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BRIEF COMMUNICATION

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Granulation tissue-derived mesenchymal stromal cells: a potential application for burn wound healing in pediatric patients

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Abstract

Objective: Multipotential cells are mobilized into peripheral blood in response to trauma, in particular in severe burns. These cells migrate to the site of injury in response to chemotactic signals to modulate inflammation, repair damaged tissue and facilitate tissue regeneration. We evaluated the possibility of isolating and *in vitro* expand mesenchymal stromal cells (MSCs) from granulation tissue (GT) during debridement of a burn wound, as a persective strategy to improve skin regeneration.
Methods: GT obtained from a 12-month-old burn patient was *in vitro* cultured. Expanded MCSs were characterized for morphology, immunophenotype, differentiation capacity and proliferative growth. Antifibrotic features were also evaluated.
Results: It was possible to isolate and *in vitro* expand cells from GT with the morphology, phenotype, proliferative and differentiation capacity typical of MSC, these cells were defined as GT-MSC. GT-MSCs exhibited antifibrotic features by releasing soluble factors, this activity was superior to that observed in BM-MSC.
Conclusions: Successful isolation and expansion of MSCs from GT is reported. Considering their functional characteristics, GT-MSCs could be considered a good candidate adjuvant therapy to improve burn wound healing, particularly in pediatrics.

Key Words: Mesenchymal stromal cells; Granulation tissue; Children; Burn; Wound regeneration

Introduction

Pediatric burns are a major form of injury, affecting millions of children worldwide, and may be caused by scald incidents, fire injury or child abuse[1]. Burn scars often create great functional problems and their disfigurement can cause serious psychosocial problems [2-4].

Mesenchymal stromal cells (MSCs) have shown promise as a tool in cell therapy to treat different conditions, including wound regeneration[5-10]. MSCs are an outstanding tool for cell therapy applications, not only because of their multipotent nature, but also due to their ability to home to and engraft in damaged tissues, release trophic factors, promote neovascularization, manage oxidative stress and trigger anti-inflammatory responses[5,9-12]. Accumulating evidence suggests that MSC act through a combination of paracrine cell signalling and cell trans-differentiation, enhancing wound regeneration and improving angiogenesis[12, 13]. Recently, it has also been reported that multipotent cells mobilize to the peripheral blood after burn incidents and migrate to the site of injury in response to chemotactic signals where they modulate inflammation, repair damaged tissue and facilitate tissue regeneration[14, 15].

The most recognized source of MSCs is the bone marrow, however, other sources have been described such as adipose tissue, teeth, bone, muscle, placenta, liver, pancreas, umbilical cord and cord blood[16]. Spyrou *et al*[17]. described the granulation tissue (GT) as an abundant source of cells with important therapeutic efficacy in wound healing and tissue repair.

We describe the isolation and *in vitro* expansion of cells from granulation tissue (GT) obtained during debridement of a burn wound in a child. Based on these results, we propose that MSCs expanded from GT, may be considered a perspective strategy to improve skin regeneration in burn wound care.

Methods

GT was obtained from a 12 month-old boy undergoing burn treatment 15 days post-injury (severe thermal burn with full-thickness injury). The patient's GT was used for *in vitro* cell expansion, after obtaining written informed consent from parents.

Isolation and culture

Isolation and expansion of cells from GT, were performed following standard MSC culture procedures, as previously described[18, 19]. Briefly, tissue samples were incubated at 37°C for 30 minutes, in serum-free α-minimum essential medium (αMEM) (Gibco, Life Technologies, Paisley, UK) supplemented with antibiotics and collagenase type II (Sigma Aldrich). Next, collagenase activity was blocked with complete medium (αMEM+10% FBS, Euroclone, Milan,

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Italy). The cell suspension was then collected and plated at 160,000/cm^2 in non-coated T175 flasks (Corning Costar, NY, USA) in D-MEM+10% FBS and incubated at 37°C, 5% CO_2 for 48-hours. Medium was then changed twice a week.

At confluence, MSCs were trypsinized (Trypsin EDTA, Lonza, Milan, Italy) and replated at 4,000 cells/cm^2, for expansion. Cells were propagated to reach senescence phase and kept in culture for additional 8 weeks.

