CROSSTALK BETWEEN UMBILICAL CORD WHARTON'S JELLY-DERIVED-MESENCHYMAL STEM CELLS AND HUMAN SKIN FIBROBLASTS:

IMPLICATIONS IN WOUND HEALING, FIBROSIS, ANTI-AGING AND BURNS.

by

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DOCTORAL DISSERTATION- SURGERY DEPARTMENT- FACULTY OF MEDICINE

Supervisors: Dr Armengol Carrasco M, Dr Barret Nerin JP, Dr Jeschke MG.

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1. ABSTRACT:

INTRODUCTION: Mesenchymal stem cells (MSCs) have emerged as a promising wound healing, anti-fibrotic and anti-aging regenerative medicine therapy. Paracrine signalling is considered one of the main underlying mechanisms of MSC action. Umbilical cord Wharton's Jelly derived-mesenchymal stem cells (WJ-MSCs) arise as a particularly efficient and advantageous mesenchymal stem cell source. However, to date there are no reports regarding the effects of human WJ-MSCs either on human keloid, burned or normal skin fibroblasts.

This study aims to investigate the effect of WJ-MSCs on the different aforementioned types of human skin fibroblasts and determine the mechanism of action, through varied culture conditions (WJ-MSC conditioned media, transwell cultures and direct co-cultures) and characterizing fibroblast responses at the genetic expression level. Moreover, this study will examine the translational *in vivo* application of WJ-MSC paracrine signalling in a mouse normal wound healing model.

MATERIALS AND METHODS: Human umbilical cords and skin samples were obtained, and WJ-MSCs and human keloid, burned and normal skin fibroblasts were isolated and cultured. Three types of co-culture systems were investigated to analyze WJ-MSC paracrine and direct cell-cell contact signalling effects. An *in vivo* preclinical translational mouse wound healing model was investigated.

RESULTS: Plasminogen activator inhibitor- I (*PAI-1*), hypoxia inducible factor-1-α (*HIF-1-α*) and transforming growth factor- β2 *(TGF-β2*) transcripts were upregulated in keloid fibroblasts cultured with WJ-MSC-conditioned medium (WJ-MSC-CM) and co-cultured with an insert transwell system, while showing lower *TGF-β3* gene expression and higher fibroblast growth factor-2 (*FGF-2*) and vascular endothelial growth factor (*VEGF*) in the WJ-MSC-CM-treated group ($p \leq 0.05$ and $p \leq 0.01$). In this latter culture system, IL-6 and IL-8 protein expression was also enhanced ($p \leq 0.05$).

Opposite results were shown in keloid fibroblasts co-cultured directly with WJ-MSCs. The WJ-MSC-CM-treated keloid fibroblasts showed higher proliferation rates than their control keloid fibroblasts with no change in apoptosis rate or migration ability.

In the burned skin fibroblast studies, WJ-MSC-CM had no effect on fibroblast genetic expression and therefore no modification in the transcript expression of *TGF-β2, PAI-1, HIF-1-α,* and *VEGF* was found, when culturing burned fibroblasts with WJ-MSC-CM. When co-culturing them through an insert (two-way paracrine signalling), *PAI-1* mRNA levels were upregulated ($p \leq 0.05$).

On the contrary, the direct application of WJ-MSCs in burned fibroblasts downregulated *TGF-β1* and *CTGF (Connective Tissue Growth Factor)* ($p \leq 0.01$), and lowered the signal of the other unaltered aforementioned gene transcripts. WJ-MSC-CM enhanced human burned skin fibroblast proliferation, migration and wound closure *in vitro* ($p \leq 0.05$ and $p \leq 0.001$, respectively).

As for human WJ-MSC-CM-treated normal skin fibroblasts, expression of re-epithelialization (*TGF-β2*), neovascularization/anti-aging (*HIF-1-α*) and fibroproliferation (*PAI-1*) promoting genes was enhanced ($p \leq 0.05$). Other wound healing and anti-aging-related transcripts were not significantly affected, although they also showed an upregulated trend (*FGF-2, CTGF, VEGF, collagen I*, *collagen III* and *SIRT-1*).

WJ-MSC-CM enhanced normal skin fibroblast proliferation ($p \leq 0.001$) and migration ($p \leq 0.05$) *in vitro*, and accelerated wound healing *in vivo* in a full-thickness excisional murine model.

DISCUSSION AND CONCLUSIONS: WJ-MSCs may play a paradoxical role in keloid management. Under our culture conditions, the indirect application of WJ-MSCs on keloid fibroblasts (via conditioned-media or transwell inserts) enhanced their pro-fibrotic phenotype, while the direct cell-cell contact interaction resulted in opposite effects. Human WJ-MSC paracrine signalling may enhance fibrosis and keloid phenotype, while WJ-MSCs might directly help to treat keloid scars.

Human WJ-MSCs may appear to promote burn wound repair by paracrine signalling, but the direct cell-cell contact effect of WJ-MSCs on human burned skin fibroblasts may downregulate fibroproliferation and avoid burn excessive scarring.

The WJ-MSC secretome might elicit an anti-aging phenotype.

Under our culture and experimental conditions, WJ-MSCs enhanced normal skin wound healing *in vitro* as well as in an *in vivo* mouse model through paracrine signalling.

Together, these results suggest that human WJ-MSCs hold promise and might become a new technology for further preclinical and clinical studies to enhance burn and wound healing in general, with anti-aging and anti-fibrotic potential applications as well.

If the reported immunoprivileged character and safety of WJ-MSCs in experimental and first clinical models is further scientifically proven, WJ-MSCs might represent a feasible, universal and off-the shelf technology to enhance wound healing to improve patient survival and quality of life.

2. INTRODUCTION:

2. 1. LITERATURE REVIEW:

Wound healing because of burn or other injuries, scarring and aging are intimately related [1]. Together, they represent a clinical and health care challenge, with predicted growing prevalence and high costs. Wound healing impairment, excessive scarring and age-related-diseases may cause mild to severe morbidity, and even death in the worst case scenario [2, 3].

2. 1. 1. SKIN:

Human skin is one of the most precious materials that plastic surgeons, dermatologists, researchers and other health care providers work with. Skin is the first "letter of recommendation" we offer to others, even without any prior introduction or any social intention, as opposed to eye contact or smiling. Hence, skin is a sensitive organ that plays an important neurosensitive and psycho-affective role; besides that, it accomplishes many vital functions such as barrier protection, immune function, thermoregulation, and repair [4].

Anatomically, skin is divided into two parts or layers: epidermis and dermis, which lie over the hypodermis or subcutaneous fat tissue [5] (Figure 1). The basement membrane or epidermal-dermal junction binds the epidermis and dermis [6].

The epidermis is the outer skin layer and has a thickness ranging between 0.4-1.5 mm, depending on the anatomical area [4, 7]. This ectoderm-derived layer constitutes an avascular poly-stratified, squamous and keratinized epithelium. It contains four or five different layers or strata depending also on the anatomical region [4]. Keratinocytes, melanocytes, Langerhans cells and Merkel cells are the predominant cells found in the epidermis. Keratinocytes represent the most abundant epidermal cell type [7]. Melanocytes synthesize melanin, the pigment which is transferred to keratinocytes and is responsible for human skin colour, and it is important to note that the colour depends not on the amount but on the activation of melanocytes [8].

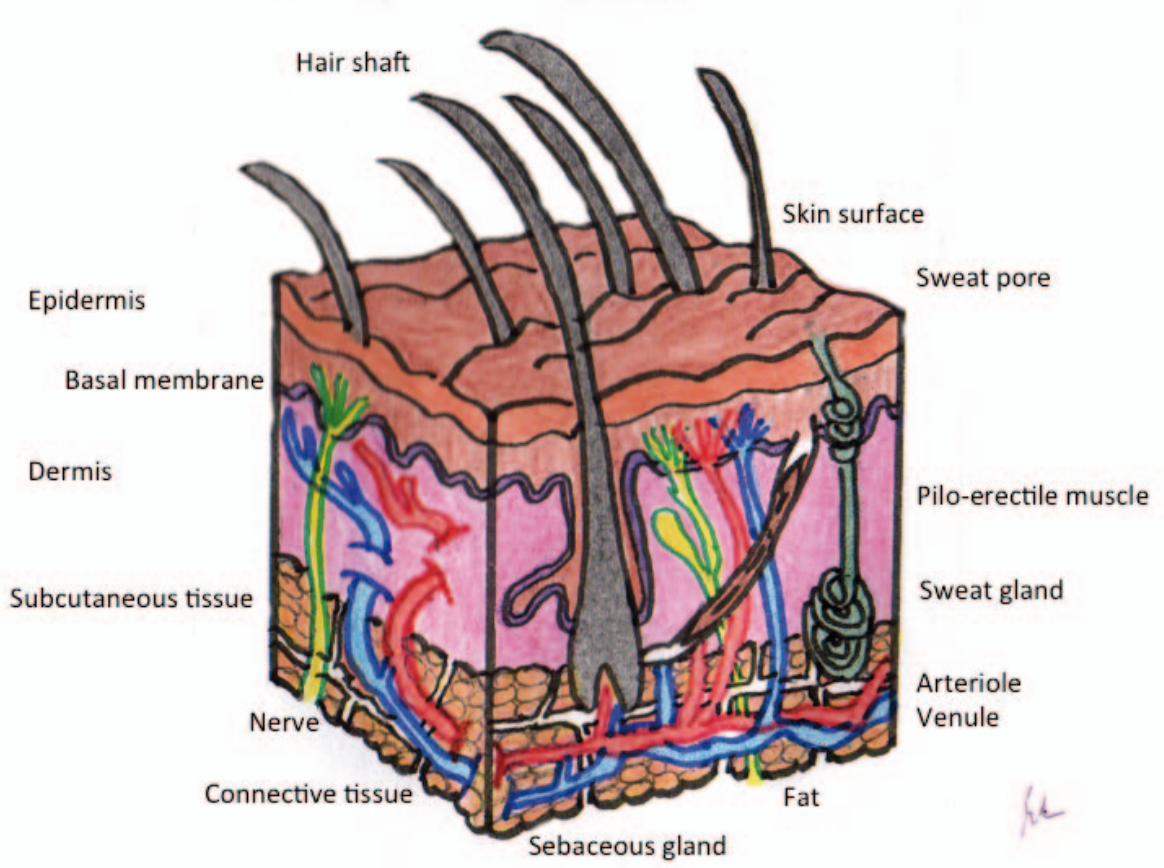

Figure 1: The composition and layers of normal skin.

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In contrast to the epidermis, the dermis originates from the embryologic mesoderm and is a vascular skin layer (Table 1). The dermis is further subdivided into two parts: a) the papillary or superficial dermis, which is loose and highly vascular, and b) the reticular or deep dermis, which is denser and less vascular. This fibroelastic dermal tissue is composed of different fibre types (collagen, elastic and reticular fibres), glycosaminoglycans, cells (fibroblasts, mastocytes and macrophages), vessels, nerves and adnexa (hair follicles, eccrine and apocrine sweat glands, and sebaceous glands) [4, 7]. Fibroblasts represent the most abundant dermal cell type.

SKIN LAYERS	ORIGIN	DESCRIPTION	MAIN CELLS
EPIDERMIS	ectoderm	It is an avascular keratinized stratified epithelium	keratinocytes melanocytes
DERMIS	mesoderm	Contains vessels*, skin appendages and extracellular matrix	fibroblasts adipocytes macrophages

Table 1: Main characteristics and differences between epidermis and dermis skin layers.

* Dermis is subdivided in superficial or papillary dermis, which is highly vascular and lax, and deep or reticular dermis, which is dense and less vascular.

Skin also normally contains stem cells, which are responsible for its continuously renewing properties and which also act as a reservoir of cells to aid in tissue repair following injury [9]. The vast majority of resident skin stem cells are located in the hair follicle bulge [10]. It has been suggested that these multipotent stem cells not only produce skin, but can also produce other cell types, such as nerve and bone cells [11].

The aforementioned description corresponds to normal non-aged human skin. With age, skin changes. Aging skin is characterized by general skin functions deterioration due to morphological dynamics, leading to a more fragile skin [1]. Briefly, there is diminished sensation, decreased vitamin D3 production, reduced sebum secretion and increased dryness. This is associated with an overall decrease in skin cell number, including Langerhans cells, melanocytes, keratinocytes, fibroblasts and macrophages.

Keratinocytes migration from the basal layer to the skin surface is slowed down. Skin stem cells also display functional impairment with age, with reduced mobilization and response to proliferative signals [12]. The dermis becomes less vascularized and there is a loss of mechanical tension in the extracellular matrix (ECM), with fragmented collagen and less matrix components. Together, this results in decreased skin elasticity, major risk of chronic wounds and ischemic ulcers, and delayed wound healing [1, 2].

2. 1. 2. WOUND HEALING:

Human cutaneous wound healing is a complex, multistep physiological process, which eventually aims to repair, but not regenerate, skin. The restoration of skin continuity after injury involves ectodermal and mesodermal repairing processes, including epithelial resurfacing or re-epithelialization, synthesis of connective tissue and biomechanics and wound contraction to reduce the tissue's gap [6]. Wound healing has three phases: Inflammation, proliferation and remodelling [6, 13] (Figure 2). These steps include fibrin clot formation, cell migration, ECM deposition, dermal reconstitution, and re-epithelialization [14]. Platelets, polymorphonuclear neutrophils, macrophages, lymphocytes, fibroblasts and myofibroblasts are the main cell types involved.

Histamine, platelet-activating factor, bradykinin, nitric oxide and prostaglandins [13], platelet-derived growth factor (PDGF) and TGF-β [6, 13, 15] are some of the players that orchestrate collagen synthesis pathways and the wound healing process. The remodelling wound healing phase lasts 6 to 15 months. During this time period, fibroblasts and myofibroblasts cause wound contraction, and vascularity decreases [13]. This last phase represents the maturation of the resulting scar, which is the final product of normal wound healing or repair processes [16].

Scar thickness and formation is correlated with injured skin depth. Lesions involving epidermis and superficial dermis, such as donor sites or superficial partial-thickness burns, heal spontaneously by migration of epithelial cells from the wound edges, and from intact skin appendages. The scarring will be minimal, especially in areas where skin appendages are numerous. Deep partial-thickness or full-thickness burns may heal after 3 weeks or they may not heal at all, and they are associated with a high risk of infection and hypertrophic scarring. Therefore, these deep lesions usually require surgical intervention in order to aid in the natural wound healing process [6, 17].

Scars are less noticeable in aging skin. However, the elderly display delayed wound healing rates (20-60%) [2]. Aging-related wound healing impairment factors include malnutrition, immobilization, psychological stress and dementia, sex hormones decrease, comorbidities such as diabetes, peripheral arterial disease and chronic venous insufficiency, as well as side effects of commonly used medications at old ages (like corticoids, or multitherapies).

The high prevalence rate of comorbidities with aging appears to play the most pivotal role in wound healing alterations. This fact has to be added to the independent age-induced wound healing impairment risk factor that accompanies individuals >60 years old [2].

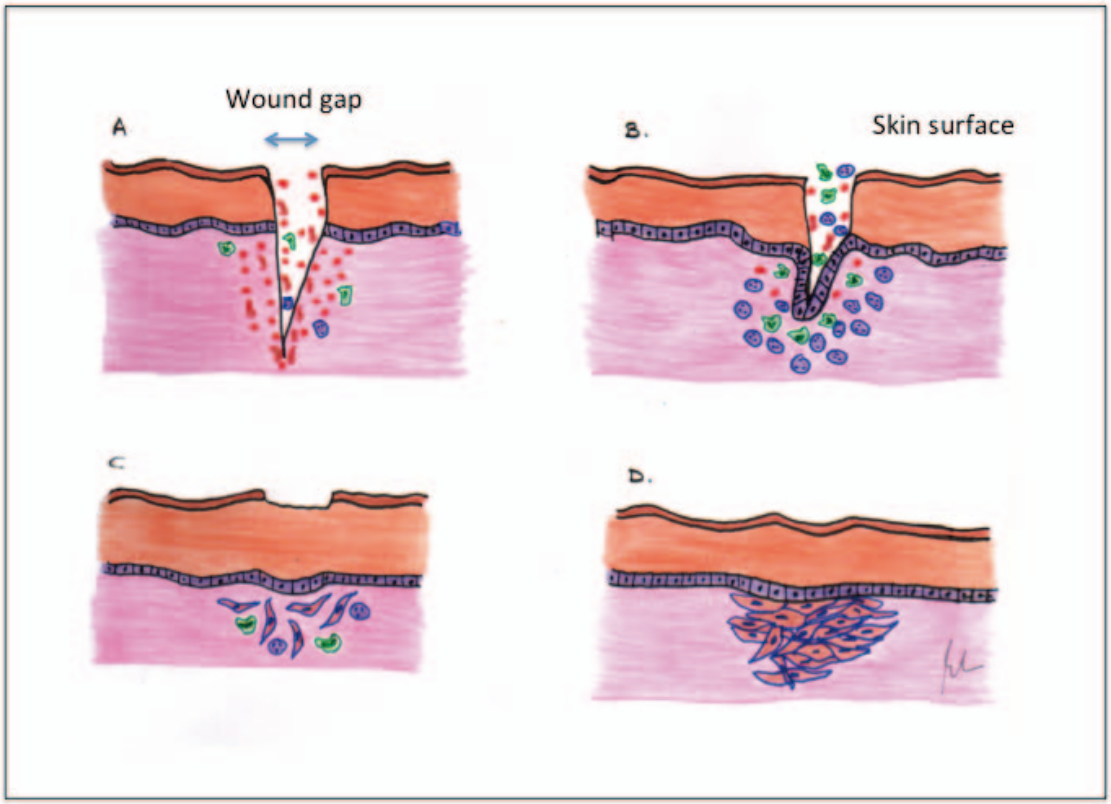

Figure 2: Phases of wound healing.

Wound healing has three phases: Inflammation (A,B), proliferation (C) and remodelling (D). The inflammatory phase begins with hemostasis or fibrin clot formation (A). Platelets (small red dots), red blood cells (bilobular red structures), polymorphonuclear neutrophils (purple rounded structures), macrophages (represented in green colour), fibroblasts and myofibroblasts (fusiform nuclear structures, in brown) are the main cell types involved.

2. 1. 3. STEM CELLS:

2. 1. 3. 1. DEFINITION:

Stem cells (SCs) are defined by two main characteristics: their capacity of prolonged self-renewal (proliferation) and multilineage differentiation (asymmetric replication) [14, 18]. These characteristics are more pronounced in younger sources [19]. By asymmetric replication, after every cell division, one cell retains its self-renewing capacity, while the other (transit-amplifying or TA cell) enters a differentiation pathway and joins a mature non-dividing population [20]. When an unspecialized stem cell differentiates, it assumes characteristics of a specific tissue [21].

SCs are pluri-, multi- or unipotent [22]. The zygote is the only mammalian cell capable of producing all cells and tissues of an organism and thus is considered totipotent [23] . Pluripotent cells produce cells and tissues belonging to all three germ layers: ectoderm, mesoderm and endoderm [24]. Multipotent cells produce more than one cell lineage, within a closely related family of cells. Unipotent cells only differentiate into a single cell phenotype [25].

Plasticity describes the phenomenon whereby SCs from one tissue produce cell types of a completely different tissue [26]. SCs can remain undifferentiated, in which state there is risk of uncontrolled proliferation and tumour formation [19]. SCs have a slow- cycling nature *in vivo*, high proliferative potential and participate in tissue regeneration and repair, during both fetal development and adult wound healing [9].

2. 1. 3. 2. CLASSIFICATION:

When classified by origin, there are two types of stem cells: embryonic (ESC) and non-embryonic stem cells. The latter are also referred to as adult (ASC) or somatic stem cells (SCs derived from the fetal-derived tissues, such as placenta or umbilical cord, are also considered ASCs) [27, 28]. Embryonic germ cells are derived from the primitive gonadal ridges of the developing embryo or fetus (6-9 weeks gestation in humans) and have many of the pluripotent properties of ESCs [29].

2. 1. 3. 2. 1. Embryonic Stem Cells (ESCs):

ESCs are pluripotent stem cells derived from the inner cell mass of an early stage embryo known as a blastocyst [18] that give rise to all cells of the three embryonic germ layers: endoderm, mesoderm and ectoderm. Human Embryonic Stem Cells (hESCs) are derived from excess developing pre-implantation embryos (5 day-old embryos, 4-8 day-old morula, or inner cell mass of blastocysts) that have usually been fertilized *in vitro* at a fertilization clinic [30]. They are considered an immortal epiblast derivative [29]. Derivation of human embryonic cell lines is controversial because it requires destruction of an embryo [19], may develop teratocarcinomas (tumours composed of tissues from all three germ layers [31]), are immunogenic and show genetic instability *in vitro* [23]. Accordingly, adult stem cell research is currently favoured [32-34].

Apart from being used in regenerative medicine, ESCs may be used to perform developmental, genetic (through knock-out technology) and pharmacological research. hESC-based *in vitro* studies of drug toxicity have proven to be an accurate alternative to experimental animal models [35]. Indeed, ESCs are able to undergo unlimited self-renewal in an undifferentiated state [29] *in vitro* using either feeder layers or extracellular stimuli (e.g., cytokines or growth factors) [36].

2. 1. 3. 2. 2. Induced Pluripotent Stem Cells (iPSCs):

Induced pluripotent stem cells (iPSCs) are artificially derived from non-pluripotent cells, typically differentiated adult cells (mostly fibroblasts of murine or human origin), most frequently by epigenetic re-programming and also by nuclear transfer or cell division [37], where expression of transcription factors characteristic for undifferentiated embryonic stem cells is induced (such as OCT4 -also known as POU5F1, being the most important one-, SOX2, c-MYC, KLF4, Lin28 and/or NANOG [18, 23, 34, 38, 39]).

Broadly, we could imagine iPSCs as "artificial ESCs". iPSCs represent stable lines of embryonic-like pluripotent stem cells [40]. They constitute a widely available, non-controversial, non-restricted and practically infinite source of pluripotent cells. Nonetheless, they still share with classic ESCs the critical disadvantage of malignancy transformation [18].

To lower this risk, a multitude of protocols for iPSCs generation have been developed in recent years, spanning across different mouse and human donor populations and varying in the number, identity and delivery of the reprogramming factors [41-44].

iPSCs may be used for establishing *in vitro* disease models, drug or toxicity screening, and basic gene research [37]. In contrast to ESCs, human iPSCs can be derived from the patient to be treated (for autologous cell therapy), reducing the risk of HLA mismatching and immune rejection [45] (although Zhao et al have raised concerns regarding their immunogenicity in syngeneic recipients [46]). Regardless of their flaws, iPSCs show great potential for regenerative medicine. The field of iPSC research is still very young and only through future studies will the true clinical impact of these pluripotent cells be determined [47].

2. 1. 3. 2. 3. Adult Stem Cells (ASCs):

SC clinical studies have increased during the past two decades in almost every field of medicine; including, haemato/immunotherapies [48-51], diabetes mellitus, chronic degenerative illnesses (e.g., in the field of rheumatology) [52-55], cardiovascular diseases [56], multiple sclerosis and other neuropathies [57], corneal repair [58] and wound healing [59].

ASCs were discovered in the mid-1950s; they are present in low abundance in almost all adult tissues and in high abundance in the umbilical cord [18].

They are found in special regulatory niches as self-renewing progenitor cells that are able to produce one or more specialized cell types. ASCs are usually considered to be tissue specific, self-renewing populations of cells, which can differentiate into cell types associated with the organ system in which they reside [60].

Slowly replicating and bromodeoxyuridine-label-retaining, ASCs are under strict regulatory control of their mobilization and differentiation [29]. ASCs are less potent (usually only uni- or multipotent) and have lower differentiation potential than ESCs. Distinct from ESCs, ASCs are not capable of unlimited expansion *in vitro*. The potency and plasticity of ASCs is still in contention, though [19, 61, 62].

ASCs include mesenchymal stem cells (MSCs), hematopoietic stem cells (HSCs), epithelial and neural stem cells, among others. HSCs and MSCs originate in the bone marrow (among other tissues; MSCs may have additional origins, as we will describe later) and differentiate into endothelium, liver, bone, muscle, skin and others [19] .

2. 1. 3. 2. 3. 1. Mesenchymal Stem Cells:

Ideally, stem cells for regenerative medicine should be abundantly available, accessible by a minimally invasive procedure and then safely and effectively transplanted to either an autologous or allogeneic host [34].

As previously mentioned, tumorigenicity and ethical considerations have impeded the widespread clinical use of ESCs [19]. Instead, most regenerative medicine research is focused on iPSCs, ASCs and in particular adult mesenchymal stem cells (MSCs) [62, 63].

Mesenchymal stem cells (MSCs) describe a population of multipotent adult cells capable of differentiating into a variety of mesenchymal progeny. They are also known as "multipotent stromal cells". Human mesenchymal stem cells (hMSCs) are characterized by three criteria: (1) plastic-adherent under standard culture conditions; (2) capacity to differentiate into at least three mesenchymal lineages: bone, fat and cartilage; (3) express CD73, CD90, CD105 and lack the expression of CD11b, CD14, CD34, CD45 and HLA-DR cell surface markers [30, 47, 63-65].

