrated in mesenchymal stromal cell cultures.[58] Therefore, we believe that platelet activation is undesirable for applications as cell culture where the slow release of growth factors from gradual platelet degranulation is required. We have thus enabled here a safe system, with the advantages of a long coculture of NHDF with different concentrations of autologous nonactivated platelets instead of their supernatants (e.g., platelet lysate) or activated PRP. We reduce the risk inherent to cell manipulation while bringing a more cost-effective method for larger scale applications, such as large tissue injury.

In line with other studies, we showed that cell proliferation depends on the dose of PRP: media supplemented with

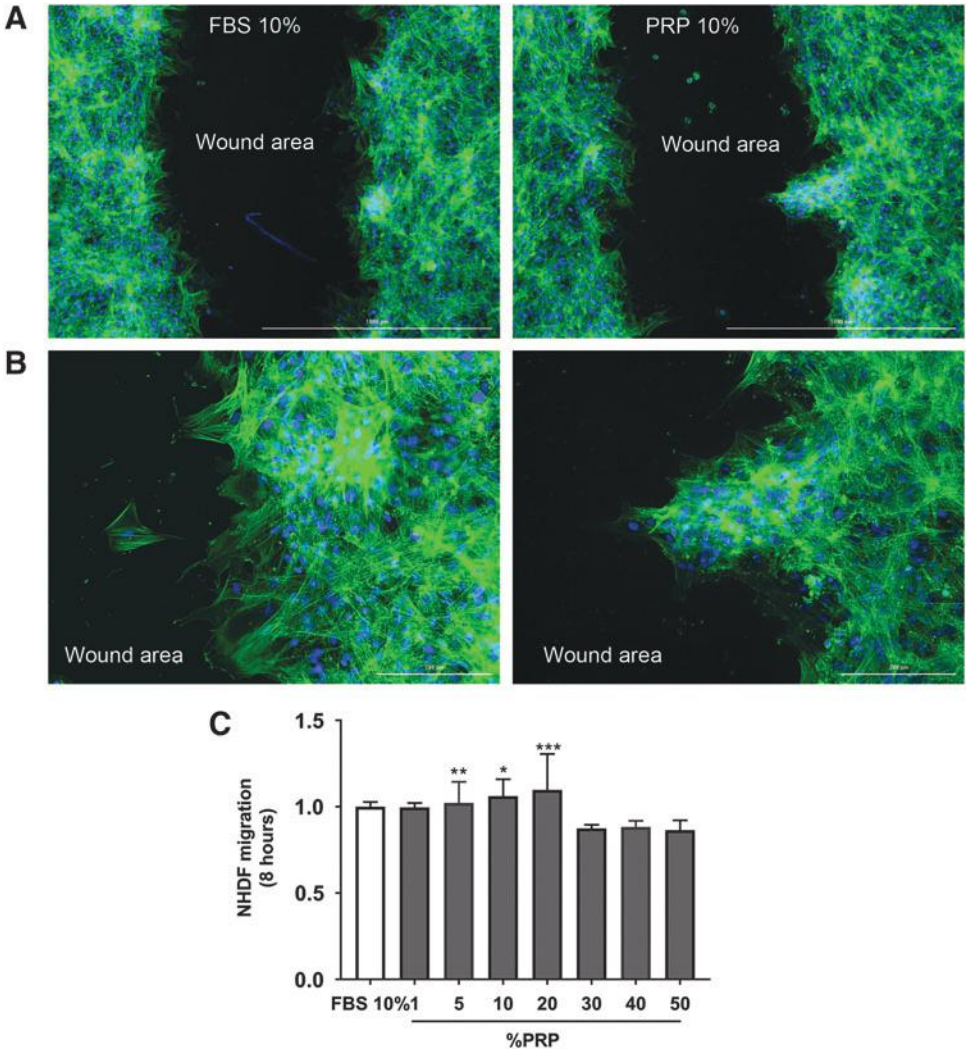

FIG. 9. Comparative cellular effects of PRP 10% treatment on cell migration in NHDF cultures. **(A)** Migrating fibroblasts narrowed the width of the scratch zone as evidenced after 8 h with an immunofluorescence staining with phalloidin. The cell migration front is equally distributed along the scratch border in FBS 10%, while it is less homogeneous in PRP 10%-treated cells. **(B)** Zoom in pictures showing isolated cell migration in FBS 10% cultures and collective cell migration in PRP 10% cultures. **(C)** Effect of 8 h treatment with increasing concentrations of PRP on NHDF migration in a wound healing assay. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