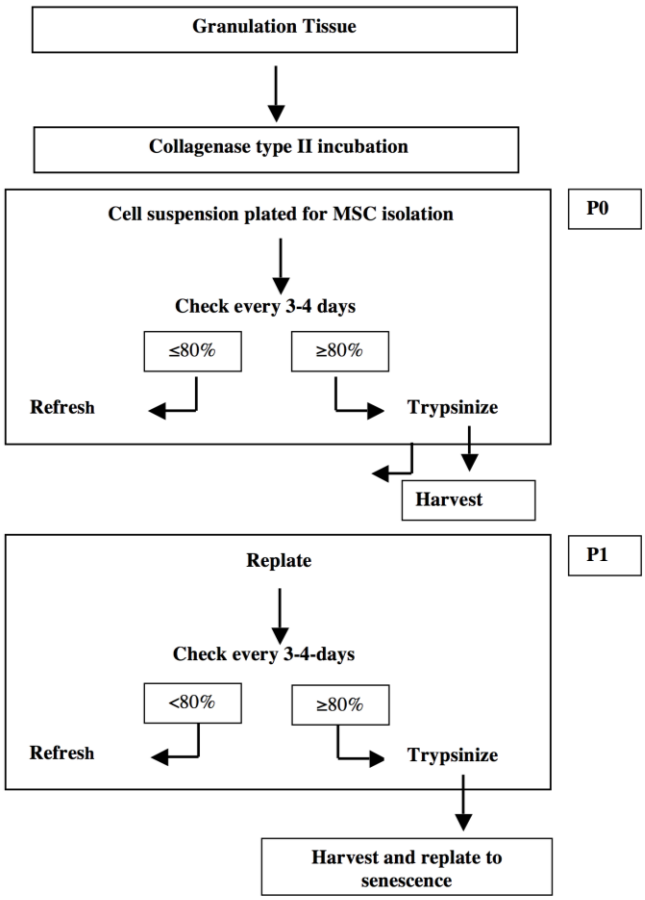

Figure 1. Scheme of the GT-MSCs isolation and expansion

Figure 1 illustrates the GT-MSCs isolation and expansion.

Characterization of ex-vivo expanded GT-cells

Proliferative capacity

Proliferative capacity was defined as cumulative Population Doubling (cPD) calculated with the following formula: PD=log (n. of harvested cells/n. of plated cells)/log2.

Phenotyping

Cells were characterized by flow-cytometry. Fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated monoclonal antibodies specific for CD73, CD34, CD90, CD14, CD45, CD31, CD105, class I-HLA and HLA-DR, (Beckman Coulter, IL, Milan, Italy) were used. Appropriate, isotype-matched, antibodies were employed as controls. Analysis was performed by direct immunofluorescence with a FACS Navios flow-cytometer (Beckman Coulter).

Differentiation capacity

The osteogenic and adipogenic differentiation capacity of GT-cells was assessed at P2-4 as previously described[18]. For osteogenic differentation complete medium supplemented with 10^{-7} M dexamethasone and 50 mg/ml L-ascorbic acid and 5 mM ß-glycerol phosphate (Sigma-Aldrich, St Louis, MO) was used while for adipogenic differentiation complete medium with 10^{-7}M dexamethasone, 50 mg/ml L-ascorbic acid, 100 mg/ml insulin, 50 mM isobutyl methylxanthine, 0.5 mM indomethacin (Sigma-Aldrich) and 5 mM b-glycerol phosphate was used. After two week incubation, cells were stained for alkaline phosphatase (AP) activity using Fast Blue (Sigma-Aldrich) and for calcium deposition with Alizarin Red (Sigma-Aldrich) to evaluate osteogenic differentation. Adipogenic differentiation was assessed by staining of fat droplets awith Oil Red O (Sigma-Aldrich).

Senescence assay

Senescence was defined by β-galactosidase (SA-β-gal) staining Kit (Cell Signaling Technology, Danvers, MA), according to the manufacturer's instructions.

Co-culture experiments

To assess the paracrine effect of GT-cells, dermal fibroblasts were co-cultured using a transwell system. Briefly, GT-cells ($1.5x10^4$ cells/well) were plated on Transwell membrane (0.4-mm pores, Corning Costar) with fibroblasts ($6x10^4$ cells/well) in the lower chambers and cultured for 4 days in RPMI 1640 (Euroclone, Milano, Italy) 10% FCS (Euroclone). Co-cultures were stimulated with 100 ng/mL of Fibroblast Growth Factor (FGF, Sigma, Milan, Italy) or not stimulated. For cell proliferation evaluation, fibroblasts were then harvested and viability was calculated using 0,2% Trypan Blue (Sigma). Cultured fibroblasts alone were used as controls. In addition, the culture medium was collected at 72 h to examine the concentration of TGFβ. Fibroblasts co-cultured with BM-MSCs were used as a control.