MSCs are derived mainly from bone marrow, blood and adipose tissue [66, 67], the most studied MSCs sources, and to a lesser extent, placenta [68], amniotic fluid [62], umbilical cord [69] (Figure 3), dental pulp [70], tendon [71], trabecular bone [72] and synovia [73], among others. Actually, MSCs may reside in all post-natal tissues. Many researchers consider that bone marrow (where they were first identified) and adipose tissue constitute the main sources of MSCs for cell therapy, due to high expansion potential and reproducible isolation protocols [74]. Nonetheless, the umbilical cord is gaining popularity.

Cord blood-derived CD34+ hematopoietic stem cells are indeed the most widely studied and they represent the first FDA (U.S. Food and Drug Administration) clinically approved source for stem cell therapy [20, 74].

MSCs release various cytokines and growth factors that influence the microenvironment by either modulating the host immune response or stimulating resident cells [75]. Mediators involved in MSC-mediated immunomodulation include interferon-γ, toll like receptors, tumor necrosis factor-α, IL-1α, IL-1β, indoleamine 2,3-dioxygenase, leukemia inhibitory factor, HLA-G5, IL-10, TGF-β1, hepatocyte growth factor, heme oxygenase1, IL6, IL-1 receptor antagonist (IL-1RA) and prostaglandin E_2 [74, 76-81]. MSCs also stimulate the proliferation of other progenitor cell populations within target organs to promote endogenous repair [78].

MSCs have a great potential in tissue engineering and may serve to treat chronic inflammatory and degenerative disorders due to their immunosuppressive properties [76, 82]. They are currently being tested in several clinical trials for osteoarthritis, osteogenesis imperfecta, articular cartilage defects, osteonecrosis and bone fracture [83, 84], just to name a few.

Regarding wound healing, BM-MSCs have received the most attention as a therapeutic tool, based on their role in native tissue repair and remodelling; however, their isolation is invasive and there is a significant decrease in available BM-MSCs with donor age [85].

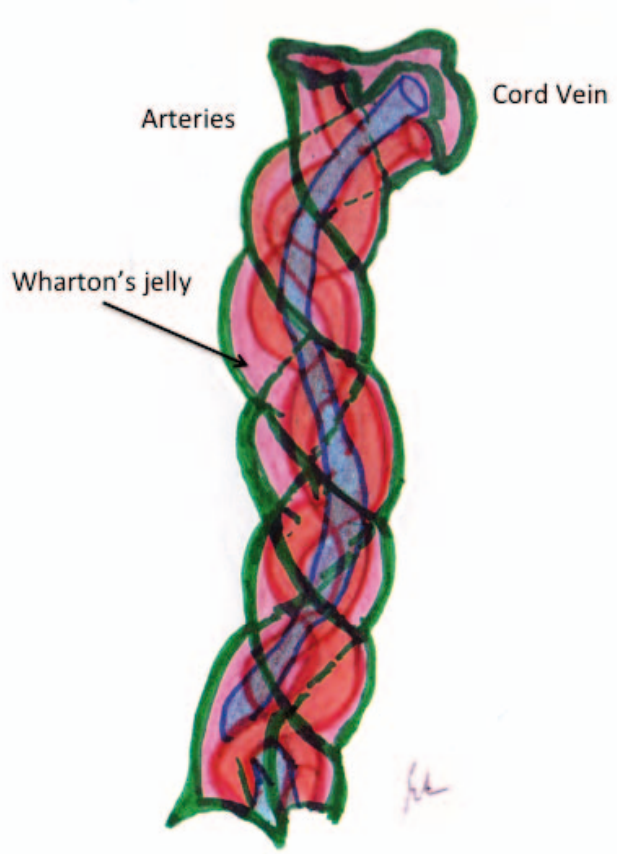

Figure 3: The composition of the umbilical cord.

Schematic representation of the umbilical cord, which contains 2 arteries and one vein. The outer cord layer gives rise to the cord lining epithelial stem cells, whereas the Wharton's jelly is a gelatinous inner part from which mesenchymal stem cells are derived. Cord blood-derived stem cells are isolated from the cord venous blood. Image: © Copyright by Ana Isabel Arnó Clúa 2013.

As an alternative to BM-MSCs, phenotypically similar multipotent cells have also been isolated from amniotic and umbilical cord tissues [86-88]. Since these tissues are usually considered biological waste following parturition, they are considered a valuable untapped source of MSCs.

Moreover, umbilical cord-derived MSCs have been reported to have higher proliferation rates and a greater capacity for expansion in vitro than BM-MSCs, further supporting their potential for therapeutic use [89].

MSCs are considered ‘immunoprivileged’ by many researchers and may permit allo-transplantation without immunosuppressive therapy, which would become particularly useful in treating acute injuries [90, 91]. Having said that, animal studies have shown that intramyocardial injection of MSCs may differentiate into encapsulated structures with calcifications and ossifications, raising the possibility of malignant transformation [78]. However, MSCs may also display anticancer properties. Further scientific evidence for specific MSCs types is needed [92, 93]. Besides biosafety and legislation, other challenges facing MSCs therapies include delivery of stem cells with scaffolds, films or wound dressings [30]. As with iPSCs, only future preclinical and clinical research will unravel the potential of this new regenerative medicine technology.

2. 1. 4. SCARS: the keloid clinical challenge.

Keloids and hypertrophic scars occur anywhere from 30-90% of patients, and are characterized by pathologically excessive dermal fibrosis and aberrant wound healing. Patients with keloids or hypertrophic scars may suffer a severe impairment of quality of life, by causing both physical, psychological and social sequelae [94]. Even normal visible scars may represent an important stigma [95]. Burns represent the clinical paradigm of excessive skin scarring. Hypertrophic scars after burn injury have a prevalence of approximately 65%. They constitute the main complication in burn survivors [96]. Excessive scars after burn not only affect physical cosmesis, causing mild to severe disfigurement, but also produce limitations on range of motion and other possible dysfunctions, such as ectropion with corneal exposure and dry eye risk, issues with deglution because of microstomia, growth abnormalities, psychological trauma, pain and anxiety, social disturbances, and generalized and long-lasting impairment of daily life activities [97].

Despite the high morbidity and possible severe sequelae of excessive skin scarring, no efficacious treatment is available to date. Cutaneous scar management has relied heavily on the experience of practitioners rather than on the results of large-scale randomized, controlled trials and evidence-based techniques [98]. Intralesional corticotherapy, silicone gel sheeting and pressure garments are the most widely used, current scar-management strategies after burn.

Other popular classical treatment and preventive options for keloid and hypertrophic scars include massage therapy, adhesive tape support, cryotherapy and radiotherapy, among others [98, 99].

Due to the still unfavorable results achieved with these classical treatments, the focus of contemporary research is directed at studying the biomolecular pathophysiology of keloids and hypertrophic scars in hopes of developing more efficacious therapies. Common research efforts are focusing on botulinum toxin, antineoplastic drugs (of which some of them are related to TGF-β pathways) and lasers, yet, another novel medical armamentarium –stem cell therapy- has not been properly studied in the context of scarring [99, 100]. Accordingly, there is a lack of scientific evidence in this field and much controversy surrounding the topic.

Whereas mesenchymal stem cells (MSC) may emerge as an antifibrotic and immunosuppressive therapy [63], with the first clinical trials showing great promises in treating several kinds of soft-tissue fibrosis [101], and epidermal stem cells appear to be partially lacking in scar tissue [102], keloid-derived stem cells have also been described [103], though their function is largely unknown. In this regard, some scientists hypothesize that hypertrophic scars, and specially keloids, are abnormally highly-proliferative lesions because they may actually be benign, stem cell-derived tumors; therefore, stem cells may be the trigger of these invasive, but benign, fibroproliferative disorders [104, 105].

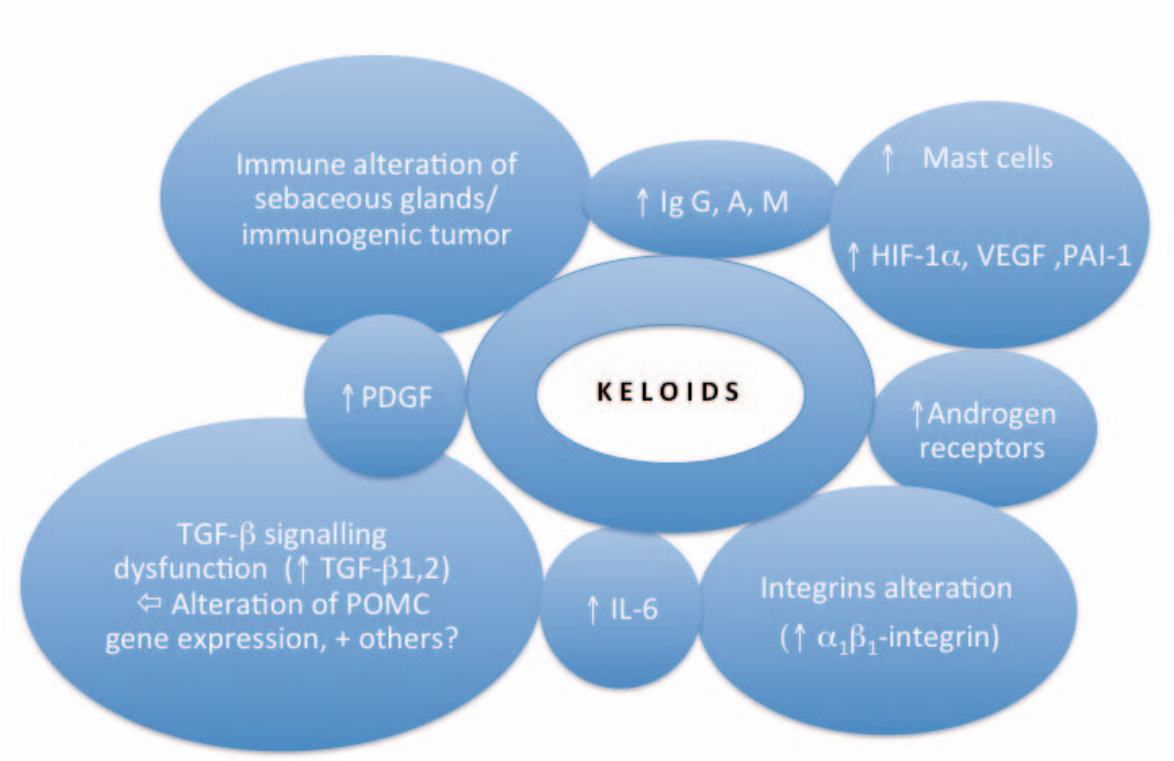

Table 2: Pathophysiology of keloids.

POMC = Proopiomelanocortin

It is important to note that the formation of a scar is the normal physiologic response to wounding in adults. However, an alteration of ECM metabolism -an imbalance between its destruction and deposition-, may lead to excessive scarring [106]. A prolonged or excessive inflammatory phase is believed to be the onset of excessive scarring, with hypertrophic scars and keloids as minor and major clinical signs (Tables 2, 3). Both entities have different clinical and histochemical characteristics, and unfortunately still represent a great challenge for clinicians due to lack of efficacious treatments. More detailed description about both types of excessive scarring is summarized in Tables 4 and 5.

Further reviews about keloid management and the role of TGF-β and other novel molecular strategies for wound healing applications can be found in manuscripts [99, 100] submitted to the *Burns* journal by the author; [99] is currently in the peer review process and [100] has just been accepted.

↑ Inflammation (↑ Th2 cells, ↑ IL-4, ↑ IL-5, ↑ IL-6, ↑ IL-13, ↑ IL-21
↓ IFN-γ, ↓ IL-12)

↑ ECM production (↑ myofibroblasts) and altered ECM remodelling

(↓ decorin, ↑ expression of both collagen types I and III, cutaneous profibrotic pathological crosslink of collagen – pyridinoline type with ↑LH2b-, ↓ collagen degradation ,↓ MMP and ↑ TIMP)

↑ neovascularization

↑ time of re-epithelialization

↓ apoptosis

Altered hemostasis (↑ chronic fibronectin deposition, ↑ PAI-1)

Platelets
Macrophages
T-lymphocytes
Mast cells
↑ Langerhans cells
Keratinocytes (↑ time of activation)

Activation of myofibroblasts

Table 3: **Etiopathogenesis of hypertrophic scars** [72].

KELOIDS	HYPERTROPHIC SCARS
Grow beyond the borders of the original wound	Remain confined to borders of the original wound
Pruritic and extremely painful	Less pruritic and rarely painful
Predominant anatomical sites (earlobes, chest, shoulders, upper back, posterior neck, cheeks, knees)	No predominant anatomical sites (but commonly occur on extensor surfaces of joints or when skin creases at a right angle)
Posttraumatic or spontaneous	Only posttraumatic
Not associated with contractures	Associated with contractures
Do not regress spontaneously	Regress spontaneously
Do not improve with time; there is continuous growth.	Improve with time (regress or stabilize)
Develop later:	Develop sooner:
Appear at 3 months or later after initial scar, then gradually proliferate indefinitely	Generally appear within 1 month, grow for 6 months, then regress often within 1 year
More common in darker skin types	Less association with skin pigmentation
Genetic predisposition (autosomal dominance inheritance susceptibility loci on chromosomes 2q23 and 7p11; may also be recessive)	Less genetic predisposition
Thick collagen fibres	Fine collagen fibres
Absence of myofibroblasts and α-SMA	Presence of myofibroblasts and α-SMA
Collagen type I > III	Collagen type III > I
COX-2 overexpression	COX-1 overexpression
High recurrence rates following excision (although often recur late, 6 months up to 2 years after surgery). If excised, combined treatment needed (corticosteroids better than radiation)	Low recurrence rates following excision
Rare incidence	Frequent incidence

Table 4: Differential diagnosis between keloids and hypertrophic scars.

Abbreviations: α-SMA = α-Smooth Muscle Actin; COX = Cyclooxygenase.

* Classical studies show that keloid scars are characterized by large, thick, wavy, randomly-oriented and closely or loosely packed collagen fibres and no collagen bundles, whereas hypertrophic scars present fine, wavy, well-organized and parallel-oriented collagen fibres and bundles. However, recent research proposes that both types of excessive scarring show parallel and separated collagen fibers, in opposed to normal skin (with higher distance between collagen bundles in keloids). Actually, histomorphology of each scar not only differs from patient to patient, but also among scars from the same patient and among areas of the same scar, complicating even more the differential diagnosis [99].

Table 5: Epidemiology of keloids.

2. 2. FOREWORD AND JUSTIFICATION OF THE STUDY:

Skin wound healing impairment underlies many conditions that eventually may compromise patient prognosis, causing mild to severe morbidity or even death in the worst case scenario. Growing aging population and concomitant aging-related diseases, such as diabetes, contribute to the high prevalence rate of chronic non-healing wounds and subsequent elevated health care costs [107]. On the other hand, systemic injuries such as major burns and other trauma may put any patient lives at risk independently of age, because of challenging skin coverage requirements.

Postnatal human wound healing repairs skin via scar formation, which is a physiological form of skin fibrosis. However, if any scar grows too much, it might become a hypertrophic scar or even a keloid, which represent less and more severe forms of excessive scarring or pathological fibrosis, respectively [106].

Nowadays, many people are concerned about healthy living in order to achieve an appropriate and long-term quality of life. Regular exercise practice, adequate nutrition, ultraviolet (UV) protection, chronic stress prevention and optimism are all well-known health, well-being, and anti-aging promoting factors [108-112].

Further prevention, education, and especially individual action to maintain a healthy lifestyle has led to an increasing demand in cosmetic and anti-aging strategies to look younger and prettier for a longer time even in non-pathological conditions.

This search for eternal health and "youth-like" state underlies behind the recent explosive business of new medical drugs and products to not treat, but prevent physiological conditions which may bring harmful consequences as time progresses [113].

Blood might resemble the light of a candle that expires with age. Not only aging, but also wound healing, fibrosis, and burns are all linked with angiogenesis and neovascularization pathways. Plastic surgery reconstruction is extremely compromised by lack of vascularization [114]. A newer therapeutic armamentarium provided by tissue engineering technologies emerges as a promising and revolutionary field to enhance regenerative medicine, but it may also be challenged by physiological vascularization limitations [115, 116]. There is a growing body of evidence to suggest that MSCs in general arise from vascular pericytes and this fact may explain why they stimulate angiogenesis and provide a microvascular network necessary to promote wound closure [117]. Indeed, MSCs emerge as a wound healing promoting and anti-fibrotic therapy, although this latter with controversy [117, 118]. Increasing scientific evidence points out that paracrine signalling is the responsible mechanism underlying those effects [101]. However, a promising, universal and advantageous stem cell type, the umbilical cord-derived-WJ-MSC, has yet not been studied in human skin.

Therefore, this research project aims to study a preliminary but not previously reported application of human WJ-MSCs in the 3 main branches of plastic surgery: reconstruction (focusing on wound healing and fibrosis or keloid scars mainly, and indirectly to ischemia-reperfusion injury or flaps), burns (independently, for their importance and particular characteristics which make them constitute a separate surgical field), and aesthetics (anti-aging, basically).

Briefly, anti-aging and wound healing share similarities. Burn wound healing is a subtype of wound healing, and angiogenesis/neovascularization is one of the wound healing phases. Keloids represent an aberrant wound healing result, which usually remains as a chronic incurable sequel. Together, the aforementioned facts explain why the experiments of this dissertation were mainly dedicated to study keloids and normal wound healing, and why an *in vivo* animal model was used to examine the wound healing effects of WJ-MSC paracrine signalling.

Next, detailed justification under the appropriate subheadings of this work (WJ-MSCs in keloids, WJ-MSCs in burns and wound healing, and WJ-MSCs in anti-aging) is provided.

2. 2. 1. WJ-MSC in keloids:

Scar formation is the physiological response to wound healing in postnatal mammalian skin [119]. To date, there is no treatment to erase scars completely in humans, and they remain as sequel to most skin injuries. The physical and psycho-social discomfort patients suffer varies from mild to severe [120]. Hypertrophic scars and especially keloids are aberrant excessive forms of pathological wounding with an excess of ECM, which appears to be mainly driven by fibroblasts [121]. Keloids are considered to be a complex polygeneic disorder whose progression is influenced by aberrant cell signalling pathways, probably as a result of both genotype and phenotype factors [122]. Keloids and hypertrophic scars represent the main clinical challenge responsible for scar-related cosmetic and functional dysfunctions, and have an incidence and prevalence of 4.5-16% and 30-90% in trauma, burn and other surgical patients respectively [106, 123, 124].

Recent reports suggest that MSCs represent a new anti-fibrotic treatment strategy [117, 125, 126]. They attenuate wound inflammation and reprogram resident cells to favor tissue regeneration and inhibit fibrosis [117]. They influence host cells and regulate the stem cell niche through differentiation and/or paracrine signalling mechanisms [127, 128]. Accumulating evidence suggests that paracrine signalling, the secretion of trophic or immunomodulatory factors or “secretome,” may represent the most pivotal underlying mechanism of MSC effects [101, 128, 129].

It has also been well documented that the MSC secretome is extremely influenced by the MSC microenvironment or stem cell niche, and cell-cell communications [127].

Between the several possible sources of MSCs, umbilical cord-derived WJ-MSCs (Wharton's jelly derived mesenchymal stem cells) appear to offer the best clinical utility because of their unique beneficial characteristics [130]. WJ-MSCs represent a fetal or birth-derived adult, efficient stem cell source with many advantages, such as anti-fibrotic and anti-cancer properties [131], with no reported teratoma formation or rejection in animal models [130]. Their isolation is relatively easy and involves no relevant ethical concerns. They represent a low cost technology and a universal cell source. Furthermore, the advantages of WJ-MSCs also include ever-lasting availability (considering births are still happening by natural or sometimes artificial means, and women donate their umbilical cords for banking or research purposes), high number of cells (more than the umbilical cord blood and bone marrow [132]), and a higher degree of stemness and self-renewal compared to BM-MSCs [133].

Last but not least, WJ-MSCs exhibit high engraftment rates with successful functional outcomes in *in vivo* animal models [130], and multiple potential clinical applications, including skin regeneration [133, 134].

It has been shown that concentrated MSC-CM can modulate wound repair without MSCs ever being present in the wound, which has the advantage of minimal risks compared to any cell therapy [101, 128]. BM-MSC paracrine signalling has been reported to promote skin fibrosis in normal fibroblasts *in vitro*, but the effect of WJ-MSC paracrine signalling has not yet been defined in keloid fibroblasts.

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2. 2. 2. WJ-MSC in burns, and wound healing:

Non-healing or chronic wounds represent an increasingly prevalent and costly public health issue [135]. As a consequence of longer life-spans due to improvements in acute care, the number of patients who suffer diabetes and other chronic aging-related diseases is growing [107, 136]. There is a link with aging-associated diseases and wound healing impairment [1, 137-139]. On the other hand, other wound healing challenges also arise at any age because of accidents, burns and other traumatic injuries. Particularly burns may represent a severe systemic injury where compromised wound healing may be most detrimental and may lead to patient death. Major burns exceeding 60% TBSA require the use of skin substitutes, but despite recent improvements, wound coverage still represents a clinical challenge [140].

MSCs appear to emerge as a promising wound healing therapy [129, 141-143]. Paracrine signalling [144], such as the release of factors that promote angiogenesis [145], immunomodulation [143] and recruitment of endogenous tissue stem/progenitor cells [146], as well as differentiation [145], have been described as possible mechanisms underlying the promoting wound healing effects of MSCs. Among the different available sources of MSCs, the umbilical cord represents a cost-effective, productive, feasible, accepted, and universal source to isolate MSCs [147, 148], which is considered advantageous compared to bone marrow-derived-MSCs and adipose-derived-MSCs for some researchers [147].

Previous studies with umbilical cord Wharton's jelly-derived-MSCs (WJ-MSCs) have demonstrated that they represent a high yield source of young, non-tumorigenic [149, 150] and immunomodulatory [151] cells which may be allotransplanted to regenerate liver [133], heart [152], bone [153], cartilage [154], fat [133], pancreas [155], neural [156], vascular/endothelial [157] and skin components [148, 158]. WJ-MSCs isolated from goats have been demonstrated to accelerate wound closure in animals from the same species, while minimizing granulation tissue and inflammation [159]. Human WJ-MSCs decrease lung [160], kidney [161] and liver [162] fibrosis, and have been shown to be able to differentiate into sweat gland-like cells and may therefore promote skin regeneration [163]. WJ-MSCs secrete pro-angiogenic and wound healing promoting factors, such as TGF-β, VEGF, PDGF, IGF-I, IL-6, IL-8, among others. Indeed, some researchers consider WJ-MSCs to be perivascular precursor cells which may represent a tailored-cell therapy for ischemic disease [164], such as burns and non-healing chronic wounds, but no evidence exists yet. Paracrine effects appear to be responsible for the wound healing promoting effects of WJ-MSCs, at least in mice [165]. However, to date, there are no reports regarding the use of human WJ-MSCs in human skin wounds or burns.

The novelty of this study lies in the use of a promising stem cell type, the WJ-MSC, which has not yet been studied in the context of human skin, and investigating its *in vitro* effect on human skin fibroblasts, as a means to develop a new therapeutic strategy to aid in wound healing.

Parts of this chapter have been or will be submitted for publication.

2. 2. 3. WJ-MSC and anti-aging:

Aging is the rise of mortality with chronological time, and/or the fall of fecundity [166, 167]. Aging has also been defined as the result of the declining force of natural selection with age [168]. Aging represents a chronic inflammatory state with enhanced cell senescence, impaired natural selection force and decreased sirtuin activity, which eventually evolves to tissue structure and function disruption, and possible malignant degeneration or even death.

Recent scientific, medical and pharmacological advances have improved current acute care. Furthermore, easily available mass-media information about health and disease, as well as prevention campaigns from public health and safety authorities, have globally elicited longer human life expectancies. These effects are especially prevalent in the developed world, and together they lead to more lasting and progressive aging, with a rise in the elderly population, and subsequent augmentation in the prevalence of age-related diseases [2].

Most recognized theories on aging include the original genetic ones from the 1950's - the mutation accumulation and the antagonistic pleiotropy-, and others of a phenotype character, like the disposable soma. This latter defends that aging is the result of accumulating damage due to a lack of maintenance, understanding that maintenance efforts consist of investments to preserve tissue function. This evolutionary theory states that the required resources for maintenance are invested in reproduction.

It is believed that aging is caused by the maintenance gap, that is, the result of maintenance requirements minus maintenance effort. Evolution naturally acts to lower the maintenance requirement to prevent aging. However, organism growth (investment that creates or adds functions) paradoxically rises this requirement [166].