20% of PRP showed the best NHDF proliferation, while higher PRP concentration was less effective. Interestingly our previous studies for *in vitro* ADSC expansion concluded that the best PRP concentration was also the media supplemented with 20% of nonactivated PRP.[16] In a set of preliminary experiments (data not shown), we demonstrated the same potent proliferative effect of autologous PRP on human epidermal keratinocytes, where media supplemented with 20% of nonactivated PRP promoted a 10-fold increase of keratinocyte proliferation. Other groups also showed that media supplemented with higher PRP concentration (50% or 75%) were less effective than those with a moderate concentration (5% or 10%).[57,59] Some others, as in our study, demonstrated that high platelet content PRP can even provoke cell differentiation into myofibroblast.[60,61] Although PRP was defined historically as a plasma containing 4–5 times more platelets than whole blood,[62] it is now admitted that high platelet concentration is no better than moderate numbers and could even be harmful. For example, in a 3D anterior cruciate ligament fibroblast culture, a PRP containing the same platelet concentration as whole blood presented the highest cell metabolism and the lowest apoptosis rate compared with higher platelet concentrations.[63] Another study with oral fibroblast showed that increased platelet concentration in PRP (>2.5×) resulted in a reduction of the proliferation.[64] The PRP used in our study contained 1.53 times more platelets than the whole blood. Even the platelet recovery rate in PRP from the whole blood was 96%, this relatively low platelet concentration was because the platelets were suspended in the whole plasma over the gel separator of CuteCell-PRP. It is important to underline that the research in platelet concentration efficacy has overlooked the value of plasma and neglected to appreciate that plasma is a source of elements essential to cell survival such as nutrients, electrolytes, hormones, and growth factors (e.g., IGF-1). Thus, a sufficient plasma volume is a source of nutrients for both the platelets and the cells in culture.[65,66] Therefore, the harmful effect of high platelet PRP concentration in PRP or/and high PRP concentration in culture media could be due to higher growth factor concentration that may provoke a downregulation of cell receptor by a negative feedback loop. This mechanism could also explain why nonphysiologically activated PRP containing a high secreted growth factor concentration is less efficient in some applications than nonactivated PRP. Further investigations are required to elucidate this hypothesis. While growth factors present in PRP trigger cell division,