TGFβ quantification by ELISA

The quantification of TGF-β1 levels in FGF activated fibroblast co-culture supernatants were tested in order to define the role of this soluble factor in scar formation. Briefly, 96-well plates were coated with anti-human TGF- β1 (Endogen Tema), in carbonate/bicarbonate buffer, pH 9.6 overnight at room temperature. After three washes, a post-coating was performed for 1 hour at room temperature. After samples activation with 1N HCl for 10 minutes and neutralization with 1.2 N NaOH/0.5 M HEPES, they were incubated for two hours at room temperature. Detection antibody, specific for TGF-β1, was added and plate incubated at RT. After washing, Streptavidin-conjugated horseradish peroxidase was added and plates were further incubated for 20 minutes at room temperature. Plates were washed again three times and substrate solution (hydrogen peroxide and stabilized tetramethylbenzidine) was added. After 20 minutes in the dark. Absorbance was read at 450 nm (Lettore Titertek Plus MS 212 - ICN). Cytokine concentrations (expressed in pg/ml) were calculated using the standard curve.

Statistical analysis

A comparison of the groups was made with Kruskall-wallis non parametric ANOVA followed by two- by-two subgroup analysis corrected for multiple tests. Statistical significance was defined as $p < 0.05$. Data analyses were performed with the STATA statistical package (released 14.2, 2012, Stata Corporation, College Station, Texas, USA).