Similarly to the paradoxical and antagonistic pleiotropy evolutionary theory of aging, there concomitantly exists a general major population concern to keep a healthy lifestyle to live longer and happier, and to look younger. This search for eternal youth has launched skin rejuvenation anti-aging research and products for a new but growing industry [113].

Skin aging is classified as intrinsic (genetic or chronological), or extrinsic (promoted by the environment, pollutants, tobacco, malnutrition and mainly UV radiation). Intrinsic and extrinsic aging are cumulative processes that occur simultaneously, and over time result in photoaging [169]. This latter process has also been defined as “accelerated chronological aging” or “aging caused by UV radiation”.

Aged skin is more atrophic and laxer, with uneven skin tone and irregular pigmentations and telangiectasias, wrinkling, coarseness, and elastosis. It usually shows a wide array of skin tumors, either benign or malignant.

Current skin anti-aging treatments used in the clinic include retinoic acid, CO_2 laser resurfacing and hyaluronic acid. Besides that, pilot clinical studies have shown that growth factors and cytokines may revert the skin aging process and be used coadjuvantly [169].

Indeed, there is a general consensus that growth factors and cytokines that promote wound healing, and especially ECM remodelling, like TGF-β1, would also serve as anti-aging drugs [169]. These products are usually derived from cultured human neonatal or fetal fibroblasts. Growing evidence shows that some of these factors might be indeed delivered via stem cells. For instance, conditioned medium from ADSCs (ADSC-CM) stimulated both collagen synthesis and migration of dermal fibroblasts, reduced cutaneous wrinkles, and accelerated wound healing in animal models [170]. Another group described that stem cells from a dental origin showed promise in photoaging treatment (stem cells from human exfoliated deciduous teeth or SHEDs) [171]. However, there are no reports regarding WJ-MSC effects on aging or rejuvenation.

Wound healing and aging are inversely correlated: aging is characterized by delayed wound healing. WJ-MSCs are immunomodulatory and obtained from child-bearing women, who physiologically are in a pre-menopausal state and represent a young cellular and niche microenvironment.

Therefore, it may well be possible to hypothesize that WJ-MSCs might promote the release of wound healing cytokines, like TGF-βs, CTGF, HIF-α, VEGF, and FGF-2, promote collagen synthesis, and increase one of the key master reported molecules in counteracting the aging process, like sirtuin-1 (SIRT-1).

2. 3. SPECIFIC AIMS:

2. 3. 1. Skin fibrosis (keloid scars):

2. 3. 1. 1. Primary aim: Investigate the effects of human WJ-MSC paracrine signalling on keloid skin fibroblasts *in vitro*.

2. 3. 1. 2. Secondary aim: Compare the effect of paracrine and direct cell-cell contact on keloid fibroblast gene expression.

2. 3. 2. Burn wound healing and burns:

2. 3. 2. 1. Primary aim: Investigate the wound healing effects of human WJ-MSC paracrine signalling on human burned skin fibroblasts.

2. 3. 2. 2. Secondary aim: Compare the effect of paracrine and direct cell-cell contact on human burned skin fibroblast gene expression.

2. 3. 3. Normal wound healing and anti-aging:

2. 3. 3. 1. Primary aim: Analyze the wound healing and anti-aging related effects of human WJ-MSC paracrine signalling on human normal skin fibroblasts *in vitro*.

2. 3. 3. 1. 1. Investigate the application of WJ-MSC-CM in an *in vivo* murine wound healing model.

2. 3. 3. 2. Secondary aim: Compare the effect of paracrine and direct cell-cell contact on human normal skin fibroblast gene expression.

3. MATERIALS AND METHODS:

3. 1. TISSUE SOURCES:

Tissue specimens (Tables 6, 7 and 8) were collected following the Declaration of Helsinki Principles, Toronto Academic Health Sciences Network (TAHSN) and University of Toronto-affiliated Sunnybrook Research Institute and Sunnybrook Health Sciences Centre Institutional Ethics Review Board approval, and patient signed informed consent. For scar samples, clinical diagnosis of keloid versus hypertrophic scar (HTS) was made before surgery based on physicians' assessments.

Patient	Age	Sex	Race	Keloid cause	Keloid location	Comorbidities
1	31	M	Afro-american	Other trauma	Face	None remarkable
2	50	M	Asian	Other trauma	Neck	None remarkable
3	27	M	Asian	Other trauma	Earlobe	None remarkable
4	53	F	Caucasian	Major burn	Neck	Smoker
5	55	M	Caucasian	Major burn	Chest	None remarkable
6	35	F	Afro-american	Severe acne	Face	None remarkable

Table 6: "Keloid skin" patient's epidemiological data.

Patient	Age	Sex	Race	Burn TBSA and thickness	Burn location	Comorbidities
1	45	M	Caucasian	18% full thickness	Head, trunk and limbs	Hyperlipidemia, depression, alcohol and tobacco use
2	57	M	Caucasian	4.5% deep second	Head, R hand	IV drug abuse, alcohol and tobacco use, hyperalgesia, psychosis
3	61	M	Caucasian	23.5%	Trunk and limbs	Cardiovascular

Table 7: "Burned skin" patient's epidemiological data.

Patient	Age	Sex	Race	OR diagnosis	Location	Comorbidities
1	50	F	Caucasian	Breast reconstruction	Abdomen	Breast cancer
2	55	M	Caucasian	Scar correction	Unknown	None remarkable
3	36	F	Afro-american	Hernia repair	Abdomen	None remarkable
4	30	M	Asian	Scar correction	Unknown	Schizophrenia
5	55	F	Caucasian	Breast reconstruction	Abdomen	Breast cancer

Table 8: "Normal skin" patient's epidemiological data.

Abbreviations: M:Male; F: Female.

3. 2. CELL CULTURE:

Primary human fibroblasts were obtained from skin tissue samples of patients undergoing acute burn (for burned skin fibroblasts) or reconstructive surgeries (for keloid and normal skin fibroblasts). In this latter group, reconstructive surgeries were performed several years after trauma, mainly burn, or as dermolipectomies. Surgical procedures were performed at the Department of Plastic Surgery at Sunnybrook Health Sciences Centre, University of Toronto. WJ-MSCs were isolated from umbilical cords obtained from planned C-sections at the Obstetrics and Gynecology Department from the same health centre.

Full-thickness skin was dissected to remove any subcutaneous adipose tissue, and cut in small pieces of 2-4 mm. Human skin fibroblasts were obtained from outgrowth of those explants cultured in small dishes (Figure 4). After trypsinizing, fibroblasts were further subcultured in 75 cm^2 tissue culture flasks at a density of 4,500 cells/cm^2.

WJ-MSCs were isolated from umbilical cords by gentle dissection of previous sectioned small cord pieces, discarding the outer or epithelial layer, according to earlier described methods [172] (Figures 5 and 6).

Explants and cells were further subcultured in petri dishes at a density of 3,200 cells/cm^2 (Figure 7). When fibroblasts and /or WJ-MSCs reached 70% confluency, usually within a week, they were trypsinized with 0.05% trypsin/0.025% EDTA v/v in preparation for subculture.

Tissue culture plasticware were purchased from BD Falcon™ (Bedford, MA), and all tissue culture media and supplements were obtained from Wisent Inc (St-Jean-Baptiste, QC, Canada), unless otherwise stated.

Fibroblast culture media consisted of DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% FBS (fetal bovine serum) and 1% antibiotic-antimycotic solution. WJ-MSC culture media consisted of CMRL (Gibco, Carlsbad, CA), with 10% FBS, 2% antibiotic-antimycotic solution and 1% L-glutamine. Media was changed every 48 hours. Collected tissues and cells were cultured at 37°C in a humidified atmosphere with 5 % CO_2.

Figure 4: Human skin fibroblast isolation.

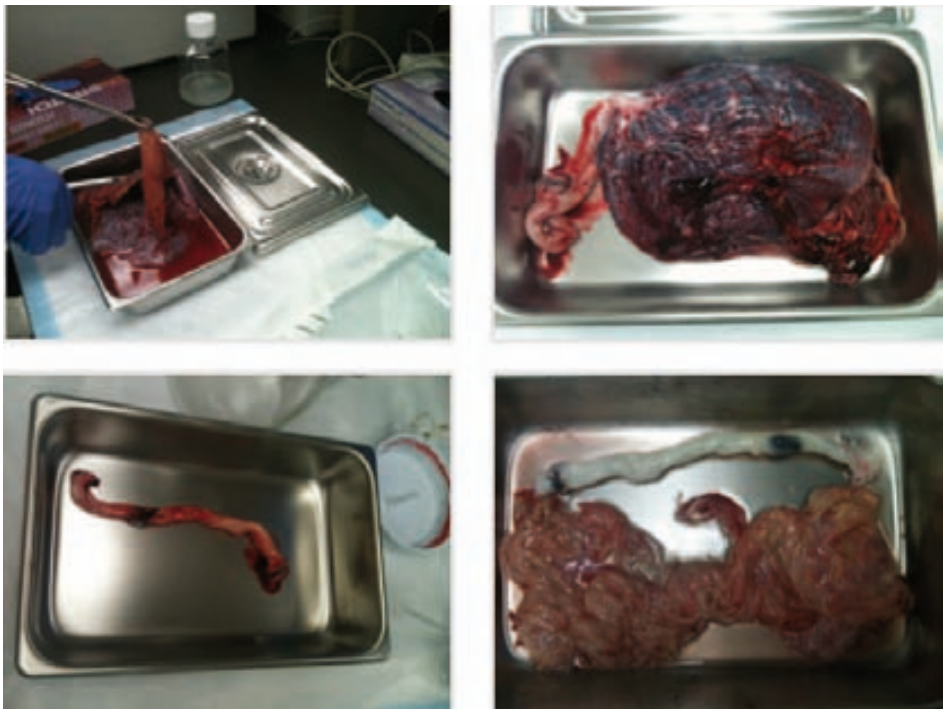

Figure 5: Human umbilical cord dissection.

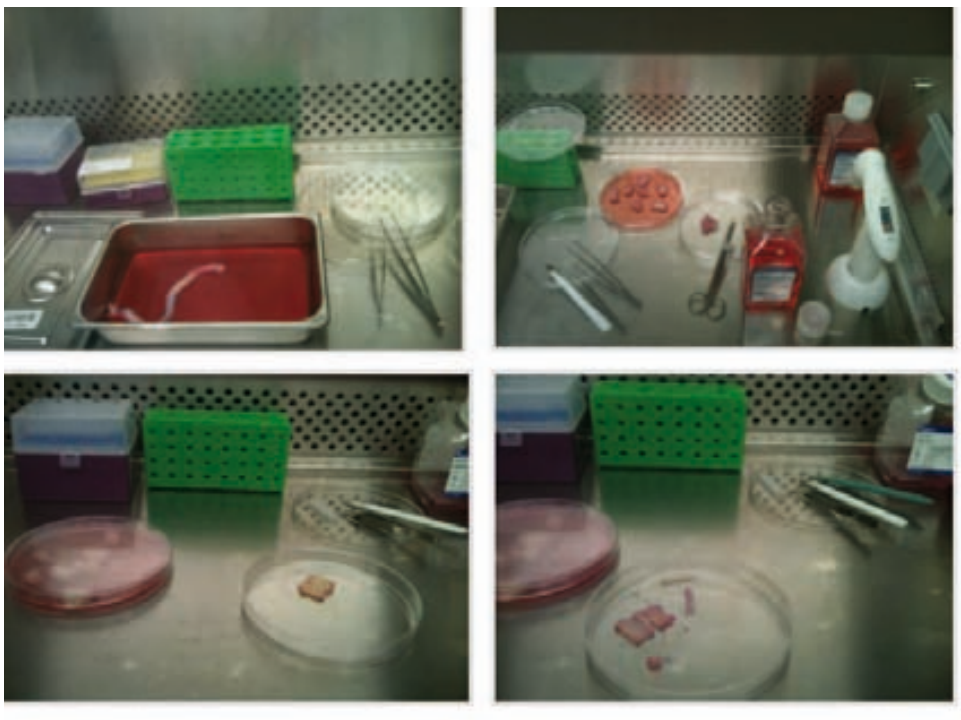

Figure 6: Umbilical cord Wharton's jelly-derived-MSCs isolation.

3. 3. CHARACTERIZATION OF HUMAN WJ-MSCs:

Cells isolated from the Wharton's jelly of the umbilical cord were studied to confirm their MSC characteristics. They were cultured and grown in plastic plates (Figure 7), and flow-cytometry for MSC cell surface markers (CD90+, CD73+, CD105+, CD45-, CD14-, CD19-, CD34-, and HLA-DR-) [30] was performed (Figure 8 A-D). Cells were differentiated into adipogenic, osteogenic and chondrogenic lineages (Figure 8 E-G).

Figure 7: Umbilical cord Wharton's jelly-derived-MSCs attached to plastic surfaces.

For adipogenic differentiation, cells were seeded at a density of 3,000 cells/cm^2 in 24-well plates (BD) with low-glucose DMEM medium supplemented with 10% FBS, 1% of antibiotic-antimycotic solution, 1mM 3-isobutyl-1-methylxanthine (Sigma-Aldrich, Saint Louis, MO), 10 µg/ml insulin (SAFC, Saint Louis, MO), 60 µM indomethacin (Sigma-Aldrich, Saint Louis, MO), and 1µM dexamethasone (Sigma-Aldrich, Saint Louis, MO). Cultures of cells in low-glucose DMEM medium supplemented with 10% FBS served as a negative control. Lipid accumulation was identified by oil red O staining (0.3 g of oil red O, Sigma-Aldrich, Saint Louis, MO) dissolved in 100 mL of isopropanol (Sigma-Aldrich, St. Louis, MO), and diluted to 60% with distilled water.

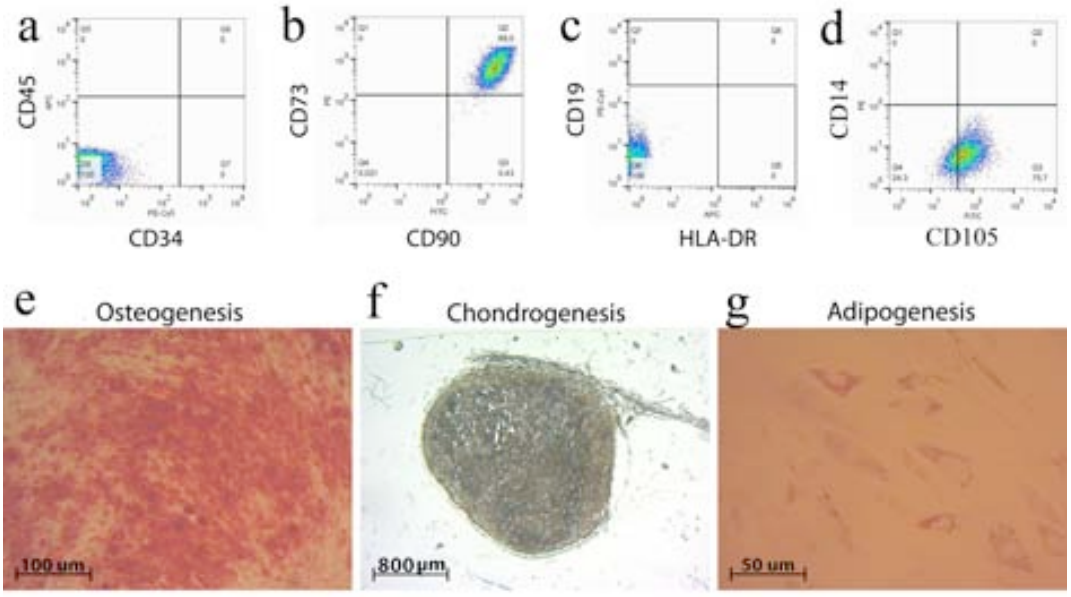

Figure 8: WJ-MSC characterization.

Flow-cytometry markers expressed by human WJ-MSC harvested from umbilical cords (a-d), and further grown on plastic plates. Cells were able to differentiate into 3 mesenchymal cell lineages: osteocytes (e), chondrocytes (f) and adipocytes (g). Images shown after oil red (e), safranin O (f) and alizarin red (g) stainings, respectively.

For osteogenic differentiation, cells were also seeded at a density of 3,000 cells/cm^2 in 24-well plates with low-glucose DMEM supplemented with 10% FBS, 1% antibiotic-antimycotic solution, 0.05 mM ascorbic acid-2-phosphate (Wako Pure Chemicals Industry Ltd., Osaka, Japan), 10 mM beta-glycerophosphate (Sigma-Aldrich, Saint Louis, MO), and 100 nM dexamethasone (Sigma-Aldrich, Saint Louis, MO). Alizarin red staining (Sigma-Aldrich, Saint Louis, MO, USA) was used to identify bone cells (2 g alizarin red dissolved in 100 ml of distilled water).

For chondrogenic differentiation, cells were seeded in 15 ml polypropylene tubes BD Falcon^{TM} (Bedford, MA) ($2x10^5$ cells per tube) with low-glucose DMEM supplemented with 10% FBS, 1% antibiotic-antimycotic solution, 1 mM sodium pyruvate (Sigma-Aldrich, Saint Louis, MO), 0.1 mM ascorbic acid-2-phosphate (Wako Pure Chemicals Industry Ltd., Osaka, Japan), 1% insulin-transferrin-selenium (ITS) (Cellgro, Manassas, VA), 100 nM of dexamethasone (Sigma-Aldrich, Saint Louis, MO, USA), and 10 ng/ml TGF-β3 (Shenandoah Biotechnology, Inc., Warwick, PA). Chondrocyte pellets were identified with Safranin O staining (0.1 g safranin O, Sigma-Aldrich, Saint Louis, MO, dissolved in 100 ml of distilled water).

3. 4. HUMAN SKIN FIBROBLASTS AND WJ-MSC CO-CULTURES AND INTERACTIONS:

Three different *in vitro* interaction culture systems were used in this study: 1) WJ-MSC-CM (conditioned media) treated fibroblast cultures, or "indirect one-way paracrine signalling co-culture"; 2) indirect-insert, porous membrane or transwell co-culture; and 3) direct cell-cell contact co-culture.

All experiments were performed with low-passage cells (less than P5), and in triplicate (unless otherwise stated). Media was changed every 2 days. On day 5 of culture, the amount of FBS in the medium was reduced from 10 to 2%, to avoid TGF-β1 false measurements. On day 7, culture medium was collected for protein studies, and total RNA extraction was started for further gene expression studies.

First, primary human fibroblasts were seeded into 6-well plates (Grenier-Bio-One Cellstar, Frieckenhausen, Germany), at a density of 22,000 cells/cm^2 with DMEM media.

For the WJ-MSC-CM experiment or "one-way indirect co-culture system", the WJ-MSCs were separately seeded at the same cell density in the upper 3 wells of a 6-well plate, with CMRL media; the lower 3 wells of the same 6-well plate were filled with CMRL media alone. When refreshing the media, the 6-well plate containing the fibroblasts was filled with the media from the WJ-MSC 6-well plate: the 3 upper wells (treatment wells) with the WJ-MSC-CM, and the 3 lower wells (control wells) with the CMRL media alone.

In the indirect-insert co-culture method, 24h after seeding the fibroblasts, WJ-MSCs were seeded into 3 μm-pore inserts (at a density of 18,000 cells/cm^2) and put on top of the 3-treatment fibroblasts wells, or on top of CMRL only-filled wells, with no fibroblasts (WJ-MSC insert controls). The 3 lower wells with fibroblasts were filled with inserts containing the same amount of CMRL as the treatment wells, but no WJ-MSCs.

In the direct cell-cell contact co-culture, 24h after seeding the fibroblasts, WJ-MSCs were seeded into the 3 fibroblast-treatment wells at a density of 22,000 cells/cm^2; the 3 lower wells were filled with CMRL media alone.

For WJ-MSC-CM further studies, skin fibroblasts were seeded at a density of 1,000 cells/cm^2 in 8-well chamber culture slides for BrdU/Ki67 proliferation test (in monoreplicate) and scratch wounding analysis (in duplicate). For TUNEL apoptotic staining, 4-well chamber culture slides (duplicate) were used in a similar manner. For cyquant proliferation and Live/Dead viability assays, both skin fibroblasts and WJ-MSCs were seeded in separate wells of a 96-well plate at a density of 1,500 cells/ cm^2 and these experiments were performed in triplicate.

3. 5. RNA ISOLATION AND REAL-TIME QUANTITATIVE POLYMERASE CHAIN REACTION:

For RNA isolation, cells were lysed using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and the RNeasy MicroKit was used (Qiagen, Inc., Valencia, CA) according to the manufacturer's instructions. The total RNA yield was determined using a NanoDrop-2000 spectrophotometer (ThermoScientific, Waltham, MA). 10 μg of RNA were used for cDNA synthesis using high capacity cDNA synthesis reverse transcription kit (AB Applied Biosystems, Foster City, CA) and thermocycler (AB Applied Biosystems, Foster City, CA). RT-PCR was conducted using SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA) to relatively quantify the mRNA transcript products of the following genes of interest: *TGF-β1, TGF-β2, TGF-β3, CTGF, PAI-1, HIF-1-α, VEGF, FGF-2, collagen type I, collagen type III,* and *SIRT-1. 18S* was used as housekeeping gene. Used primer sequences of the above genes are listed in Table 9.

Amplification and analysis of cDNA fragments were carried out using the StepOnePlus RT-PCR System (AB Applied Biosystems, Foster City, CA). Relative gene expression was measured as cycle threshold (Ct) and normalized with individual housekeeping gene control Ct values. qPCR was loaded in duplicates, and Ct values from the triplicates of the same treatment group sample were averaged. The delta-delta Ct method was used to report qPCR results.

Gene	Forward primer sequence	Reverse primer sequence
TGF-β1	GGCTTTCGCCTTAGCGCCCA	CTCGGCGGCCGGTAGTGAAC
TGF-β2	CTTTGGATGCGGCCTATTGCT	AGCTGTTCAATCTTGGGTGTTT
TGF-β3	GCTGAGACCCCACGTGCGAC	GTGTTTCCCGAGGAGCGGGC
CTGF	TGCCCGGGAAATGCTGCGAG	CAGTCGGTAAGCCGCGAGGG
PAI-1	TGGCACGGTGGCCTCCTCAT	TCCTGTGGGGTTGTGCCGGA
HIF-1-α	GATCACCCTCTTCGTCGCTT	AAGGAAAGGCAAGTCCAGAGG
VEGF	ACGAAAGCGCAAGAAATCCC	CTCCAGGGCATTAGACAGCA
FGF-2	CTGGCTATGAAGGAAGATGGA	TGCCCAGTTCGTTTCAGTG
Decorin	CGCCTCATCTGAGGGAGCTT	TACTGGACCGGGTTGCTGAA
Type I Collagen	GGCCAAGACGAAGACATCCCACCAA	TGCCGTTGTCGCAGACGCAGAT
Type III Collagen	GAGGTGGTGCAGGTGAGCCTGGTAA	GATCCATCCTTGCCATCTTCGCCTT
18S	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG

Table 9: Primer sequences for RT-PCR.

3. 6. PROTEIN DETERMINATION:

Protein levels of human TGF-β1, TGF-β2 and TGF-β3, and the inflammatory and pro-fibrotic cytokines IL-6 and IL-8 were measured in aliquots of WJ-MSC-CM treated and untreated human skin fibroblasts using Luminex Multiplex technology (Millipore, Billerica, MA). Protein values in culture supernatants were measured as pg/ml.

3. 7. PROLIFERATION AND VIABILITY ASSAYS:

3. 7. 1. LIVE/DEAD ASSAY:

Both human skin fibroblasts and WJ-MSCs were cultured in different wells at a cell density of 1500 cells/cm^2 in five 96-well culture plates, one for each time point of the viability study (days 0, 1 , 3 , 7 and 14). Media was refreshed every 2 days. At each time point, medium was removed, fibroblasts and WJ-MSC were washed with phosphate buffered saline (PBS), and 200 µg of staining solution -a mix of 10 µl ethidium bromide and 2.5 µl calcein-AM (both from Invitrogen, Carlsbad, CA) in 15 ml PBS- was added to each well. After 15 minutes of incubation, cells were imaged on a confocal microscope (Laser Scanning Microscope –LSM- 510, Axiovert 100 M, Zeiss, Oberkochen, Germany). The number of live (stained green) and dead (stained red) cells was counted with Zeiss software program.

3. 7. 2. BrdU PROLIFERATION ASSAY:

Human skin fibroblasts and WJ-MSCs were cultured in different 8 well-chamber culture slides at a cell density of 1000 cells/cm^2 for 7 days. Study design and medium change was similar to the WJ-MSC-CM or "one-way paracrine co-culture" system previously described. On day 6, cells were labelled with 50 µM bromodeoxyridine (BrdU) per well for 24 hours. DNA incorporation of BrdU was measured to determine fibroblasts proliferation.

3. 7. 2. 1. Immunocytochemistry:

After fixing and washing, cells were pre-incubated for 30 minutes in PBS with 1% bovine serum albumin (BSA) followed by incubation with mouse anti-human BrdU (1:200, Cell Signaling, Boston, MA, USA) overnight at 4ºC.