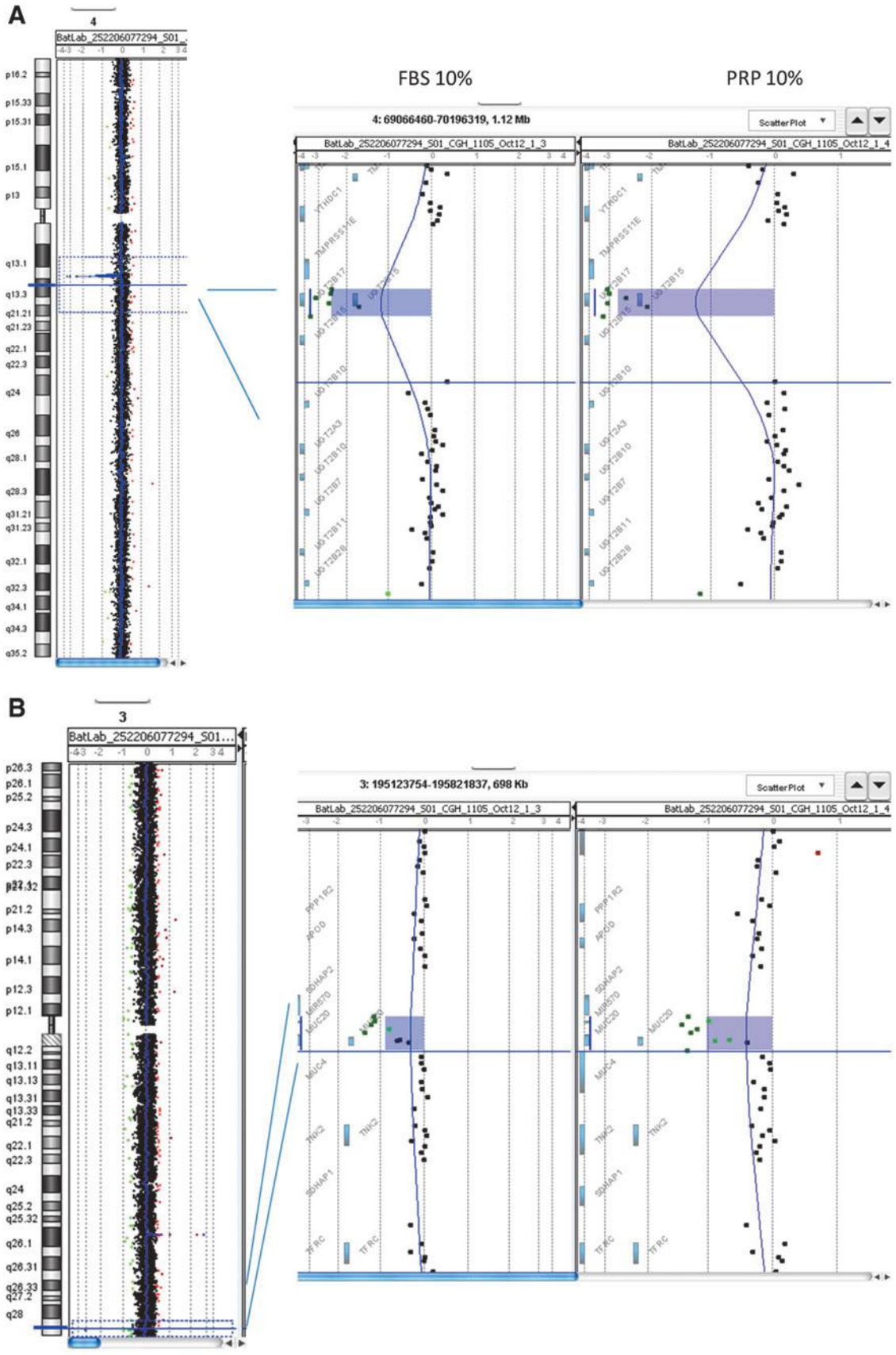

FIG. 10. Array CGH profile of NHDFs treated with media containing FBS 10% or PRP 10% for 4 days. Example of comparable homozygous deletion on chromosome 4 in q13.2 region **(A)** and comparable benign heterozygous deletion on chromosome 3 in q29 region **(B)**. CGH, comparative genomic hybridization.

they also exert other pleiotropic activity on fibroblast. Although few studies deciphered PRP mechanisms of actions in cell culture,[47] we assessed cellular functions and key factors that may influence early wound healing. The metabolic activity of NHDF, quantified by formazan production from MTT, was increased with the media containing 20% PRP. MTT added to the culture media is taken up by cells by endocytosis and reduced to formazan in the endosomal/lysosomal compartment. The amount of formazan production indicates the reductive potential of the cytoplasm and thus cell viability, proliferation, and metabolic activity.

PRP also modulated NHDF adhesion capacities (e.g., reduced cell adhesion on laminin and collagen type I), and elicited a collective and faster cell migration during the scratch assay. The cytoskeleton was also reorganized. Under 20% PRP treatment, vimentin expression, recognized as a proliferation coordinator in wound healing,[67] was enhanced. Expression of alpha-SMA (myofibroblast marker) was only triggered by high PRP concentrations (>40%). This is in line with the recent study of Chellini *et al.,* demonstrating that low PRP concentration was not able to stimulate the myofibroblast phenotype acquisition.[68]

There is some debate in the scientific community about the potential proneoplastic effect of long-term PRP treatment,[69] and some studies raise questions about genetic stability during massive cell expansion.[70] Therefore, we checked the genetic variations using CGH between cells grown in FBS versus PRP, and demonstrated no unbalanced chromosomic rearrangement with PRP treatment. This demonstration is of prime interest for translating PRP-cultured NHDF application into the clinical setting to meet legal regulatory requirements.

Culture media supplementation with autologous nonactivated PRP presents several advantages in comparison with FBS (Table 1). The main disadvantage of autologous PRP is the variability of the product. Due to poor standardized PRP preparation methods, affecting PRP biological identity (platelet number, growth factor concentrations, plasma nutrients, residual blood red or white cells,…), variable results were achieved in the past.[47] Kushida *et al.*[71] reported remarkable differences in GF concentrations in the activated PRP prepared with seven different commercial preparation methods. Therefore for the clinical application of PRP in GMP-cell therapy protocols, there is an urgent need of standardization and harmonization on the efficiency of PRP in correlation to its preparation.[47]

Conclusions

We have shown for the first time an autologous biological system for NHDF expansion using nonactivated PRP prepared with CuteCell-PRP devices as a safe and efficient biological supplement in culture medium without affecting the genotype of the expanded cells. The various effects of this specific PRP range from speeding up cell proliferation to modulating cell adhesion and migration without changing chromosomal stability, depending on the concentration and duration of the treatment. As this autologous technique respects GMP Guidelines and regulatory agencies standards, this PRP should be considered as an efficient, cost-effective, and safe supplement for fibroblast culture, as well as a substitute for xenogeneic or allogenic blood derivatives for the validation of future clinical protocols of *in vitro* cell expansion.

Intellectual Property

Cell culture expansion with PRP is protected by seven worldwide patents of Antoine Turzi (Switzerland): WO2008/023026, WO2011/110948, WO2016/083549, US8529957, EP2073862B, US2016158 286, and CH696752.

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