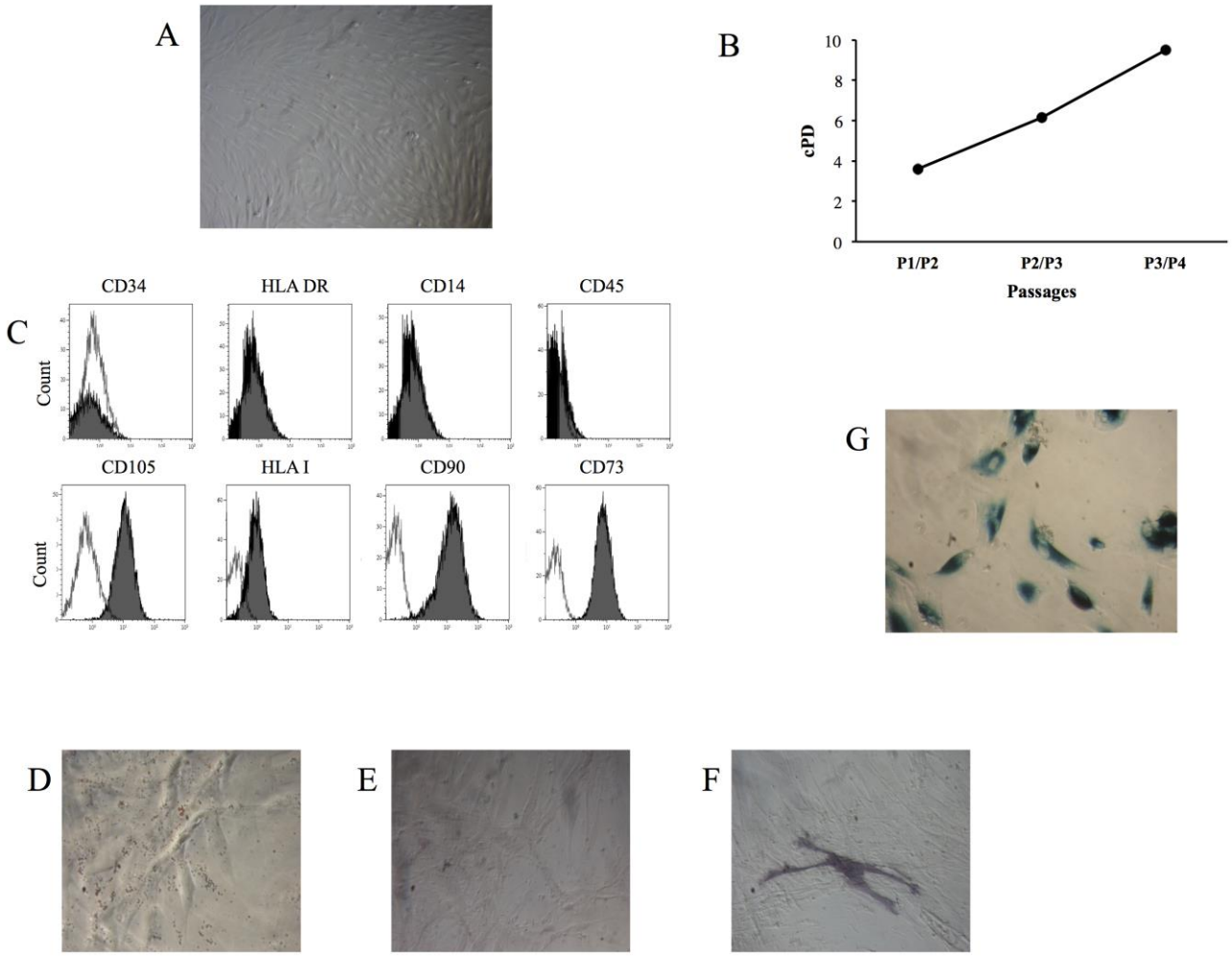

Figure 2. *Characterization of GT derived cells. Panel A: typical "spindle-shape" morphology of in vitro expanded GT-MSCs, Original 10x magnification using an inverted Leica DM-IL microscope equipped with a Nikon Digital Sight DS-Fi1 camera linked to a NIS Element F Imaging system; Panel B: Proliferative capacity (defined as cumulative Population Doubling, cPD) was obtained by tracking cultured MSCs at each passage; Panel C: Representative surface antigen analysis by flow cytometry (y axis=cell numbers (count), x axis= fluorescence intensity). Empty histogram represents isotype-matched control. Grey histogram represents stained cells. Overlapping histograms indicates absence of positive cells. Panel D-F: differentiation into adipocytes and osteoblasts. Poor differentiation capacity is revealed by the presence of small lipid droplet formation (stained with oil red O) and by poor calcium deposition (stained with alizarin red) and poor AP activity upon histological detection; Panel G: Representative evaluation of β-galactosidase activity in GT-MSC at senescence passage (P19). Senescent cells are stained blue.*

Results

Characterization of GT-derived cells

Cells were successfully isolated from GT and propagated *in vitro*. After 7 days culture, it was possible to observe the appearance of 32 colony forming units (CFU-F) in $1{,}5x10^6$ plated cells. Cells were plastic adherent and had the characteristic MSC "spindle-shape" morphology (Figure 2, Panel A) and normal proliferation capacity as demonstrated by cPD (Figure 2, Panel B). Moreover, they showed positive expression of CD73, CD90, CD105 and HLA-I and negative expression of CD34, CD14, CD45, and HLA-DR (Figure 2 C), the cells were termed GT-MSCs.

MSC isolated from GT poorly differentiated into adipocytes; this was confirmed by the presence of small lipid droplet formation (Figure 2D). MSC isolated from GT also poorly differentiated into osteoblasts, as confirmed by poor calcium deposition upon histological detection (Figure 2, Panel E); poor AP activity was noted as well (Figure 2, Panel F).

GT-MSCs were expanded up to P19, when they entered into senescence, as confirmed by typical senescence-associated β-Galactosidase staining (Figure 2, Panel G).

Anti fibrotic effect of GT-MSC

Fibroblasts cultured in the presence of GT-MSCs showed suppressed proliferation, (based on trypan blue viability score) compared with control fibroblasts both with or without inflammatory stimulation ($p<0.001$), while BM-MSCs did not induce this suppressive effect ($p=0.19$ and $p=0.10$, respectively) (Figure 3, Panel A).

TGF-β1 quantification

The quantification of TGF-β1 levels in FGF activated fibroblast co-culture supernatants showed that in the presence of GT-MSC, TGF-β1 levels were superimposable with that quantified in control wells (1460 pg/ml and 1477 pg/ml, respectively), while in the presence of BM-MSC, TGF-β1 levels were slightly increased (1651 pg/ml) compared to controls ($p=0.31$) (Figure 3, Panel B).