After washing with PBS, the secondary antibody was added in 1% BSA and left incubating for 1 hour at room temperature in the dark (Alexa Fluor 488 donkey anti-mouse, 1:500, Life Technologies, Grand Island, NY, USA). Images were taken using an Apotome Axiovert fluorescent imaging system (Zeiss, Oberkochen, Germany). Quantification was performed by counting the number of BrdU-positive nuclei in the high power field (HPF) as well as 4', 6-Diamidino-2-Phenylindole (DAPI) positive total number of nuclei.

3. 7. 3. Ki67 ANTIGEN STAINING:

Skin fibroblasts and WJ-MSCs were seeded in different 8 well-chamber culture slides at a cell density of 715 cells/cm^2 and cultured for 7 days, following methods described above.

3. 7. 3. 1. Immunofluorescence:

Cells were washed with PBS and fixed for 15 minutes in 4% paraformaldehyde (PFA) (Alfa Aesar, Karlsruhe, Germany). Fixed cells were washed in PBS and permeabilized for 10 minutes with PBS with 0.5% Triton-X-100 (PBST) solution. After another washing step, cells were blocked for 30 minutes with 1% BSA in PBST.

A monoclonal mouse anti-human Ki67 (1:100, clone MIB-1, Dako, Markham, Canada) primary antibody was added and incubated overnight at 4ºC. After washing with PBS, the secondary antibody was added in 1% BSA in PBST and incubated for 1 h at room temperature in dark (Alexa Fluor 488 donkey anti-mouse, 1:500, Life Technologies, Eugene, OR). After three final washes with PBS, slides were mounted with Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA). Cells were examined and photographed using an Apotome Axiovert fluorescent imaging system at 10X magnification (Zeiss, Oberkochen, Germany). Three images were taken per well and two wells were imaged per treatment. Quantification was performed by counting the number of Ki67-positive cells in the high power field (HPF) as well as DAPI positive total number of nuclei. Data presented are means with 95% confidence intervals of duplicate measurements for 3 different skin samples.

3. 7. 4. CYQUANT CELL PROLIFERATION ASSAY:

A cell pellet of known number of human skin fibroblasts was frozen at -80ºC for the standard curve. Both skin fibroblasts and WJ-MSCs were cultured in separate wells at a cell density of 1500 cells/cm^2 in five 96-well culture plates, one for each time point of the proliferation study (days 0, 1, 3, 7 and 14). Media was replaced as described, until cells reached the desired time-point, where plates were treated and frozen following Cyquant assay kit (Invitrogen, Eugene, OR) manufacturer's instructions. The fluorescence signal for all samples was read in a spectrophotometer (Beckman Coulter, Mississauga, Canada).

3. 8. APOPTOSIS ASSAYS: TUNEL apoptosis assay

Human skin fibroblasts were seeded into 8-chamber culture slides at a cell density of 2,000 cells/cm^2 and were cultured with WJ-MSC-CM and with non-conditioned medium for 4 days. TUNEL (terminal transferase TdT-mediated dUTP biotin end-labeling) apoptosis kit (Promega, Fitchburg, WI) was used as per manufacturer's instructions. Images were taken as in the BrdU assay. Cell quantification was performed by counting the number of TUNEL and DAPI positive nuclei, or apoptotic and live cells, respectively.

3. 9. CELL MIGRATION ASSAY: Scratch wound assay

Human skin fibroblasts were seeded into 4-well chamber culture slides (2000 cells/cm^2), with WJ-MSC-CM, or non-conditioned medium as control, for 48 hours. A pair of scratches were performed with a 200 µl pipette tip. After 24 hours, cells were fixed with 4% PFA. The staining protocol followed the same procedure as the aforementioned BrdU proliferation assay. Briefly, cells were incubated with phalloidin antibody conjugated to fluorescein isothiocyanate (FITC) (1:30, Invitrogen, Eugene, OR, USA) in blocking solution for one hour. Cells were washed three times with PBS and mounted with Vectashield mounting medium with DAPI. Images were taken on an LSM META 510 confocal microscope (Zeiss, Oberkochen, Germany) at 5X magnification. Three images were taken per scratch.

Quantification was performed using ImageJ software (National Institutes of Health, Bethesda, Maryland). A set area with a height of 0.5 mm and width spanning the HPF was placed in the centre of the scratch, and the cells within this area were counted as the cells within the scratch zone.

3. 10. *IN VIVO* WOUND HEALING MODEL:

Eight BALB/c mice (13 weeks old; male; body weight: 28-34 g) were obtained from Jackson laboratories under the guidelines of the Sunnybrook Research Institute and Sunnybrook Health Sciences Animal Policy and Welfare Committee of the University of Toronto. Animal procedures were reviewed and approved by Sunnybrook Research Institute and Sunnybrook Health Sciences Centre at University of Toronto animal care and use committee.

Animals were anesthetized and back cutaneous hair was removed. Two pairs of 4-mm diameter full-thickness skin excisional wounds were created on each side of the midline. Animals were randomly divided into 2 groups: treatment (WJ-MSC-CM and Matrigel®, BD Biosciences, San Jose, CA) and sham (non-conditioned medium and Matrigel®). Each wound topically received 100 µl of the treatment or sham mix.

3. 10. 1. Wound analysis:

Digital photographs were taken on days 1, 3 and 7. Wound measurements were recorded and wound closure was examined in a timely manner. Wounds with a complete re-epithelialization were considered as healed wounds.

Mice were sacrificed at day 7, when skin biopsies including the wound/scar and 2 mm of satellite skin were harvested for further histologic analysis. 24 hours before sacking, animals received an intraperitoneal injection of BrdU (Calbiochem, San Diego, CA).

3. 10. 2. Histologic examination:

Tissue specimens were fixed in 10% buffered formalin overnight at room temperature, preserved in 70% ethanol, embedded in paraffin and cut into 5 μm sections. Tissue specimens were cut simultaneously at different sites, the centre or midline and both sides, eliciting a cross section through the whole wound and satellite area. A serial section of the scar or healing wound was performed. The largest wound diameter or central wound section was stained for trichrome staining. Trichrome reagents were from EMF (Hatfield, PA) unless otherwise stated. Briefly, paraffin embedded slides were deparaffinized with citrosol (Fisher Scientific, Nepean, ON, Canada), followed by rehydration through 100%, 95%, 70% and 50% ethanol to water. Slides were placed in Bouin's solution (EMS, Hatfield, PA) overnight at room temperature and washed next. Hematoxylin stain (Sigma, St Louis, MO) and Biebrich scarlet-acid fuchsin solution were applied sequentially for 10 minutes. Washes were performed after each stain addition.

Slides were differentiated in phosphomolybdic-tungstic acid for 15 min, and transferred to aniline blue for 5 min. Slides were next rinsed and differentiated in 1% acetic acid for 2 min. Slides were dehydrated through 95% ethanol and absolute ethanol followed by clearing in citrosol. Slides were mounted with SHUR/Mount xylene-based liquid mounting media (Triangle Biomedical Sciences, Durham, NC). Images were acquired using Zeiss Axiovert 200 light microscope at 10X and 40X magnification. Quantification was done using merged 10X images to measure the wound bed and satellite area.

For immunohistochemistry staining, paraffin embedded skin tissue slides were deparaffinized with xylene followed by rehydration. Antigen decloaker (1X, Biocare, Concord, CA) was added to the slides in a preheated decloaking chamber for 4 minutes at 110ºC. For BrdU staining, samples were denatured with 1.5 N HydroChloric Acid (HCl) for 30 min at 37°C and neutralized with 0.1 M borate buffered twice for 5 min. Samples were blocked with 3% H_2O_2 for 10 min, then washed with washing buffer (0.05 M Tris-HCl, 0.15 M Sodium Chloride –NaCl-, 0.05% Tween 20 in deionized -DI- water). The primary antibody (mouse monoclonal anti-BrdU, 1:200, Cell Signaling, Beverly, MA) was diluted in PBS and incubated at room temperature for 1 h. Next, slides were incubated for 15 minutes first with MACH 3 mouse probe (Biocare Medical, Concord, CA), and secondly with MACH 3 rabbit or mouse horseradish peroxidase (HRP) polymer, with before and after washes. The betazoid diaminobenzidine (DAB) chromogen kit (Biocare Medical, Concord, CA) was mixed and added for 5 min or until brown stain was noticeable. The reaction was terminated with running water.

Nuclear staining was done with hematoxylin for 30 sec, followed by differentiation with 3 dips in 1.5% acid alcohol and bluing in 0.1% sodium bicarbonate for 10 sec. Sections were dehydrated through 95% and absolute ethanol to citrosol and mounted with SHUR/Mount as previously described. Images were acquired using a Zeiss Axiovert 200 light microscope at 10X magnification to image the whole section followed by 40X magnification to further focus on the wound margins and the wound centre. The higher magnification images of BrdU staining were quantified by counting using ImageJ software, and normalized to the number of cells in the HPF.

3. 11. STATISTICAL ANALYSIS:

Data are graphically expressed as the mean of the target group ± the standard error of the mean, or 95% confidence interval. The statistical comparisons between the groups were performed using an unpaired Student's t-test or a two-way-ANOVA (analysis of variance) using GraphPad Prism® software (LaJolla, CA). A two-tailed p-value ≤ 0.05 was considered significant.

4. RESULTS:

4. 1. KELOIDS:

1. WJ-MSC secretome enhanced pro-fibrotic gene expression.

In our culture conditions, WJ-MSC-CM enhanced mRNA expression of the pro-fibrotic markers *PAI-1 and HIF-1-α* ($p \leq 0.01$); *TGF-β2, VEGF and FGF-2 (*$p \leq 0.05$) (Figure 9). WJ-MSC two-way paracrine signalling (insert) also upregulated the expression level of the pro-fibrotic genes *PAI-1, HIF-1-α* and *TGF-β2 (*$p \leq 0.05$) (Figure 10). Under both of our paracrine signalling culture conditions, the expression level of *TGF-β1* did not change ($p > 0.05$) (Figures 9 and 10).

2. WJ-MSC-CM downregulated the expression of the anti-fibrotic gene *TGF-β3* in keloid fibroblasts.

In our culture conditions, WJ-MSC-CM decreased the transcript levels of the anti-fibrotic gene *TGF-β3* in keloid fibroblasts ($p \leq 0.01$) (Figure 9).

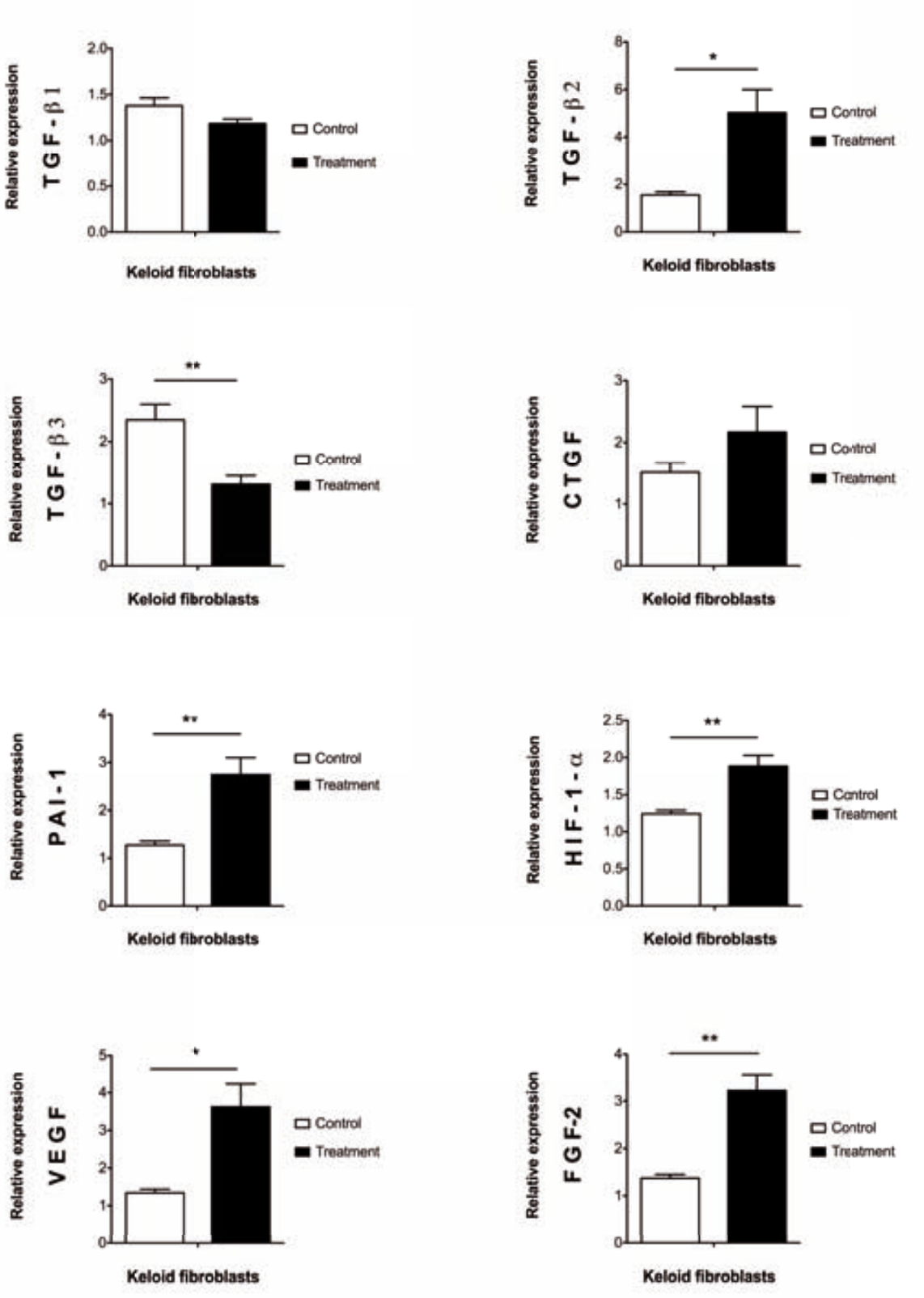

Figure 9: Human WJ-MSC one-way paracrine signalling effects on human keloid skin fibroblast gene expression.

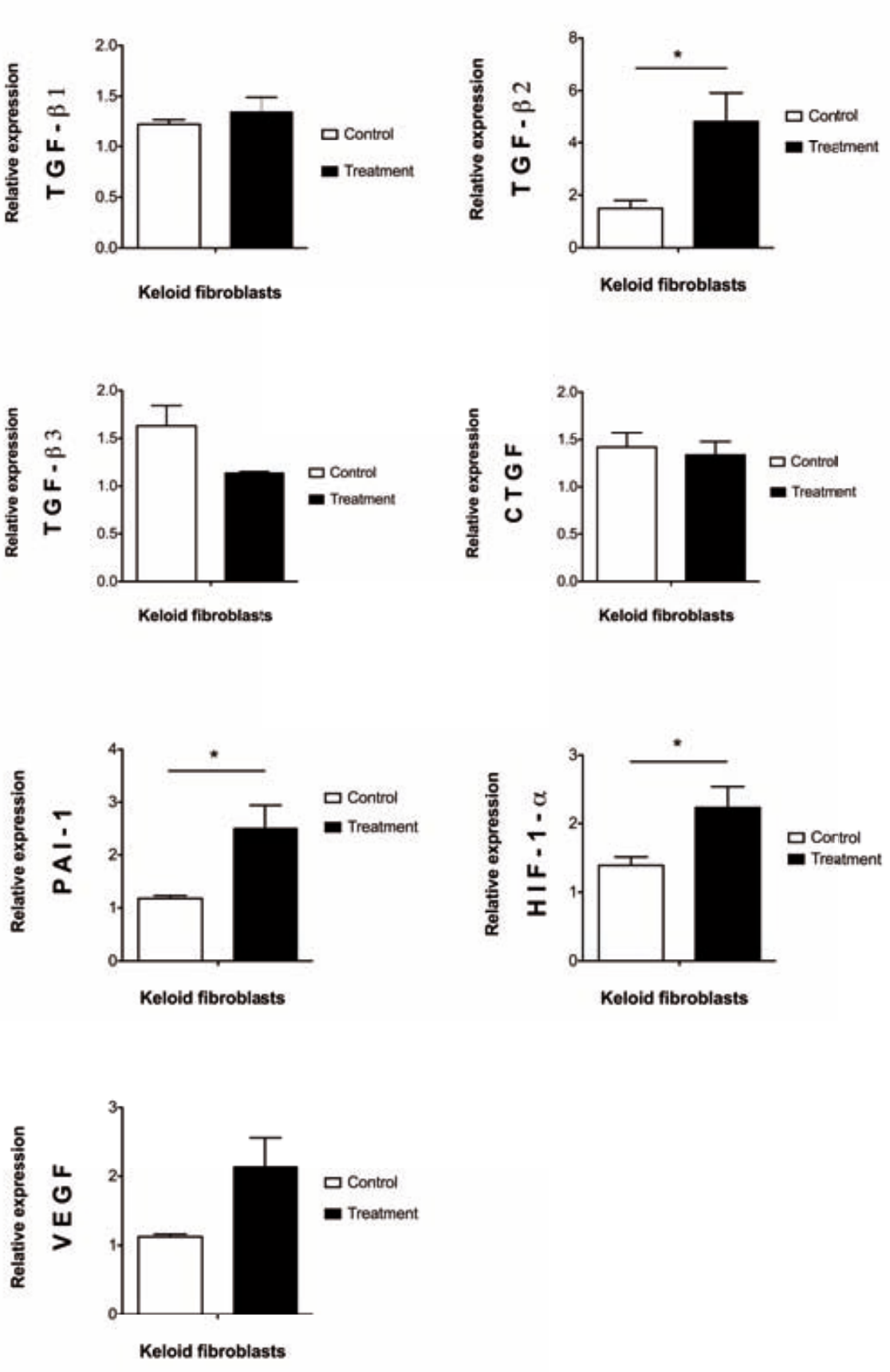

Figure 10: Human WJ-MSC two-way paracrine signalling effects on human keloid skin fibroblast gene expression.

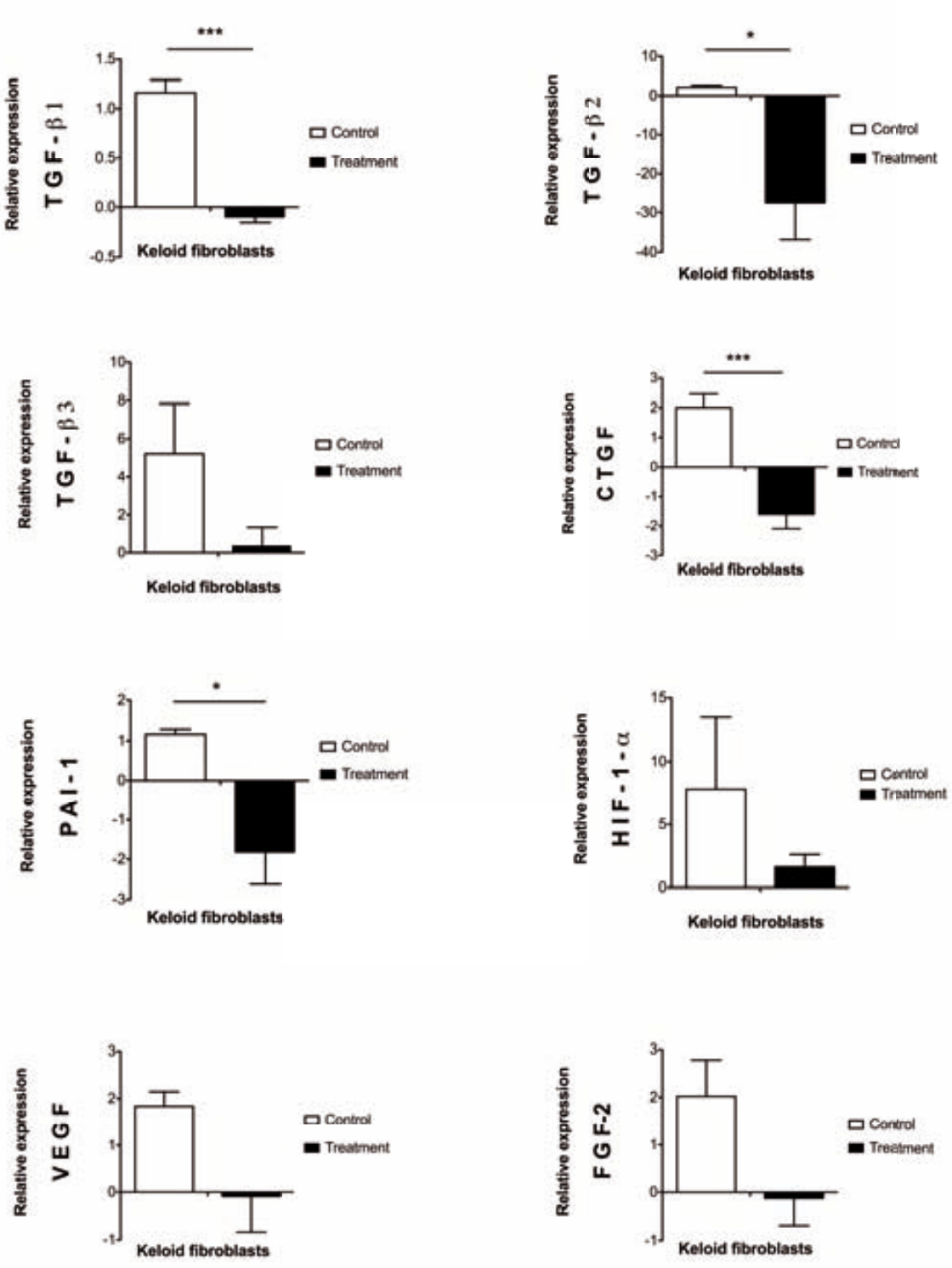

Figure 11: Human WJ-MSC direct cell-cell contact effects on human keloid skin fibroblast gene expression.

3. WJ-MSCs directly decreased pro-fibrotic gene expression.

In our experimental conditions, WJ-MSCs downregulated mRNA expression of the pro-fibrotic markers *TGF-β1* and *CTGF* ($p \leq 0.001$); *TGF-β2 and PAI-1 (*$p \leq 0.05$) (Figure 11).

4. WJ-MSC-CM increased the level of pro-fibrotic proteins in human keloid fibroblasts.

The expression of the inflammatory and pro-fibrotic proteins IL-6 and IL-8 was upregulated in keloid fibroblasts treated with WJ-MSC-CM ($p \leq 0.05$). Unlike the one-way paracrine effect, the expression of these interleukins through-an-insert co-cultured fibroblasts was not affected ($p > 0.05$). Our protein assay revealed no significant changes at the protein level for TGF-β1 and TGF-β2 in both paracrine culture systems ($p > 0.05$). Overall, our data suggested that WJ-MSC-CM upregulated the level of some pro-fibrotic proteins (Figure 12 A and B; one-way and two-way paracrine signalling, respectively).

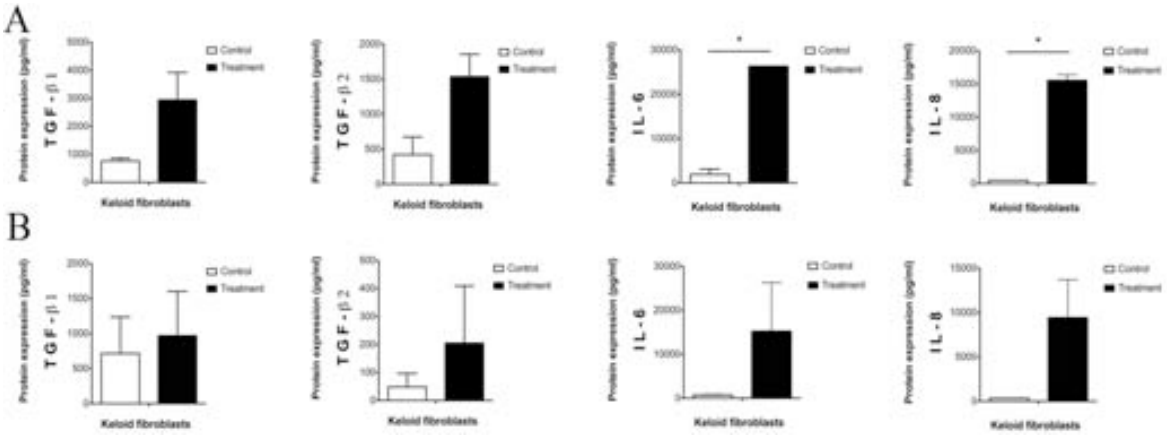

Figure 12: WJ-MSC paracrine signalling effects on keloid skin fibroblast protein expression.

5. WJ-MSC-CM promoted keloid fibroblast proliferation.

To determine the paracrine effect of WJ-MSC-CM on keloid fibroblast proliferation, cells were exposed to BrdU and were stained as elaborated in the methods section.

WJ-MSC-CM-treated human keloid fibroblasts showed a significantly higher number of viable cells as measured by DAPI staining, as well as higher number of BrdU incorporated cells ($p \leq 0.001$) in comparison with non-WJ-MSC-CM treated keloid fibroblasts at 7 days post exposure (Figure 13 A, B and C). A DNA assay confirmed the increased proliferative number of WJ-MSC-CM-treated keloid fibroblasts at day 7 ($p \leq 0.01$) (Figure 13 D).

Keloid fibroblasts remained viable after treatment with WJ-MSC-CM and proliferated during the 14 days of the experiment (Figure 14).

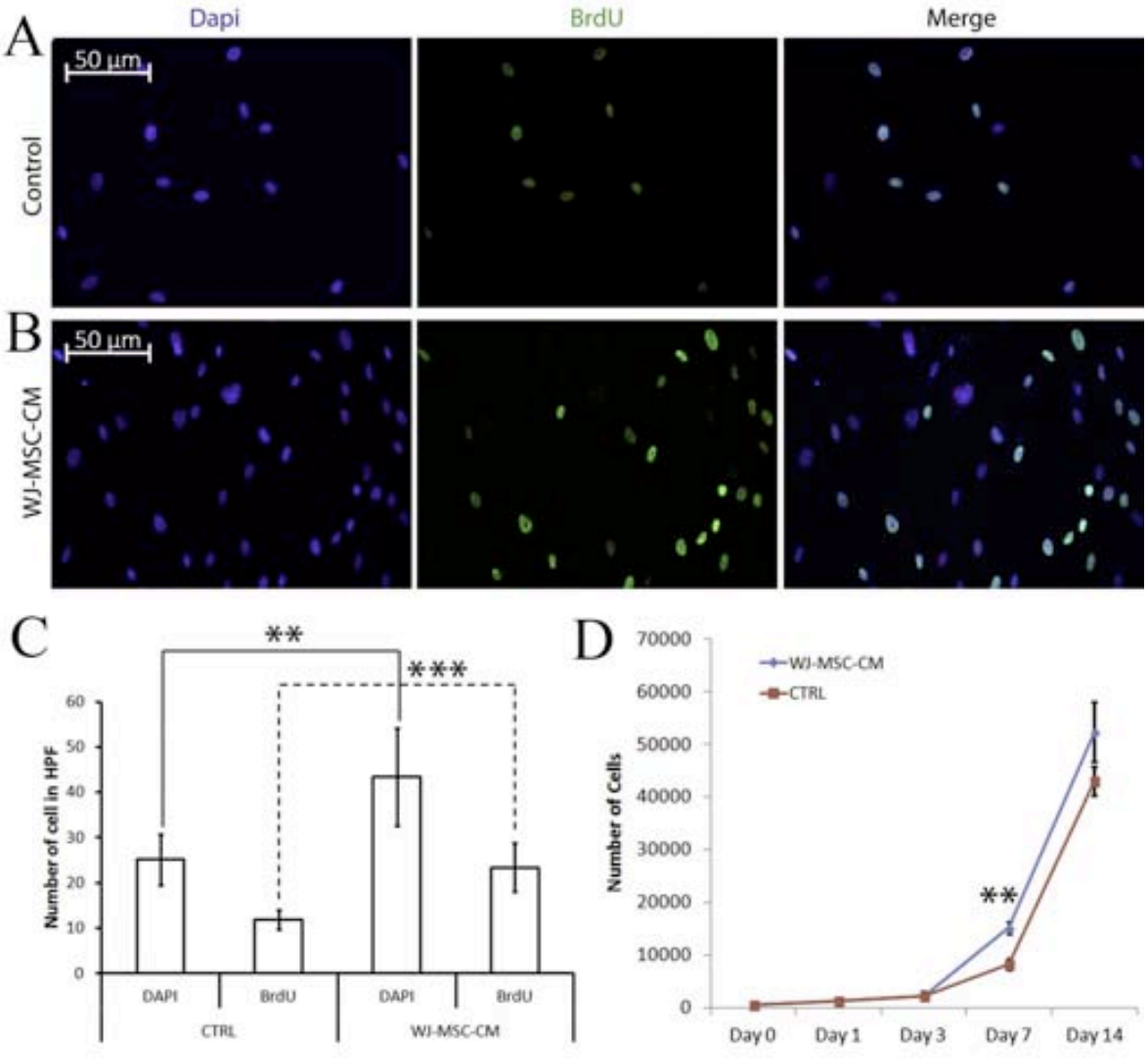

Figure 13: WJ-MSC-CM promotes keloid fibroblast proliferation.

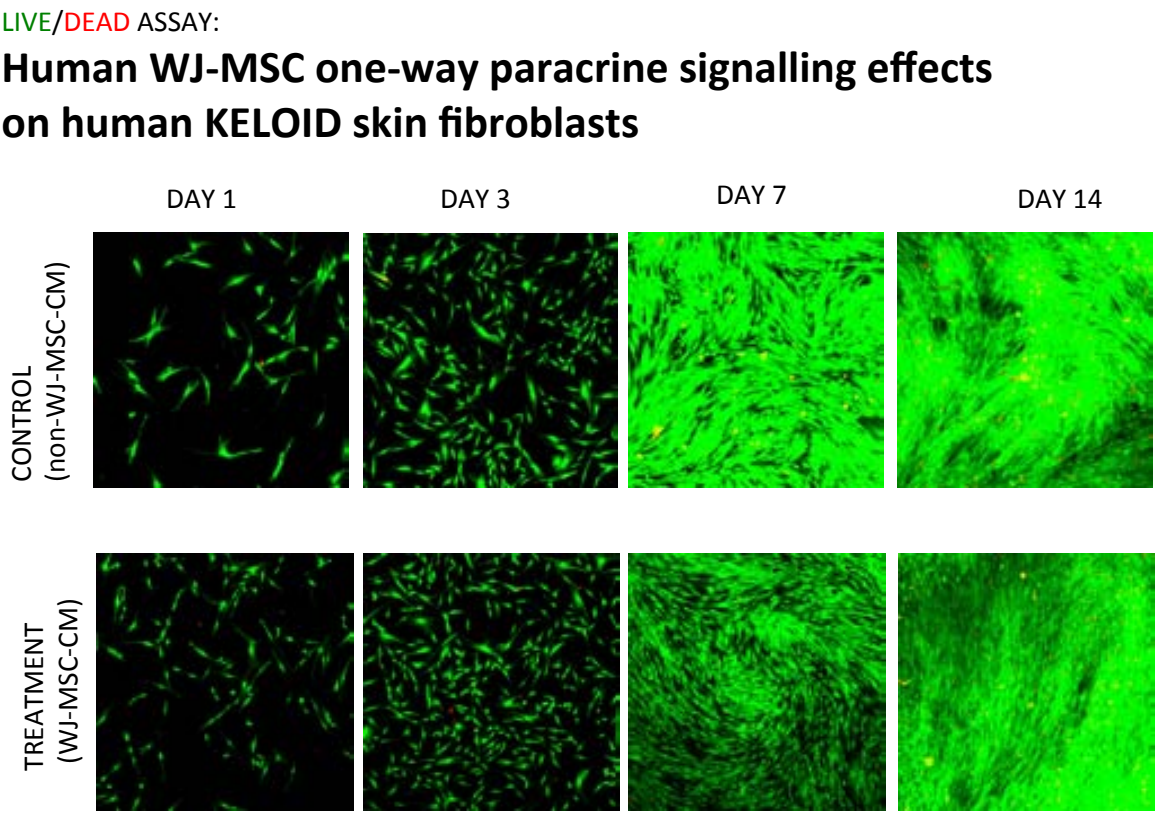

Figure 14: Human keloid fibroblasts remain viable and proliferate with WJ-MSC-CM.

6. WJ-MSC-CM did not enhance apoptosis in cultured keloid fibroblasts.

To further explore the effect of WJ-MSC-CM on keloid fibroblasts, we asked whether WJ-MSC-CM modulated the apoptosis rate of keloid fibroblasts. Under our culture conditions, the TUNEL apoptosis assay did not detect any apoptosis-related effects among human keloid skin fibroblasts following treatment with WJ-MSC-CM (Figure 15 A and B).

7. WJ-MSC-CM did not modulate keloid fibroblast migration.

Treatment with WJ-MSC-CM resulted in a slightly decreased but non-significant keloid fibroblast wound gap compared to the untreated or control group, meaning that WJ-MSC-CM did not affect keloid fibroblasts migration in this particular culture conditions ($p > 0.05$, Figure 15 C and D).

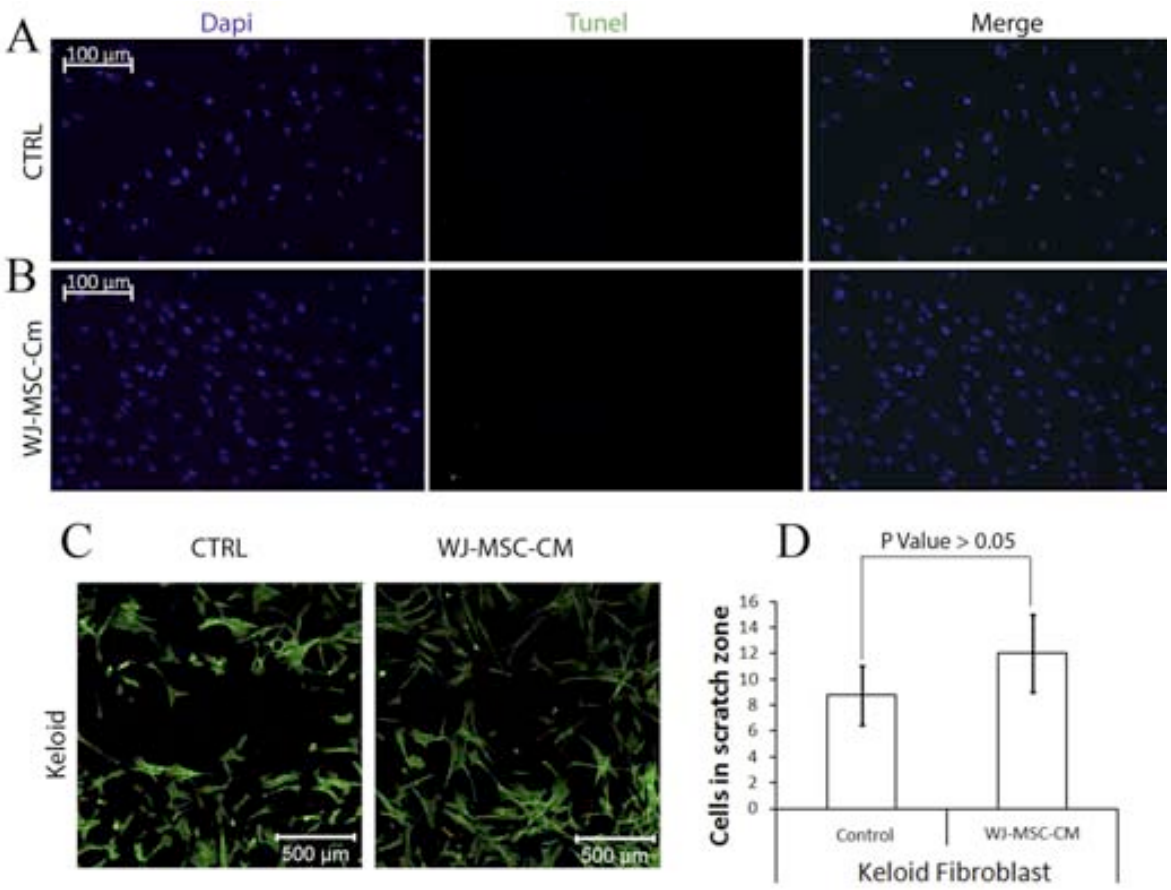

Figure 15: WJ-MSC-CM do not enhance apoptosis or modulate migration in cultured keloid fibroblasts.

4. 2. BURNS:

1. WJ-MSCs enhanced the expression of *PAI-1* transcript by two-way paracrine signalling in human burned skin fibroblasts.

PAI-1 mRNA levels were upregulated ($p \leq 0.05$) in human burned skin fibroblasts when co-culturing them with WJ-MSC through an insert transwell system, suggesting that paracrine signalling might promote fibroproliferation and burn wound repair (Figure 16). Other wound healing related markers, such as *TGF-β2, VEGF, HIF-1-α, FGF-2* and *collagen type III* showed an increased signal in burned skin fibroblasts treated with WJ-MSC-CM, but with no statistical significance and therefore no change (Figure 17). Signal for *TGF*-β3 mRNA expression was insignificantly lower ($p > 0.05$) and subsequently also not modified.

2. WJ-MSCs directly downregulated pro-fibrotic markers in burned skin fibroblasts.

In the direct co-culture conditions of this study, WJ-MSCs decreased the mRNA expression of the pro-fibrotic markers *TGF-β1* and *CTGF* ($p \leq 0.05$) in human burned skin fibroblasts. Pro-fibrotic transcripts *TGF-β2, PAI-1, VEGF* and *HIF-1-α* were not significantly modified ($p > 0.05$), although they appeared to be downregulated (Figure 18).

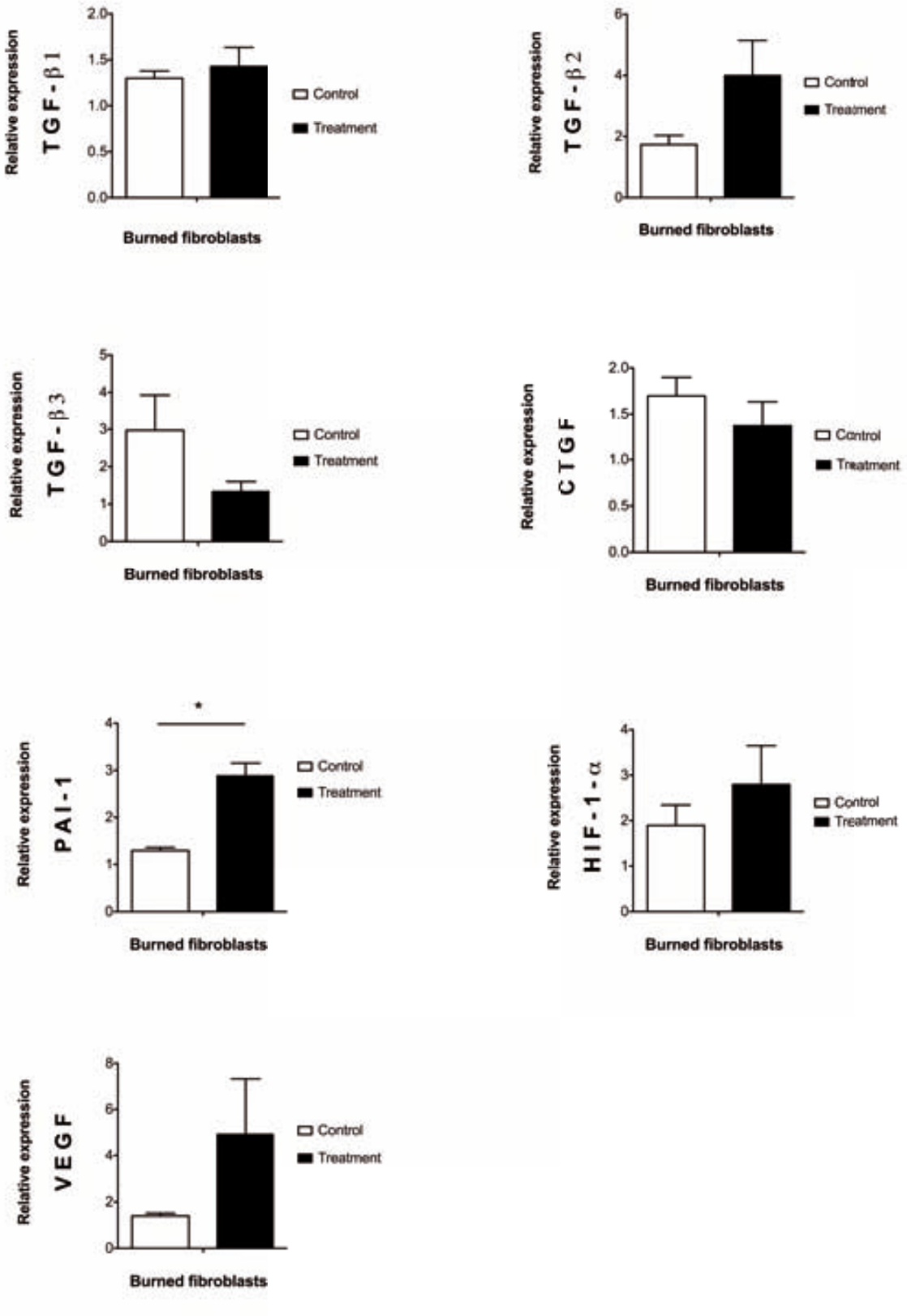

Figure 16: Human WJ-MSC two-way paracrine signalling effects on human burned skin fibroblast gene expression.

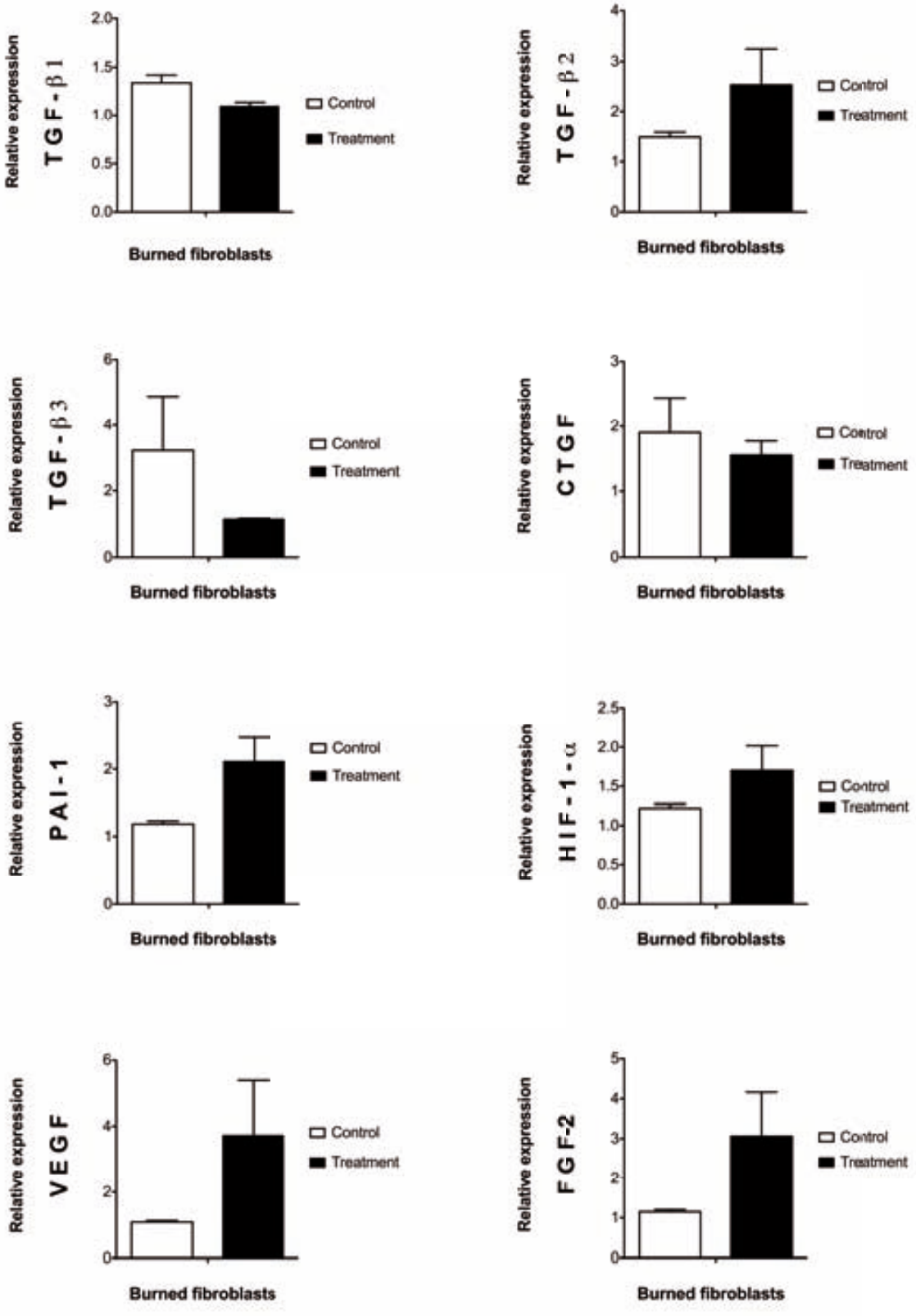

Figure 17: Human WJ-MSC one-way paracrine signalling effects on human burned skin fibroblast gene expression.

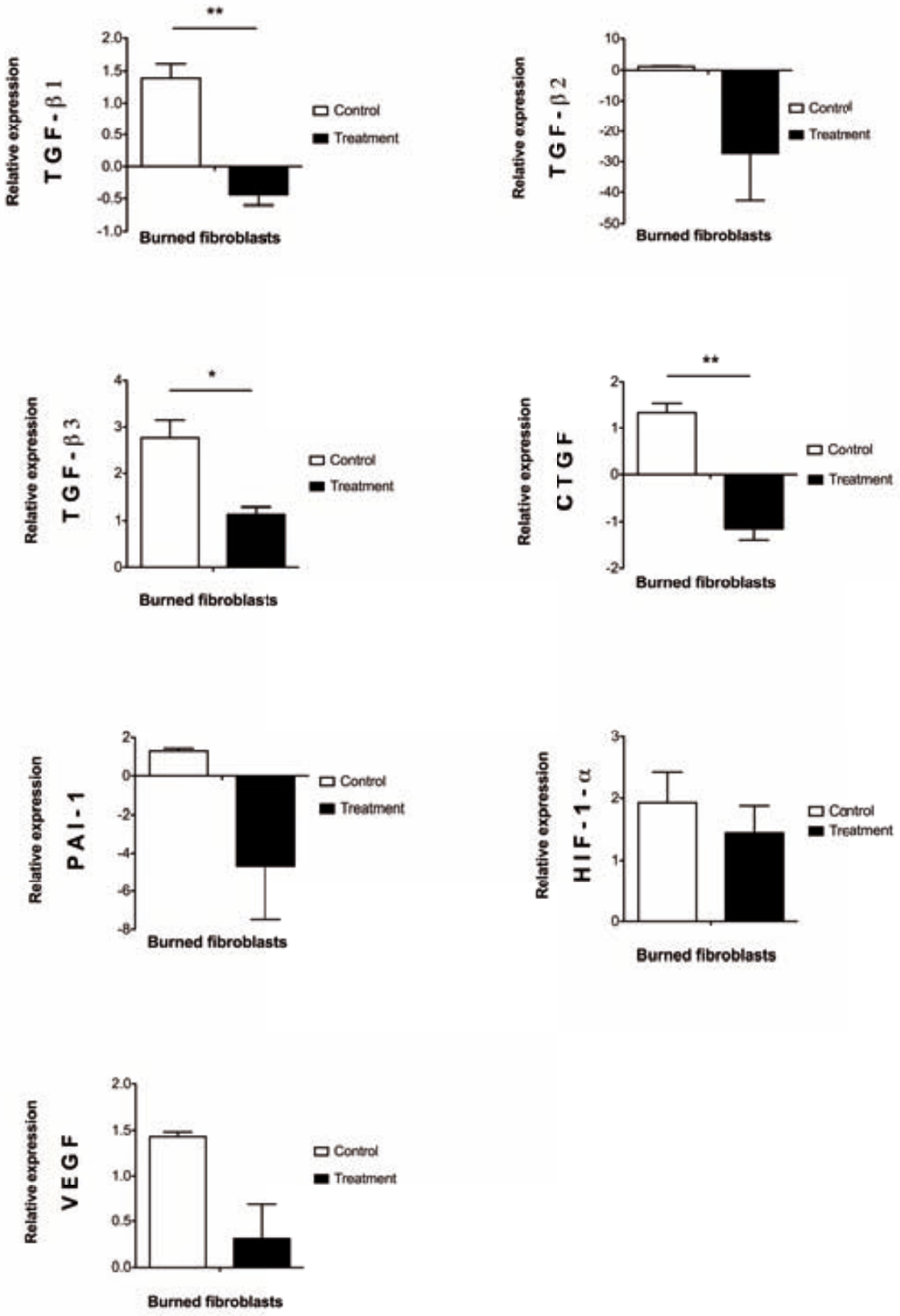

Figure 18: Human WJ-MSC direct cell-cell contact effects on human burned skin fibroblast gene expression.

3. WJ-MSC-CM enhanced burned skin fibroblast proliferation.

WJ-MSC-CM promoted human burned skin fibroblast proliferation after 3 days of culture ($p \leq 0.05$) using a DNA Cyquant assay (Figure 19). Live/Dead viability assay showed viable burned fibroblasts treated with WJ-MSC-CM (Figure 20).