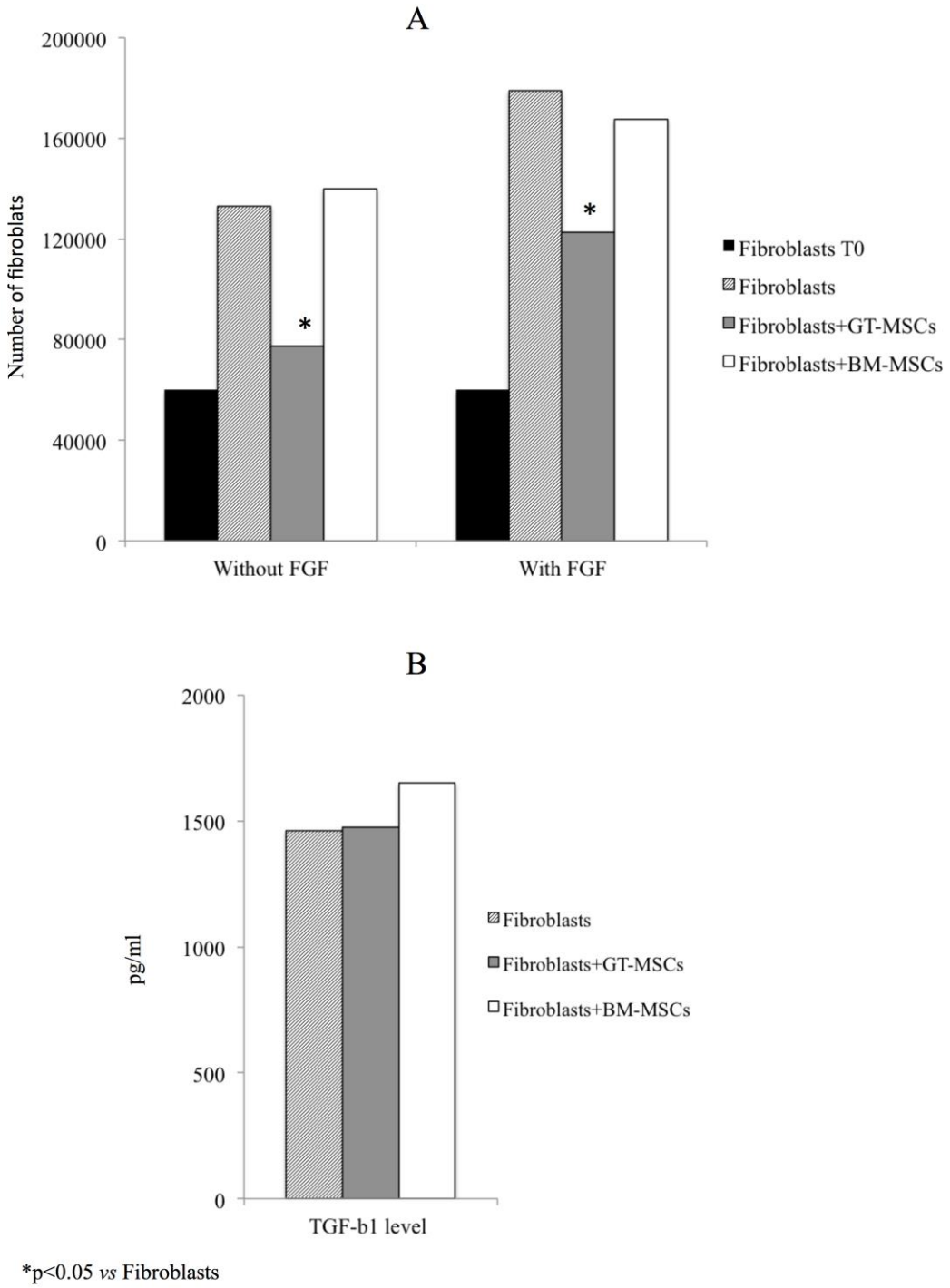

Figure 3. *Anti fibrotic effect of GT-MSC and quantification of TGF-β1.* ***Panel A:*** *Number of fibroblasts alone (lined boxes) or in the presence of GT-MSCs (grey boxes) or BM-MSC (white boxes) after 4 days incubation. Black boxes represent the number of fibroblasts plated at time 0. Proliferation is reported for fibroblasts activated or not with FGF. Viable cells were counted by Trypan Blue 0,2%.* ***Panel B:*** *TGF-β1 levels quantified in supernatants of fibroblasts activated with FGF under different culture conditions (Fibroblasts alone, Fibroblast+GT-MSC, Fibroblast+BM-MSC).*

Discussion

In this brief report we demonstrated that MSCs can be *in vitro* isolated and expanded from GT tissue of burn wounds. Cells derived from GT presented the typical MSC morphology and phenotype with regular proliferative capacity. GT-MSCs also exhibited antifibrotic features, for this reason they are an attractive alternative cell source with potential in burn wound healing and tissue repair.