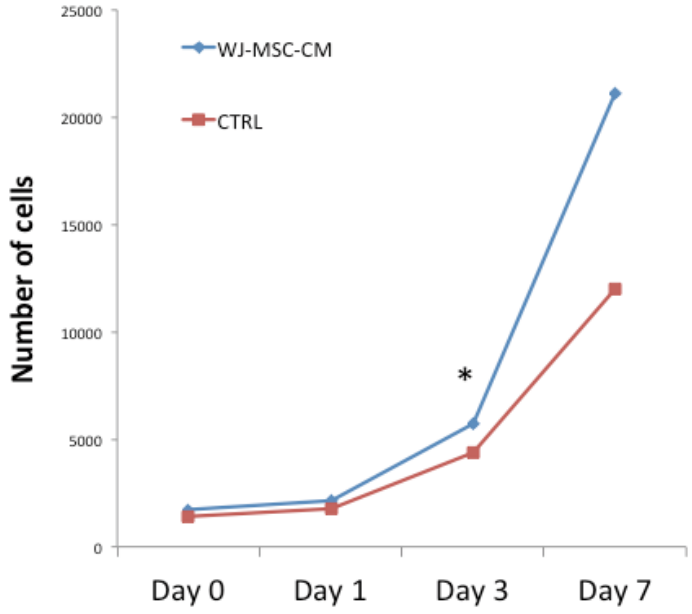

Figure 19: WJ-MSC-CM enhances human burned skin fibroblast proliferation.

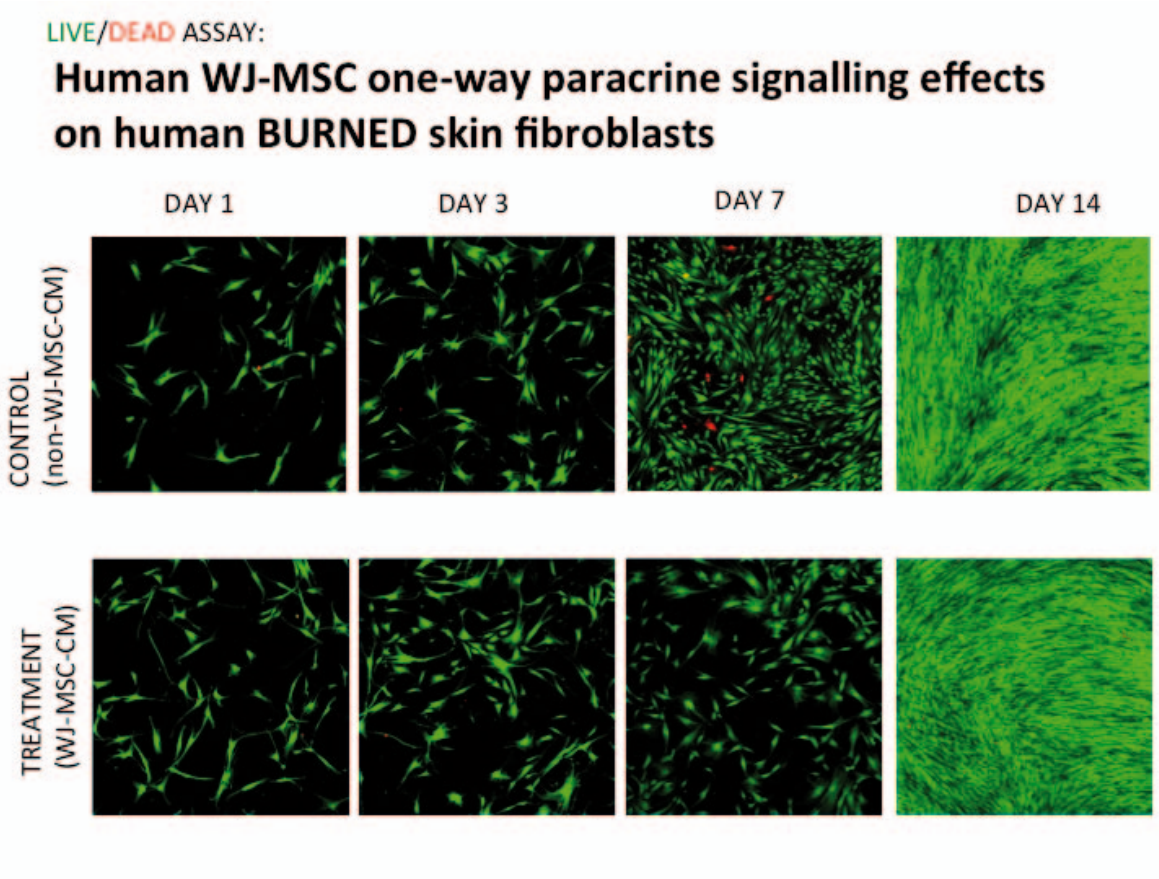

Figure 20: Human burned skin fibroblasts remain viable and proliferate with WJ-MSC-CM.

4. WJ-MSC-CM promoted burned skin fibroblast migration and wound closure *in vitro*.

In our culture conditions, human burned skin fibroblasts treated with WJ-MSC-CM accelerated wound closure compared to the burned skin fibroblasts of the control group (that is, treated with non-WJ-MSC-CM) ($p \leq 0.001$) (Figure 21).

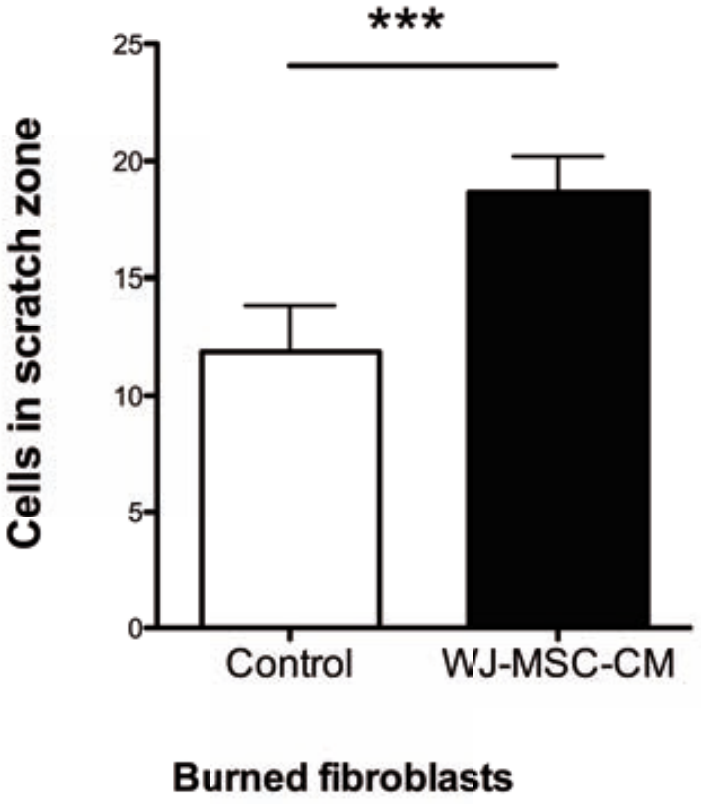

Figure 21: WJ-MSC-CM promotes burned skin fibroblast migration and wound closure *in vitro*.

4. 3. NORMAL WOUND HEALING/ANTI-AGING:

1. WJ-MSCs enhanced the expression of wound healing and anti-aging genes by paracrine signalling.

Human WJ-MSC-CM upregulated the mRNA transcript expression of *TGF-β2, HIF-1-α,* and *PAI-1* ($p \leq 0.05$) in normal skin fibroblasts in our culture conditions. Although there was also a positive trend with other genes involved in re-epithelialization, neovascularization and/or remodelling, including *VEGF, FGF-2, CTGF*, *collagen I* and *collagen III,* there were no significant changes regarding their expression. Decorin and *TGF*-β3 also remained unaffected ($p > 0.05$) (Figure 22). In the insert co-culture system, *collagen I* and *collagen III* transcript expression was enhanced ($p \leq 0.05$) (Figure 23). The direct cell-cell contact co-culture decreased *TGF-β1* and *CTGF* gene expression ($p \leq 0.05$) (Figure 24).

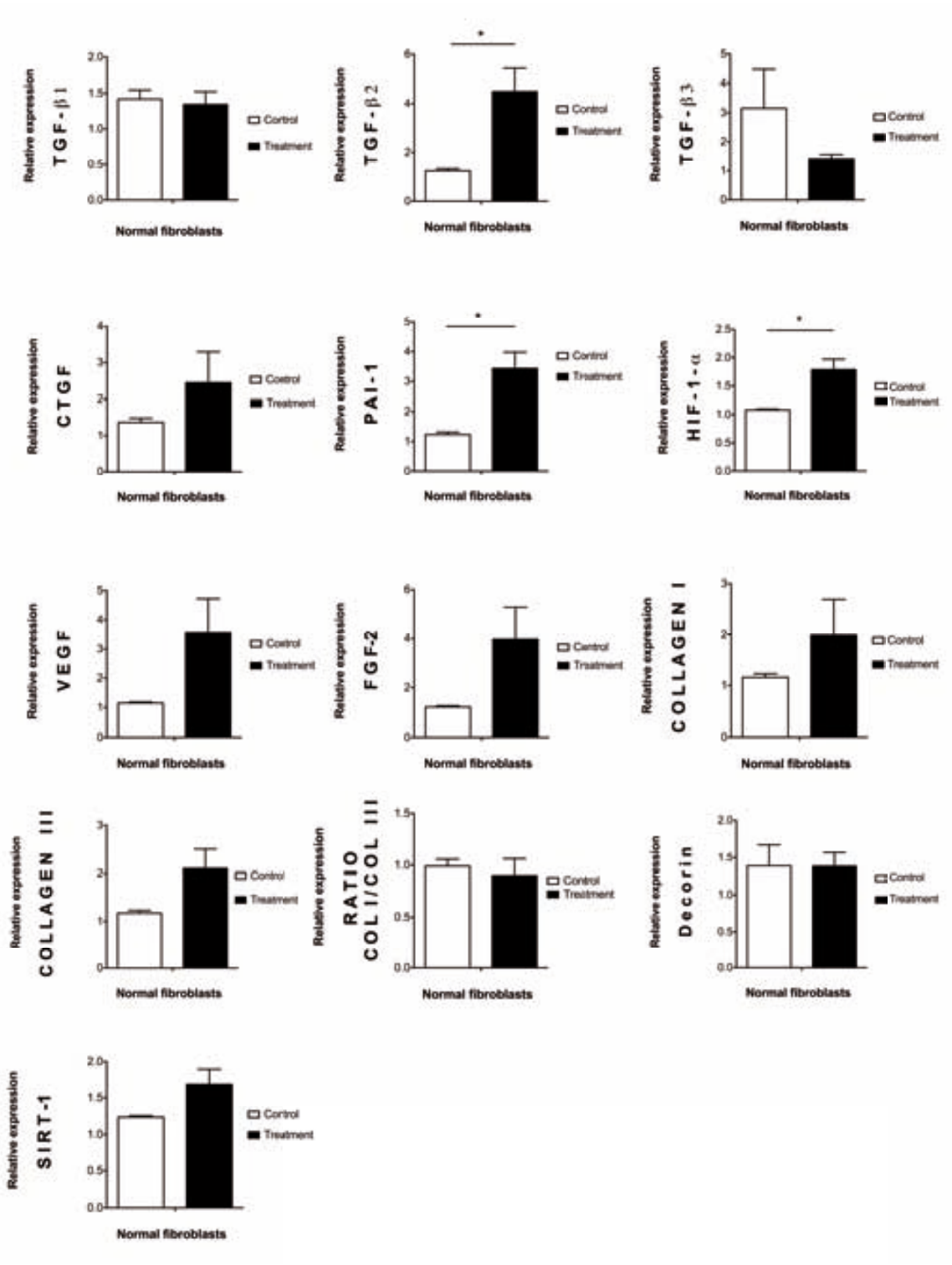

Figure 22: Human WJ-MSC one-way paracrine signalling effects on human normal skin fibroblast gene expression.

mRNA transcript expression relative to 18S after 7 days of culture of human normal skin fibroblasts with WJ-MSC-CM (treatment group) or non-conditioned medium (control group) from 5 different patients (but 4 in *FGF-2* and *Sirt-1*, and 3 in *collagen I, collagen III* and *decorin*). Overall, WJ-MSC-CM enhanced a wound healing promoting phenotype in human normal skin fibroblasts in our culture conditions.

- * = $p \leq 0.05$.

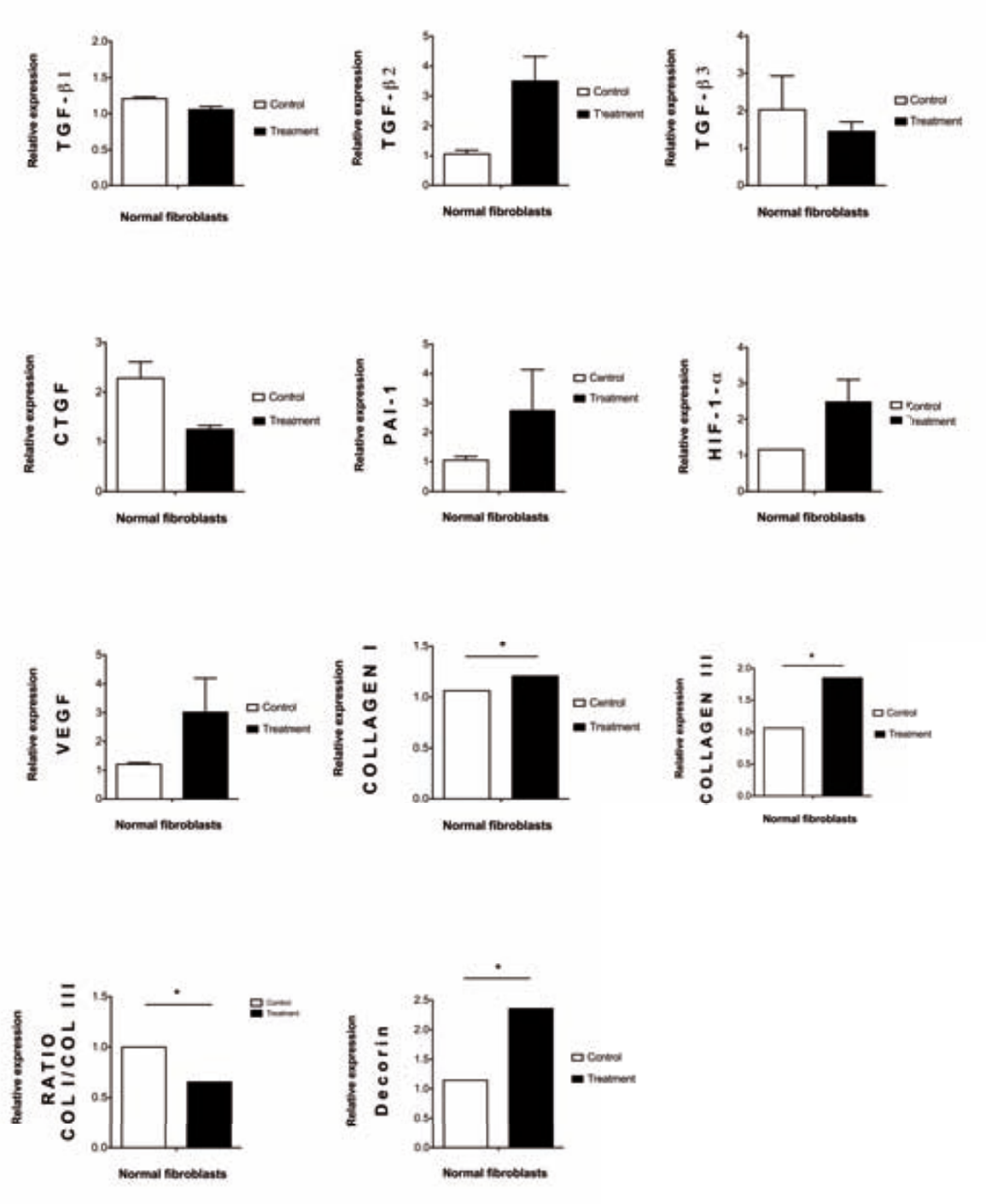

Figure 23: Human WJ-MSC two-way paracrine signalling effects on human normal skin fibroblast gene expression.

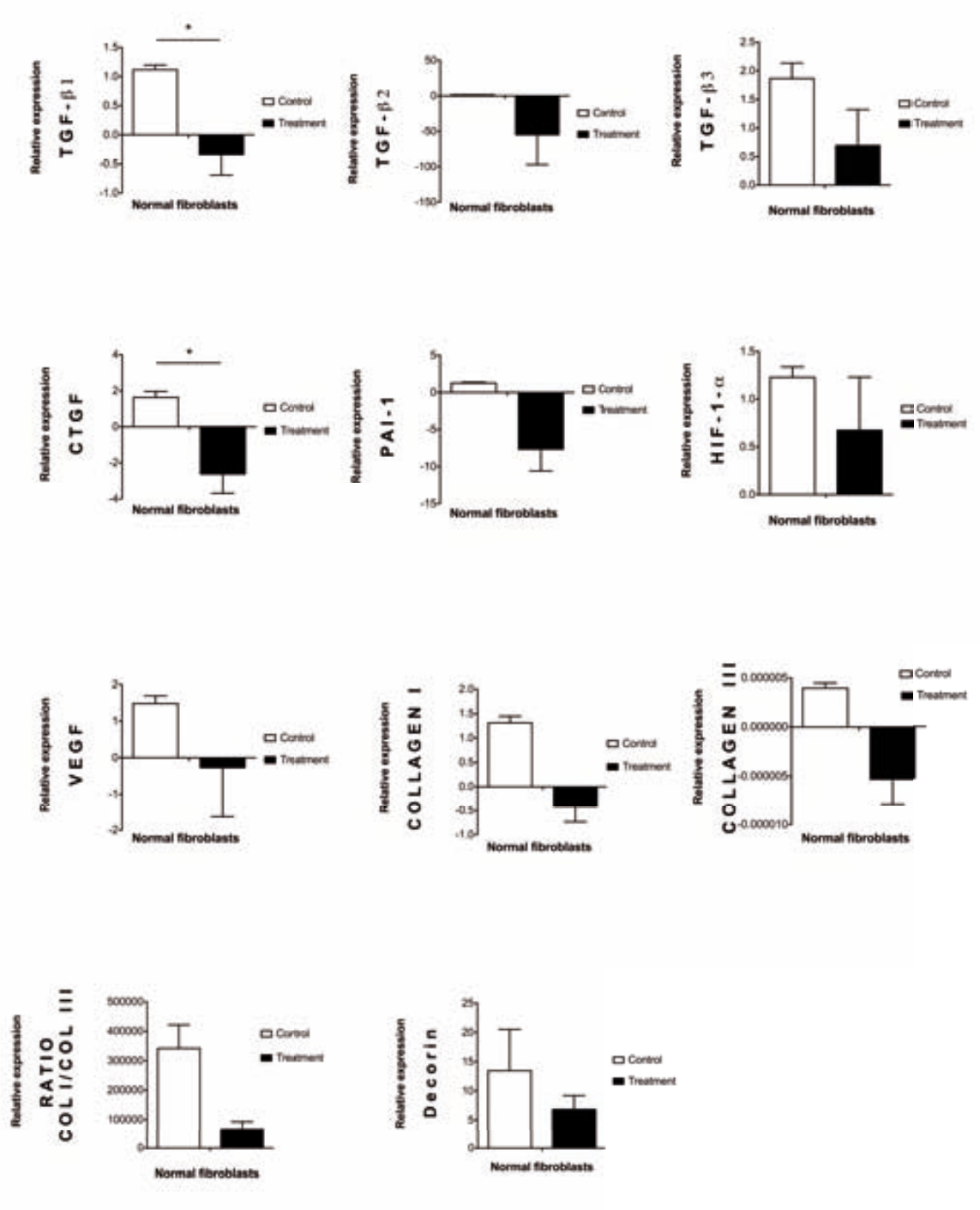

Figure 24: Human WJ-MSC direct cell-cell contact effects on human normal skin fibroblast gene expression.

2. Normal skin fibroblasts proliferated faster when treated with WJ-MSC-CM.

WJ-MSC-CM accelerated normal skin fibroblast proliferation ($p \leq 0.001$), as measured by a Ki67 proliferation assay (Figure 25 A, B and C), in the culture conditions of this study. A DNA assay confirmed the increased proliferative number of WJ-MSC-CM-treated normal skin fibroblasts at day 3 and 7 ($p \leq 0.05$) (Figure 26). The aforementioned cells remained viable and proliferative during a 14 days study period, as a Live/Dead assay showed (Figure 27).

3. WJ-MSC-CM did not affect normal human skin fibroblast apoptosis.

This study did not find any significant modulation in the number of apoptotic normal skin fibroblasts treated with WJ-MSC-CM using a TUNEL assay, suggesting that WJ-MSC-CM does not appear to affect normal skin fibroblasts apoptosis under the culture conditions of this study (Figure 25 D).

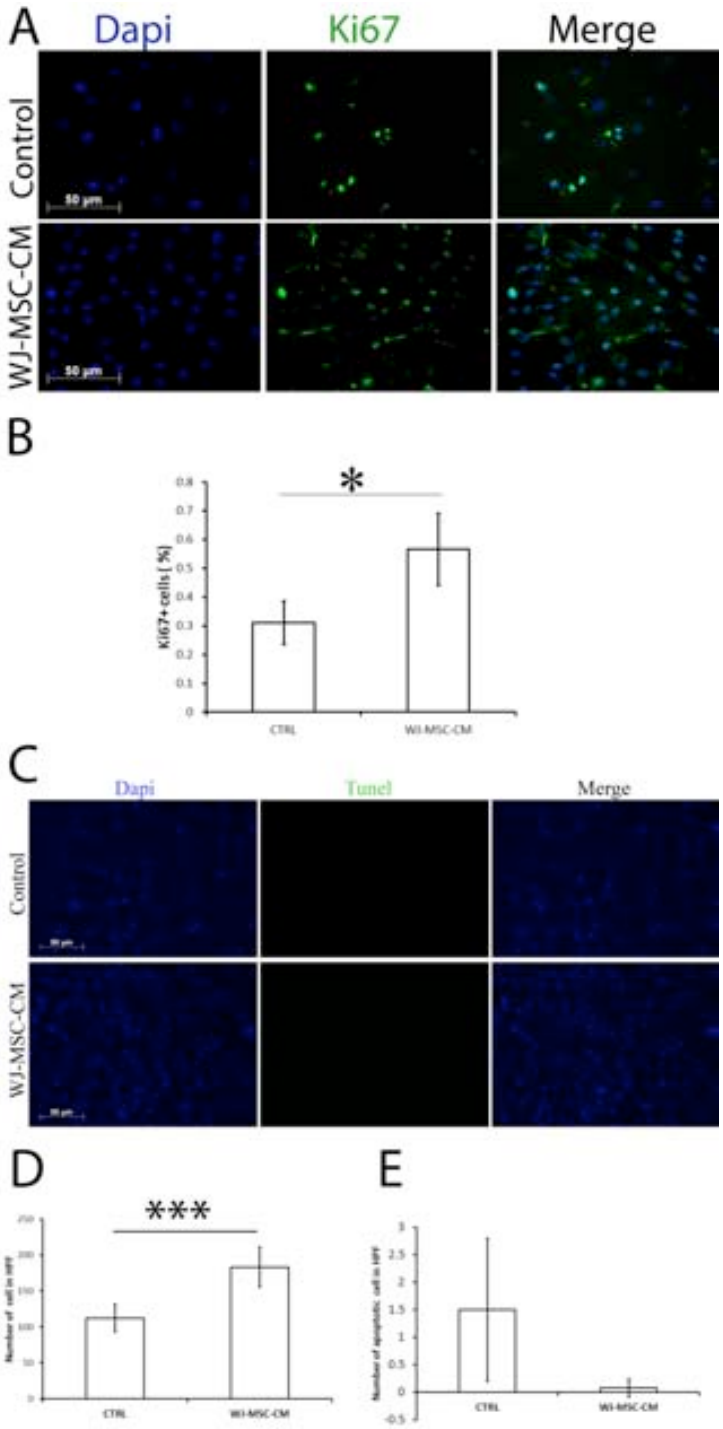

Figure 25: WJ-MSC-CM enhances human normal skin fibroblast proliferation and not apoptosis.

Cell proliferation was examined using Ki67 staining. WJ-MSC-CM-treated normal fibroblasts showed enhanced proliferative rates compared to the control group (A, quantified in B). TUNEL staining of WJ-MSC-CM-treated human normal skin fibroblasts versus control (non-WJ-MSC-CM treated) normal skin fibroblasts showed no significant difference in induction of apoptosis (C, quantified in E). Note that the total number of viable cells was significantly higher in the WJ-MSC-CM-treated cells comparing to the non-WJ-MSC-CM treated cells
(D, $p \leq 0.001$). (n=3 samples each group). *** = $p \leq 0.001$.

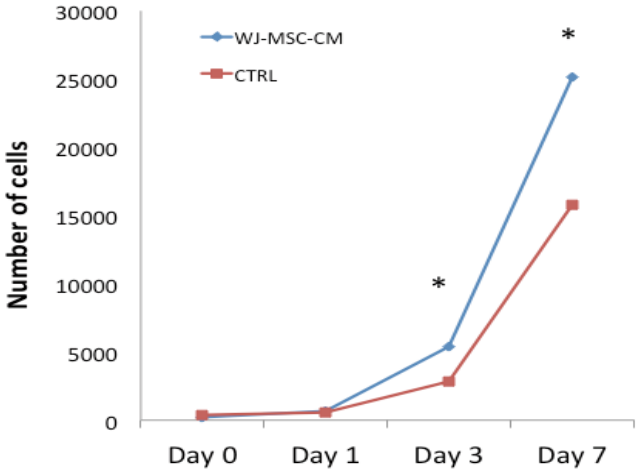

Figure 26: WJ-MSC-CM enhances human normal skin fibroblast proliferation.

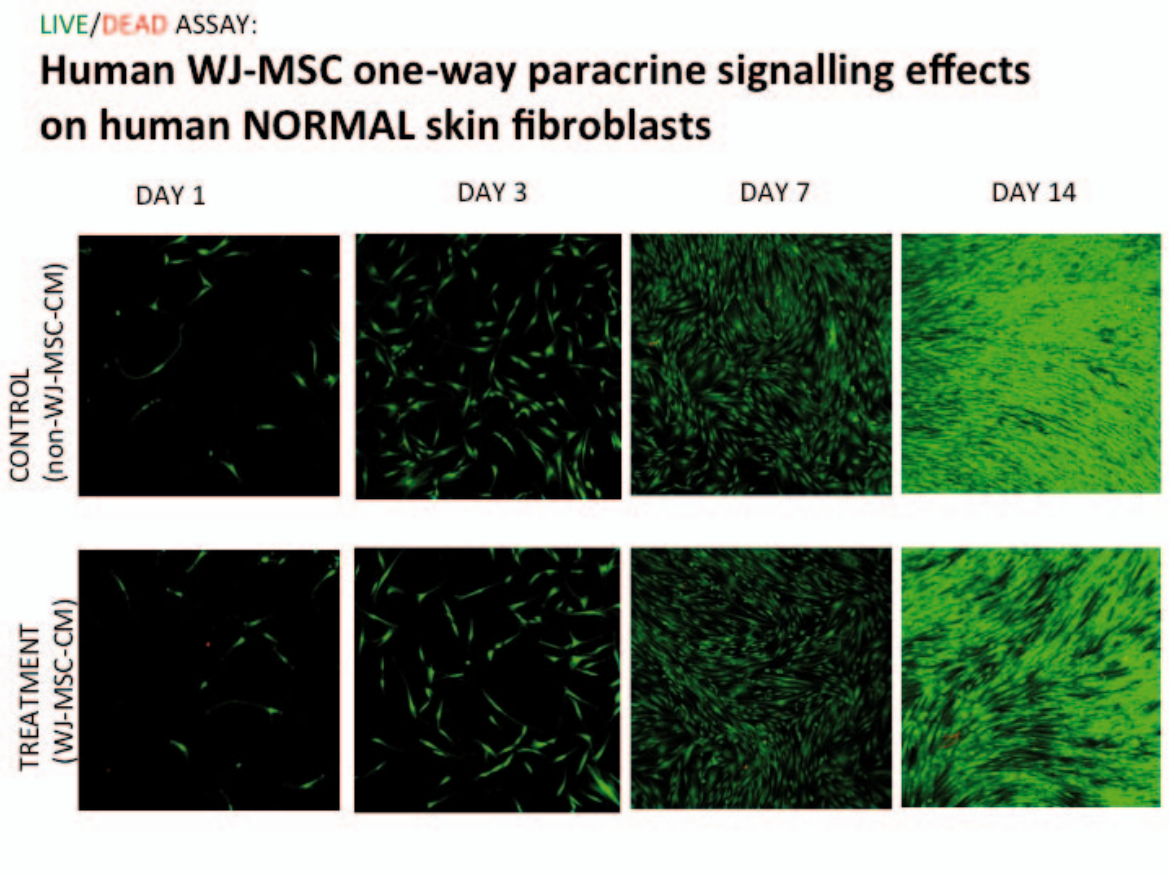

Figure 27: Human normal fibroblasts remain viable and proliferate with WJ-MSC-CM.

4. WJ-MSC-CM promoted normal skin fibroblast migration and wound closure.

To further examine if the enhanced proliferation of fibroblasts treated with WJ-MSC-CM could also promote wound closure, a wound scratch assay was performed. Under the *in vitro* culture conditions of this study, WJ-MSC-CM-treated normal skin fibroblasts coapted wound borders faster than normal skin fibroblasts from the control group (that is, treated with non-WJ-MSC-CM), suggesting that WJ-MSC-CM induced enhanced migration activity and wound closure in human normal skin fibroblasts (Figure 28 A and B).

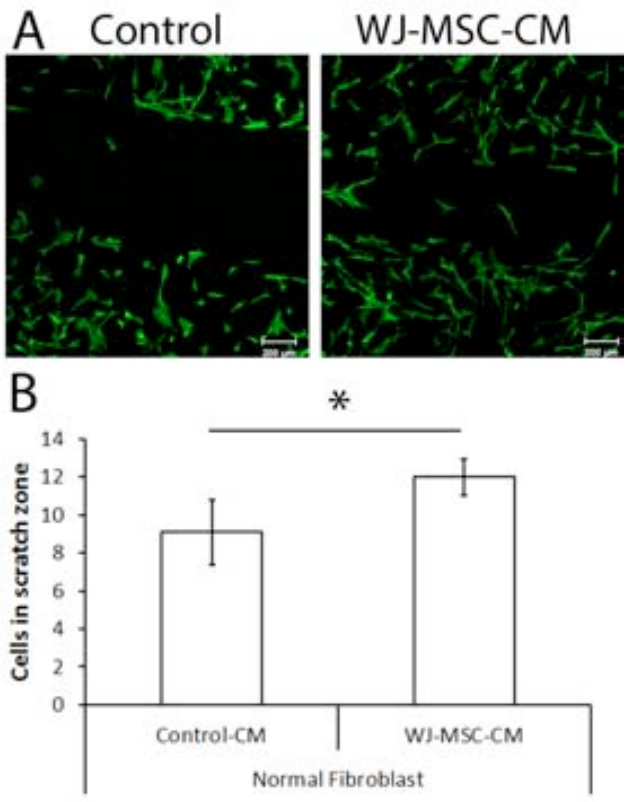

Figure 28: WJ-MSC-CM accelerates wound closure *in vitro*.

A, B) Scratch wound assay was performed to examine migration properties of WJ-MSC-CM-treated and untreated normal skin fibroblasts. The treated group showed significantly enhanced migration rates and coapted wound borders faster than the control group. * $p \leq 0.05$.

5. WJ-MSC-CM promoted wound healing and repair in a mice model.

Next, we examined if the observed *in vitro* wound healing promoting effects with WJ-MSC-CM might be translated into an *in vivo* wound healing model. BALB-c mice WJ-MSC-CM-treated wounds showed enhanced wound healing rates compared to the control mice ($p \leq 0.05$) (Figure 29 A and B, quantified in C).

In order to delineate the pro-proliferative effect of WJ-MSC-CM *in vivo*, one dose of BrdU was injected intraperitoneally. Both higher number of cells and higher amount of proliferative cells were found in the WJ-MSC-CM-treated-wounds ($p \leq 0.05$ and $p \leq 0.01$, respectively), after BrdU intraperitoneal injection (Figure 30 E and F). A microscopic wound cross-section showed higher number of positive proliferating nuclei (black arrows, BrdU positive cells) ($p \leq 0.01$) in the WJ-MSC-CM treated wounds (Figure 30 D) compared with the control ones (Figure 30 C), as well as general increased cellularity, matrix remodelling and overall wound repair. Higher magnification images corresponding to the aforementioned detailed micrographs of both control and treated wounds were shown in Figures 30 A and B, respectively.

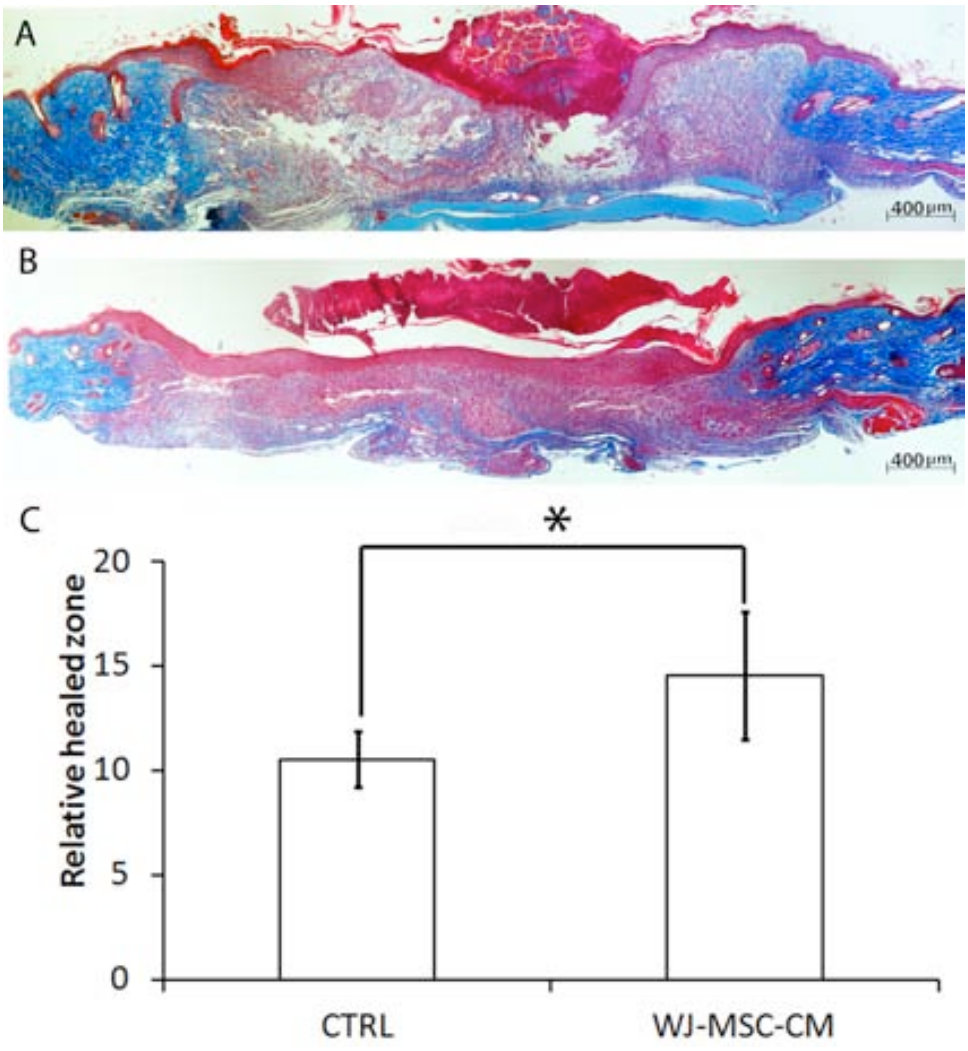

Figure 29: WJ-MSC-CM enhances wound healing in an *in vivo* mouse model.

A mice wound healing model was used, and animals were wounded and treated, as previously described. Histological sections of wounds and satellite donut area (10X) from BALB-c mice, after 1 week of full-thickness excisional skin wounding and reconstruction with WJ-MSC-CM and vehicle (matrigel®) (B), or vehicle alone (A). Photomicrographs were taken after Mason's Trichrome staining. Increased and complete re-epithelialization, higher cellularity in newly formed granulation tissue, and less random and more organized extracellular matrix were observed in the WJ-MSC-CM-treated wounds, suggesting that WJ-MSC-CM promoted wound healing and repair *in vivo* in mice.

- $p \leq 0.05$; ** $p \leq 0.01$. Error bars represent 95% confidence interval.

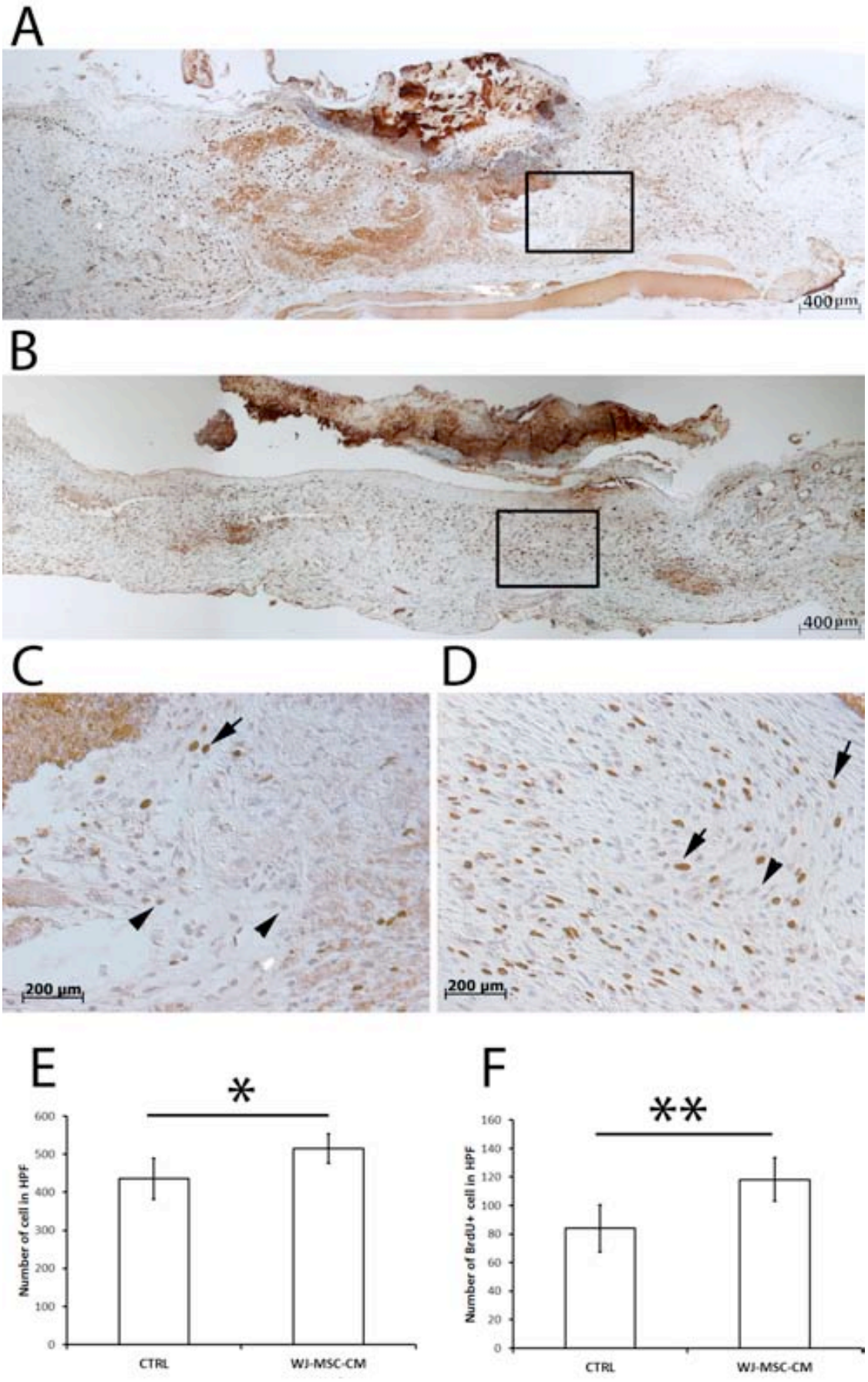

Figure 30: WJ-MSC-CM promotes cell proliferation in an *in vivo* mouse wound healing model.

A BALB-c mouse wound healing model was used and animals were wounded and treated, as previously described. Animals received one dose of BrdU intraperitoneally 24h before harvesting of wounds. Four animals were included in each group, and 4 wounds of 4 mm diameter each were performed per animal (total of 16 wounds in each group). Cutaneous tissue specimens were stained for BrdU in both groups, control (A) and treatment (B). Enhanced magnification (40X) of the above microscopic images were included for non-conditioned medium treated (C) and WJ-MSC-CM-treated normal skin fibroblasts (D) to examine in further detail the increase in cell number or stained nuclei (black arrows, BrdU positive cells) in the WJ-MSC-CM-treated wounds, compared to controls. This denoted that WJ-MSC-CM stimulated cell proliferation in vivo (F, ** $p \leq 0.01$). Together, these results suggested that WJ-MSC promoted wound healing and repair by one-way paracrine signalling in an *in vivo* preclinical model. * $p \leq 0.05$ and ** $p \leq 0.01$. Error bars represent 95% confidence interval. Arrows show BrdU+ nuclei, while arrowheads indicate BrdU- ones.

5. DISCUSSION:

5. 1. Fibrosis (Keloid scars):

The results of this study suggested that, under the current culture conditions, the indirect application of human WJ-MSCs (via conditioned media or a microporous membrane) on human keloid fibroblasts promoted gene characteristics of fibrosis, while the direct-contact application of WJ-MSC produced the opposite effects and might therefore be helpful in the management of keloid scars.

Keloids remain a clinical challenge and, as of yet, there exists no efficacious treatment and research is now focused on their pathophysiology at the molecular level [106, 173]. It has been published that human fetal scarless healing is mainly due to a predominant *TGF-β3* shift versus the pro-fibrotic sibling cytokines *TGF-β1* and *TGF-β2* [174, 175].

This study has indeed found very significant lower levels of *TGF-β3* in human skin fibroblasts cultured with WJ-MSC-CM (or "one-way indirect co-culture") and almost significant when co-culturing both cell types via an insert transwell system, suggesting that WJ-MSC paracrine signalling may actually promote fibrosis.

Along the same lines, this study also showed that *TGF-β2* expression was upregulated under the same culture conditions. However, *TGF-β1* mRNA transcript did not mimic that tendency and it turned out to show a slightly decreased signal, although it was not significantly modified.

The same trend with both TGF-β1 and TGF-β2 proteins, whose expression was not significantly changed, but an enhanced signal in both one-way and two-way paracrine signalling systems was detected. These differences could be explained by incidental culture conditions [176], disease heterogeneity even intralesionally [177] or, it might be hypothesized, that *TGF-β2* and not *TGF-β1* might appear to be the most consistent *TGF-β* fibrotic gene marker in human skin, as previous studies with keratinocytes have suggested [178]. Indeed, it has been advocated that the most upregulated *TGF-β* isoform in keloids is usually *TGF-β2* and not *TGF-β1* [179, 180], although *TGF-β1* is often also increased [122, 181] and is generally representative of the *TGF-β* superfamily and typically referred to as *TGF-β* in general.

Controversy has recently been aroused regarding the classically believed anti-fibrotic role of TGF-β3. Naitoh M et al also found higher levels of TGF-β3 protein in keloid samples [182], and Lee TY et al encountered similar levels of the mentioned peptide in 3 keloid and 3 normal fibroblast samples [183]. This could explain why despite our findings of an insignificantly low *TGF-β3* transcript signal in our cell-cell direct co-culture experiments, all the other cytokine results were pointing towards an anti-fibrotic role for WJ-MSCs.

Although *TGF-β1* is the most predominant and studied *TGF-β* isoform, and it holds the first master position in the fibrotic marker ladder in many diseases, *TGF-βs* themselves may not be ideal *specific* fibrotic markers [184-188]. TGF-βs form a complex superfamily of peptides with pleiotropic functions which are cell-context dependent. They are involved in normal embryogenesis and also in pathological states with epithelial-to-mesenchymal transition or EMT, like fibrosis and cancer [189]. TGF-β regulates many signalling pathways, and is involved in various crosstalk interactions with other cytokines and peptides [187, 190, 191].

Regarding fibrosis, TGF-β appears to act as an upstream mediator in the synthesis of CTGF, PAI-1, HIF-1-α and VEGF, all well-known promoters of pathological fibrosis, including excessive scars, i.e. keloids [188, 192, 193]. Due to the recent controversy on TGF-β isoforms, their cell-context dependency, paradoxical functions, and pleiotropic and mutual interaction with MSCs (TGF-β regulates the recruitment of MSC in tissue remodelling [194], as well as adult MSC proliferation, self-renewal, multipotency and differentiation [195, 196]), those *TGF-β* downstream targets might work as more suitable fibrotic markers when studying MSC effects. They have all been involved in both inflammation and fibrosis, and are overexpressed in keloid tissue [188, 197, 198]. Among all of them, *PAI-1* is considered to be necessary but not unique to develop a keloid scar, playing a fundamental and major role in keloid pathogenesis [192, 199-203].

This study showed that *PAI-1* levels were increased in human keloid fibroblasts when cultured with WJ-MSC-CM, or when co-cultured using a microporous membrane, suggesting the pro-fibrotic paracrine effect of WJ-MSCs. It has been reported in the literature that not only *TGF-β1*, but also *HIF-1-α,* induce *PAI-1* upregulation in keloid fibroblasts [204]. This effect is triggered by *VEGF*, a *HIF-1-α* target gene [205]. *HIF-1-α*, in turn, may upregulate *TGF-β1* expression [206], and influence MSCs. *HIF-1-α, VEGF* and *PAI-1* are all overexpressed in keloid fibroblasts [207].

This study found increased transcription levels of all of those fibrotic markers in keloid fibroblasts treated with WJ-MSC-CM.

TGF-β1, *VEGF* and *HIF*, among others, upregulate in turn *CTGF* or *CCN2*, another known pro-fibrotic marker, initially described as a "PDGF-related mitogen" [208, 209].

The observed trend of an upregulated expression of *CTGF* and most probably the increased transcript levels of *PAI-1 and TGF-β2,* promoted by WJ-MSC-CM, might explain the enhanced keloid proliferation effects observed in this study.

CTGF is activated by *TGF-β1*, *angiotensin-II (Ang-II), endothelin-1, HIF-1-α, VEGF*, and others [210, 211]. It has been described that the unfrequent co-existence of *TGF-β* activation with inhibition of *CTGF* yields to a net anti-fibrotic effect, suggesting that *CTGF* may prevail over *TGF-β* in specific conditions [122].

This may partly also explain why we found an increased trend in *CTGF* with a decreased trend in *TGF-β1* transcript expression. However, *CTGF* has also been reported to be indispensable for the *TGF-β*-induced phosphorylation of Smad1 and Erk 1/2 (extracellular signal-regulated kinase 1/2) in systemic sclerosis, another skin fibrotic condition. What seems clearly accepted is that *TGF-β1-Smad3*-induced fibrosis may occur independently of *CTGF* [212].

Although we did not study in detail further *TGF-β* canonical or non-canonical mechanisms, the latter statement would agree with the observed gene expression results in the transwell insert co-culture system. In this case, a decreased *CTGF* trend with a slightly increased *TGF-β1* trend was found. More importantly, the upregulation of other fibrotic markers, including *TGF-β2, PAI-1* and *HIF-1-α,* was observed in the same culture conditions. Furthermore, it has been suggested that *CTGF* might exert fibrotic or anti-fibrotic effects depending on the concentration [212]. Together, all of this highlights the complex molecular mechanisms underlying *CTGF* in extracellular matrix regulation [212].

FGF-2 is another growth factor involved in wound healing and angiogenesis which promotes scarring (although with controversial reports) and is overexpressed in keloid fibroblasts. It decreases *decorin* and increases *collagen* levels [188, 213, 214]. This study showed upregulated *FGF-2* transcript levels in WJ-MSC-CM-treated keloid fibroblasts.

In the culture conditions of this study, WJ-MSC-CM increased the transcript expression of the pro-fibrotic markers *PAI-1*, *HIF-1-α, VEGF, TGF-β2,* and *FGF-2*, with lower amounts of the anti-fibrotic *TGF-β3* transcript. An upregulated trend in *CTGF* transcription, and TGF-β1 and TGF-β2 protein expression, with enhanced IL-6 and IL-8 protein levels, was also found. Furthermore, enhanced keloid fibroblast proliferation was induced by WJ-MSC-CM. Together, this orchestrated and suggested a coordinated pro-fibrotic response [207, 215]. Indeed, WJ-MSC paracrine secretome induced a fibrotic phenotype in keloids, under the culture conditions of this study. This finding agrees with the pro-fibrotic paracrine signalling effect of BM-MSCs in human normal skin fibroblasts [216] previously reported by Tredget E. and colleagues.