The treatment of burns is a delicate art, especially in children and these injuries remain a major challenge worldwide. Nearly a fourth of all burn injuries occur in children under the age of 16, of whom the majority are under 5 years of age[1]. Scar tissue and skin grafts in children are subject to exagerated inflammatory responses, which may create a hypertrophic scar with an unsightly appearance and risk of impaired mobility[2-4]. To improve the quality of the scar, and diminish functional problems and disfigurement, new cell sources have been exploited for skin tissue regeneration.

MSCs have also been proposed as therapy for lost tissues, including the skin, due to their potential to differentiate into multiple cell types and replace the missing tissue[5, 9-12] . Although MSCs from bone marrow have been successfully used for therapeutic purposes and often designated as the gold standard, the accessibility and availability of these cells is limited, preventing their wider application in clinical situations. Therefore, more accessible stem cell sources have been proposed, such as peripheral blood, adult tissue such as adipose, teeth, bone, muscle, neonatal birth-associated tissues including placenta, cord blood and skin derivates[16].

To the best of our knowledge, we showed for the first time, that MSCs could also be isolated from debrided burn GT. Wound healing is a complex process that requires the interaction and coordination of a cascade of cellular responses to injury and includes three overlapping stages: inflammatory phase, fibroproliferative phase, and remodeling phase[20]. During the fibroproliferative stage, dermal fibroblasts from the wound margin proliferate and migrate into the wound, generate GT, and begin to remodel the wound matrix to create new dermal tissue. GT begins to be formed approximately four days after lesion formation, and is characterized by a high density of fibroblasts, granulocytes, macrophages and microcapillaries[20]. GT is often discarded during surgical burn treatment and thus represents a readily available and accessible source.

The first step in skin repair is wound closure, the next step is management of scar formation[20]. At this point, the skin has the capacity to stimulate the production of fibroblasts in very high quantities, especially in children, with the risk of creating a hypertrophic scar and consequent retraction or impaired articulation[1-4]. There is increasing evidence that MSCs are trophic mediators of tissue repair; these cells promote scar-free wound healing and inhibit fibrotic tissue formation[21, 22]. Paracrine effect is considered one of the main underlying mechanisms behind the therapeutic effects of stem cells[11, 13, 23]. In particular, the exosome, an essential paracrine factor for intercellular communication, plays a dominant role in cell-to-cell communication and modulates the molecular activities of recipient cells[9, 11-13, 23, 24]. Our results confirm the anti-fibrotic properties of MSC, in particular GT-MSCs. These features were demonstrated by *in vitro* suppression of fibroblast proliferation and inhibition of a the potent driver of tissue fibrosis, TGF-β1[25, 26,27]. Moreover, we confirmed their paracrine method of action since results were obtained in co-culture experiments using transwell plates. Another interesting observation was the difference found between GT-MSC and BM-MSC in terms of antifibrotic activity; the former were more efficacious.

We also observed that adipogenic and osteogenic differentiation, hallmarks of MSCs, although detectable in all experiments, were less efficient in GT-MSCs compared with BM-MSCs, as demonstrated by microscopic examination. Indeed, contrasting data are available in the literature regarding the differentiation capacity of MSCs obtained from different sources[28-30].

In conclusion, we reported on the successful isolation of MSCs from GT and their *in vitro* expansion. Although further studies are needed to confirm these observations, we speculate that GT-MSCs may be “committed” by the wound microenvironment and as such would be a logical candidate to improve burn wound management and skin repair, particularly in the pediatric setting.

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Abbreviations

AP	Alkaline phosphatase
CFU-F	Colony forming units
FITC	Fluorescein isothiocyanate
GT	Granulation tissue
MSCs	Mesenchymal stromal cells
PD	Population doubling
PE	Phycoerythrin
SA-β-gal	β-galactosidase
αMEM	α-minimum essential medium

Potential Conflicts of Interests

None

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