On the other hand, and interestingly, we have shown that the direct co-culture of WJ-MSCs and keloid fibroblasts had an opposite gene expression character, eliciting anti-fibrotic effects under the same culture conditions. Why did we find such difference between paracrine and cell-cell direct human WJ-MSCs effects on keloid fibroblasts? Although we did not study that in further detail, we hypothesize that cell contact might be the underlying responsible mechanism. Inhibition of cell contact is linked to epithelial-to-mesenchymal-transition (EMT), a normal development process that is also linked to pathological fibrosis and cancer [189], TGF-β and β-catenin signaling pathways, and cell cycle regulation [217].

Accumulating evidence supports that all the aforementioned factors play a pivotal role in keloid pathogenesis [184-188, 218]. Besides that, and from a more general point of view, it has been reported that MSCs promote wound tissue repair by paracrine signalling (that means with no direct application of cells, but through MSC-derived-products, such as conditioned medium), whereas the direct application of MSCs particularly involves tissue regeneration through differentiation [128]. However, the underlying *in vivo* mechanisms still remain largely unknown [219].

Skin scarless regeneration in humans only occurs in the initial stages of the embryologic and fetal development, and it has been reported that the fetal fibroblast, with its special secretome, is responsible for this anti-fibrotic effect. The human umbilical cord starts to develop around the fifth week of gestation. Conceivably, it might well be possible that WJ-MSCs, which represent a fetal non-embryonic (adult) MSC source, may express a more scarless-like-phenotype than other adult MSC sources and, when directly co-cultured with keloid fibroblasts, regulate the altered keloid niche and differentiate into more scarless-like fibroblasts (or, at least, into non-keloid, or normal adult skin fibroblasts, which would overexpress less fibrotic markers). Further research is needed to study WJ-MSC differentiation in keloids.

Indeed, MSCs themselves have been considered to have immunosuppressive and anti-fibrotic properties [30, 104, 220, 221]. However, this latter statement is too general and it is still surrounded by controversy.

Mesenchyme is the embryologic layer which gives rise to many cell lineages including fibroblasts, the heteregenous cell type responsible for extracellular matrix deposition [105]. Fibrosis evolves from an excess of extracellular matrix synthesis or a defect on its destruction.

A particular fibroblast type, the myofibroblast, has classically been considered as the main cell type responsible for skin tissue fibrosis. It has been shown that myofibroblasts in hypertrophic scars have decreased apoptotic rates, and therefore excessive scars are characterized by an overproduction of extracellular matrix [222]. In the WJ-MSC-CM-treated keloid fibroblasts, we did not find significant apoptosis changes. Alpha-smooth muscle actin (α-SMA) is the main myofibroblast marker, but MSCs also share this mesenchymal marker. Some researchers hypothesize that myofibroblasts could well be MSCs. The fact that α-SMA is also expressed in MSCs raises this possibility. It has been suggested that MSCs from the subcutaneous fat might be responsible for the accumulation of collagen in excessive scars [105].

Other published reports also discuss the role of MSCs in keloid pathogenesis [103, 104, 223] . Moon et al. isolated a population of keloid-derived mesenchymal-like stem cells (KMLSCs) from keloid scalp skin. They considered them to be equivalent to skin derived precursors, or fibroblasts; however, altered fibroblasts that expressed a pathological and specific cytokine milieu [103].

Iqbal et al. suggested that fibrocytes in keloid tissue may represent abnormal hybrid mesenchymal/hematopoietic cells which could function as targets for scar prevention and treatment [223]. Similarly, malignant tumors of the skin, such as squamous cell carcinomas (SCC), have been described to contain tumor-initiating cells or cancer stem cells [224]. Accordingly, benign tumors of the skin, such as keloids, may also contain keloid-derived stem cells, which would be responsible for their persistent growth and recurrence, but without long-distance invasion or metastasis [225]. In fact, Akino et al. concluded in a study that resident and human MSCs may be involved in keloid pathogenesis [104].

All these facts go in hand with our WJ-MSC paracrine signalling findings, but not with our cell-cell direct contact effects.

Although it still has to be proven if our *in vitro* results might be extrapolated to an *in vivo* setting, the first clinical trials with MSCs go in hand with our results. Indeed, all published MSC clinical trials to date hold great promise in treating several kinds of soft-tissue fibrosis [220, 221, 226, 227]. It is conceivable, therefore, to suggest that this would be in favour of the anti-fibrotic effect of the WJ-MSCs themselves, as this study showed. This leads to the important remark to differentiate between stem cell “per se” or cell-cell direct effects, and niche-driven or paracrine effects, the ever controversial but interesting argument between stem cell biologists and tissue engineers, which might be eventually translated into a personalized-regenerative-medicine therapy modulating the stem cells or the niche cytokine milieu.

This study showed that human WJ-MSC-CM significantly enhanced keloid fibroblasts proliferation and also promoted some genomic and proteomic fibrotic characteristics, with no increase in apoptosis. On the contrary, WJ-MSC direct cell-cell effects caused an anti-fibrotic phenotype in keloid fibroblasts.

If the results observed under the culture conditions of this study are corroborated in further preclinical *in vivo* models, WJ-MSCs might become a keloid management strategy, while WJ-MSC-CM per se may be detrimental.

Subsequently, manipulation of WJ-MSC-CM, through inhibition or removal of undesired fibrotic secretome components, and further induced replacement with added anti-fibrotic signals, could also become an alternative keloid therapy for those patients who don't tolerate cell therapies.

WJ-MSCs may represent a new treatment strategy to manage keloids, but further research is warranted.

CONCLUDING REMARKS:

In the culture conditions performed in this study, human WJ-MSCs had a pro-fibrotic paracrine effect on human keloid skin fibroblasts, whereas a direct cell-cell contact caused anti-fibrotic effects. Therefore, WJ-MSCs may play a paradoxical role in keloid management, becoming a double-edged sword. Future studies will confirm whether the intralesional injection of WJ-MSCs may become a regenerative medicine technology to manage keloid scars.

5. 2. Burn wound healing and burns:

The results of this study suggested that WJ-MSCs might enhance burn wound healing and repair by paracrine signalling mechanisms. Under our culture conditions, human WJ-MSCs promoted the gene expression of some wound healing factors (*PAI-1*, $p \leq 0.05$) and enhanced migration and wound healing *(*$p \leq 0.001$) in burned skin fibroblasts *in vitro*. Similarly, and as it will be described in the next section, WJ-MSC-CM accelerated the re-epithelialization rate *in vivo* in a full-thickness excisional mouse wound healing model.

Burns represent the clinical scenario with maximum wound healing requirements to guarantee patient's survival. Skin is the largest organ in the human body and its barrier function plays a vital role.

However, major burns are a form of severe systemic trauma, ischemia-reperfusion injury, endoplasmic reticulum stress and inflammasome activation where the epidermal barrier is usually lost, alterations in the inflammatory cascade are present, immunosuppression and reduced leukocyte migration to the wound bed are encountered, and profound ischemia or even irreversible necrosis is found in the burn wound itself and even sometimes in other vital organs, such as heart, liver and gut [228-229].

Restoring the natural skin barrier or providing adequate wound coverage after burn debridement improves prognosis, decreases infection rates and saves lives. However, major burn patients are deprived of enough autologous skin tissue to cover their large burned areas. Despite the use of allografts, cultured epithelial autografts (CEA) and other more modern skin substitutes and wound dressings, to date there is no complete skin replacement clinically available [30, 230].

Therefore, it is conceivable to consider other alternatives, such as seeding those debrided but still uncovered wound areas with local growth factors that promote angiogenesis (like VEGF), re-epithelialization (like FGF-2) and fibroproliferation (like PAI-1, and collagens). These growth factors and proteins may be delivered to the burn wound bed through stem cell therapy technology. Although the best approach is to replace like with like and thereby use autologous techniques whenever possible.

The fact that autologous skin and fat tissue is also burned and necrotic in full-thickness burns, and that the successful transplantation of burned skin- or burned adipose-derived MSCs appears unlikely (this still needs to be tested in patients), it might seem feasible to consider the next available and popular autologous MSC source, namely the BM-MSCs. However, the obtainment of autologous BM-MSCs in a severe burned patient would compromise his general status and might be detrimental.

Therefore, we propose the use of a reported autologous (in the case where it was kept in a cord bank for the lifetime of the patient) or homologous but immunoprivileged, universal and largely available MSC source, such as the umbilical cord (UC).

From all the different possible MSC types which might be isolated from UC, WJ-MSCs represent the one with greatest yield, among other advantages. Conceivably, we hypothesize that delivery of WJ-MSCs (and consequently their secretome) into burn wounds would promote wound healing.

It has been suggested that MSC paracrine signalling is the main mechanism responsible for enhanced wound repair. Subsequently, we first studied the paracrine signalling effect of WJ-MSCs on wound healing response markers on human burned skin fibroblasts.

This study only found upregulated transcript levels of *PAI-1* in the burned skin fibroblasts co-cultured with WJ-MSCs through a transwell insert. Although the other genes were not significantly modified, an increased trend in the expression of *TGF-β2, VEGF, PAI-1, HIF-1-α, FGF-2, type III collagen* and *decorin*, with decreased signal of *TGF-β3*, was observed when subculturing burned skin fibroblasts with WJ-MSC-CM ("one-way paracrine signalling system"). In the transwell-insert or two-way paracrine signalling co-culture model, we also studied some of the main wound healing cytokines, and found similar results, with no significant difference but albeit a consistent upregulation in the transcript levels of *TGF-β1, TGF-β2, VEGF,* and *HIF-1-α*, with decreased *TGF-β3*.

WJ-MSC-CM did not affect burned skin fibroblast apoptosis, but it increased their migration to a significant extent, accelerating burned wound healing. In the human burned fibroblasts cell-cell direct co-culture with human WJ-MSCs, we found downregulated levels of *TGF-β1, TGF-β3* and *CTGF*, with consistently decreased trends in all the other aforementioned cytokines and growth factors.

It has been previously documented that, in contrast to paracrine signalling, MSCs per se promote wound regeneration with no or minimal scarring, mainly due to MSCs differentiation, instead of wound repair [128]. According to our results, we suggest that the direct application of WJ-MSCs to wounds may aid in modulating the wound healing response and abrogate excessive fibrosis, being a "qualitative wound healing promoter", whereas WJ-MSC-CM might enhance wound re-epithelialization and closure, functioning more as a "quantitative wound healing promoter".

Although these results would need to be supported with further experiments with higher sampling rates and eventually, with *in vivo* studies, this observed difference *in vitro* could have important translational implications. It is conceivable to hypothesize that in acute burn wounds, WJ-MSC-CM might be therefore first used to ensure fast wound coverage, and later on, when the fibroproliferation phase is about to enter its mid-phase, adding WJ-MSCs directly to the wound bed might contribute to achieve adequate healing. However, optimum timing for stem cell therapy still remains rather unknown and deserves further research.

Similarly, the systemic effects of the aforementioned cytokines should also be studied *in vivo* in other important organelles and organs in burns, and not only in the wound healing setting, which was our aim for this study, due to their pleiotropic crosstalk character. For instance, during wound healing, upregulated levels of PAI-1 help accelerate wound healing. On the contrary, under pathological conditions such as keloids, PAI-1 contributes to enhance an excessive scarring phenotype [192, 199-203]. On the other hand, PAI-1 deficiency has also been reported as detrimental. PAI-1, a pivotal thrombosis regulator which is increased in multiple organ fibrosis –such as liver [231], one of the master organs that determines burn prognosis-, is also a cell senescence [232] and cardiovascular [233] risk marker, and an adipokine that plays a pivotal role in obesity, diabetes, insulin resistance and inflammasome activation [233-235].
Although it has been reported that excessive levels of PAI-1 might be downregulated with vitamin D [236], the hypermetabolic state with insulin resistance in major burns requires further metabolic studies analyzing the systemic effect of WJ-MSCs in burns, to give a complete new insight before administering this therapy in a clinical setting.

As any cell therapy, the use of MSCs themselves and not MSC-derived-products, such as CM, is associated with increased risk for human patients, and therefore it requires more costly, complex and long-lasting pre-clinical studies, special research and pharmacological development in controlled Current Good Manufacturing Practices (cGMPs) facilities, and controversial ethical and governmental health authority approval.

Therefore, we decided to first study the WJ-MSC-CM effect in a translational experimental animal model with a normal wound (simpler, easier and in this case, mandatory to carry out before getting approval from the Ethics Committee for burn wounds).

Indeed, enhanced wound repair rates were found in the WJ-MSC-CM-treated mice compared to the non-treated ones, as will be described in the next section.

This is the first report on the use of WJ-MSCs in burns, and in human burned skin fibroblasts, and it offers interesting and useful preliminary data. This pilot study suggested that WJ-MSC might hold promise in burned wound healing and repair by paracrine signalling, but further research is warranted.

CONCLUDING REMARKS:

In summary, under the culture conditions of this study, human WJ-MSCs promoted burn wound repair by paracrine signalling. Cell-cell direct WJ-MSC therapy might aid in controlling the fibroproliferative phase of wound healing, and promote scarless or minimal scar wound healing. Only with further research the role of WJ-MSCs in burns will be completely deciphered.

5. 3. Normal wound healing :

The results of this study suggested that WJ-MSCs enhance wound healing and normal wound repair by paracrine signalling mechanisms. Under these particular culture conditions, human WJ-MSC-CM upregulated the gene expression of wound healing factors in human normal skin fibroblasts, and promoted human fibroblast proliferation and migration to coapt wound borders *in vitro*. Accordingly, WJ-MSC-CM accelerated the re-epithelialization rate and promoted wound repair *in vivo* in a full-thickness excisional murine wound healing model.

Cutaneous wound repair is a complex orchestrated process that is activated upon injury and includes the multicellular overlapping and coordinated phases of inflammation, angiogenesis and formation of granulation tissue, re-epithelialization, and fibroproliferation or matrix formation and remodelling [47, 237, 238].

Decreased pro-inflammatory cytokines, compromised neovascularization and/or impairment in leukocyte recruitment might disturb and delay wound healing [239]. Patient comorbidities, including diabetes, immunosuppression and other chronic or aging-related diseases, which are highly prevalent, especially in developed countries, as well as malnutrition in the developing world, are all well known to delay wound healing [135, 239-242]. Smoking also impairs skin vascularization and wound healing [243].

Despite the current use and availability of a wide array of wound dressings, ointments and devices, wound healing still remains a clinical challenge in these particular pathological settings, but also in other conditions affecting prior healthy patients, such as massive trauma or major burns [244, 245]. Therefore, there is a need for new strategies to promote or at least coadjuvantly help in wound healing and repair.

Regenerative medicine has emerged as a new therapeutic modality with pleiotropic clinical applications, including wound healing [20, 139]. Skin MSCs populate the normal skin niche, remain quiescent and become active after injury, aiding in wound closure [47].

As lasers appeared as a new promising technology that revolutionized the medical and surgical field many years ago as a means to cut or remove undesired cells [246-250], stem cells seem to play a current similar and pivotal role, albeit to reconstruct and rebuild tissue, rather than to remove. Between them, MSCs have been shown to promote wound repair in multiple preclinical and a few recent clinical studies [47, 101, 143]. It has been suggested that MSC paracrine signalling is the main underlying mechanism for their enhanced wound repair effects [101, 128, 129, 144, 233, 251, 252]. BM-MSCs have been reported to promote wound healing, but their isolation requires an invasive and artificial method [113, 134]. Harvesting adipose-derived-MSCs also requires a surgical procedure.

Subsequently, we focused on a more advantageous MSC source, the umbilical cord-derived Wharton's jelly [113, 253]. The harvest of WJ-MSCs is not painful or invasive, as they are isolated naturally with no extra surgeries and dissected from discarded umbilical cords after birth. Indeed, caprine WJ-MSCs have already been shown to promote wound repair with minimal scarring [159], but there are no reports of human WJ-MSC on human skin wounds, to our knowledge.

WJ-MSCs represent a very efficient stem cell source with reported immunoprivileged, anticancer and antifibrotic characteristics in animal models [113, 253, 254]. Due to their reported immunoprivileged properties and universal and ever-lasting availability [148], their allotransplantion as an off-the-shelf therapy may represent an appropriate treatment strategy in the already compromised patients who suffer of recalcitrant cutaneous wounds [30]. Therefore, this study aims first to examine the effects of human WJ-MSC paracrine signalling on human normal skin fibroblasts.

In our culture conditions, human WJ-MSCs enhanced the expression of some wound healing promoting genes, including re-epithelialization, neovascularization and fibroproliferation inducing genes, in human normal skin fibroblasts. Besides altering skin fibroblasts gene expression, MSC signalling has also been shown to positively regulate cell survival, proliferation and migration [128, 255].

Under our culture conditions, although apoptotic changes were not found in normal skin fibroblasts treated with WJ-MSC-CM, significantly enhanced proliferation and migration were observed. This is important as cellular dynamics and cell migration is an essential step during cutaneous healing.

WJ-MSC-CM accelerated wound closure both *in vitro* and in an *in vivo* mouse wound healing model, suggesting that human WJ-MSC-CM may promote wound repair. Other researchers have also reported enhanced wound healing rates in murine models with other human MSC sources, such as BM-MSCs [145, 128], and umbilical cord blood-derived MSCs [256]. Blood of the umbilical cord has been reported to yield fewer MSCs than the cord itself, though [256] and therefore, Wharton's jelly may emerge as a more appropriate stem cell source.

To the best of our knowledge, no published studies regarding the use of human WJ-MSCs in human wounds have been reported, so this work is novel, and could prompt us to test if the same results would be encountered first in a more appropriate but more costly experimental model, such as the red duroc pig. Secondly, and if the results appeared to be promising, the next step would be to translate them into phase I-II clinical trials for further evaluation and development. Indeed, pilot clinical studies have so far pointed out that MSCs in general are safe *in vivo*, and they currently represent the most widely used stem cells in the clinical setting [219]. Accordingly, in the particular case of WJ-MSCs, 15 diabetic patients received systemically WJ-MSC with no documented relevant safety concerns [155].

Furthermore, many MSC clinical applications to manage intestinal fistulae, acute artery ischemic disease in diabetic patients, and periodontal defects have been reported, among others [101]. Anecdotically, one case report describes the use of autologous BM-MSCs to help to heal a scapular recalcitrant wound in a Russian burned patient [257], but no reports regarding the use of WJ-MSCs have been reported yet.

Although it has been described that WJ-MSCs secrete a more angiogenic secretome in comparison to BM-MSCs [258], and that the isolation of WJ-MSCs is more efficient, and WJ-MSCs have a higher capacity for proliferation and are less senescent than ADSCs [259], proper studies comparing the wound healing abilities of different MSC sources are still lacking in the literature.

A new preclinical and clinical research arena investigating the potential of WJ-MSCs in wounds and wound healing is born and may hold promise for future medical therapies.

CONCLUDING REMARKS:

Human WJ-MSCs promoted wound healing by paracrine signalling in our culture conditions *in vitro*, and in an *in vivo* preclinical animal model. If the reported immunoprivileged character and safety of WJ-MSCs observed in experimental and first clinical models is further scientifically proven, WJ-MSCs might represent a feasible, universal and off-the shelf technology to enhance normal wound healing to improve patient survival and quality of life.

5. 4. Anti-aging:

The results of this study suggest that, under our culture conditions, WJ-MSCs may prevent skin aging effects and repair skin by paracrine signalling.

We have shown that human WJ-MSC-CM upregulates *HIF-1-α* and increases *VEGF* signal gene expression in human normal skin fibroblasts. *HIF-1-α* and *VEGF* represent two of the main factors lacking the most in aged wounds, as well as in aging-related disease derived-skin lesions, such as diabetic wounds [260]. Other sibling downregulated factors, such as *ANGPT2, PDGF-B* and *PLGF,* have also been described in aged wounds [260]. All these cytokines and growth factors are involved in angiogenesis and vascularization, such as *FGF-2* and *TGF-β2*. *TGF-β2* transcript levels were significantly increased in WJ-MSC-CM treated normal skin fibroblasts, whereas *FGF-2* levels showed an upregulated signal. FGF-2 is a re-epithelialization promoting factor which has also been involved in ECM remodelling.

Skin aging in vivo is characterized by an alteration of ECM, due to a lack or progressive loss of mechanical tension. This further promotes fibroblast collapse, with consequent irreversible downregulation in collagen levels and enhanced metalloproteinases (MMPs) or collagen-degrading-enzymes levels. In fact, skin aging has been compared to a “fragmented” or disorganized collagen structure, where decreased CTGF levels cause downregulation of both collagen type I and collagen type III expression [110].

Under our culture conditions, all three above mentioned genes, *CTGF*, *collagen type I* and *collagen type III*, showed an upregulated signal in normal human skin fibroblasts after treatment with WJ-MSC-CM, whereas *collagen type III* was enhanced in the two-way paracrine co-culture system, suggesting another application for WJ-MSC-CM for anti-aging.

Besides the close inverse relationship between wound healing growth factors and cytokines and aging, other pathophysiologic molecular processes have been also reported to be responsible for the aging process. Among them, telomere dysfunction, enhanced reactive oxygen species (ROS) and superoxide dismutase 2 (SOD2), metabolic mitochondrial alterations, and cellular senescence are encountered [261]. It is noteworthy that the superfamily of sirtuins (SIRTs) appears to be the master modulator of all aforementioned aging triggers [262].

As for cell senescence, this term has been described as a paradoxical and complex term in the literature. Whereas there is general consensus that senescent cells accumulate with aging, there are still controversies about their effects. Some researchers believe that the secretome of senescent cells would be detrimental and should be blocked, or the cells should be cleared, to avoid age-related damage. However, others believe they are activated only upon stress to initiate tissue repair as a survival and protective response through their secretome, and hence would be beneficial to naturally select surviving individuals. Paradoxically, this effort is considered “wasted” or inefficient in elderly people, if we take into account classical aging theories.

Only if this stress self-defense response lasts too long and becomes chronic, it is considered to become detrimental, and therefore cause damage and cellular aging, tissue degeneration and cancer progression [167, 168].

PAI-1 has recently been recognized as a cell senescence marker, with complex interactions and tissue-specific and dose-dependent pleiotropic functions [263, 264]. PAI-1 is a downstream target of the anti-apoptotic and tumor-suppressor protein p53, which plays an important role in keloid, haematopoietic alterations and metabolic syndrome pathogenesis, as well as in wound healing. It is secreted by various MSCs from different sources.

The closest related sibling cytokine, PAI-2, has also been described to be released by birth-related-tissues-derived MSCs at early passages [265]. Our findings demonstrated higher PAI-1 transcript levels in the WJ-MSC-CM treated normal skin fibroblasts. PAI-1 has also been reported to be upregulated in completely closed wounds. Therefore, we suggest that these enhanced PAI-1 levels would represent higher wound healing rates. However, it might also be possible that PAI-1 senescence effects would promote autonomous cell apoptosis but proliferation of satellite cells by paracrine signalling, as some researchers have hypothesized, and globally ensure a complete wound healing and tissue repair response. Accordingly, levels of its upstream trigger, p53, have been described in the literature for forensic wound age estimation.

Uninjured skin shows no significant number of p53 expression, while wounds of at least 3 days of age show p53 positive fibroblasts, which grow in number after one week, demonstrated by immunohistochemistry. However, non-detectable p53 levels have also been reported in older wounds [266].

Recently, sirtuins have also been involved in aging research and have gained much attention, due to their beneficial health and skin wellness effects [261, 267-269]. Resveratrol, for instance, is a popular nutriceutical which activates sirtuins, among others, promoting survival upon stress [270]. Sirtuins represent a large family of NAD+-dependent molecules with ubiquitious cellular location. Sirtuins 1, 6 and 7 are considered to be nuclear, although the former one shuttles to the cytoplasm. SIRT2 is cytoplasmic, whereas the remaining SIRT3, SIRT4 and SIRT5 are located in the mitochondria.

SIRT1 is the most known of the sirtuins and it participates in a large variety of stress responses, such as hypoxic, genotoxic, heat shock, endoplasmic reticulum and inflammatory stress [261, 269]. It has been reported that SIRT1 enhances stress resistance via Heat Shock Factor-1 (HSF-1), HIF-2-α, HIF-1α and p53. It also usually inhibits apoptosis, promotes lipolysis, modulates carbohydrate metabolism, and regulates and maintains mitochondrial respiration to optimize energy harvesting.

Indeed, mitochondrial metabolism alterations play a major role in aging pathophysiology, and sirtuins are important regulators of metabolism and redox state**.** SIRT1 diminishes oxidative stress, insulin resistance, fat depots, inflammation, fibrosis, and protects against ischemia-reperfusion injury [261, 269].

Inflammation is also particularly important, since it has been linked to aging and aging related diseases. SIRT1 inhibits inflammation, one of the main triggers to aging and aging related diseases, via NF-kB suppression. Ultraviolet radiation activates NF-kB and is considered the most responsible agent for skin photoaging. Therefore, SIRT1-targeted therapies may counteract cellular photoaging or tissue damage and may be useful in the anti-aging and cosmeceutical armamentarium [269, 271]. Furthermore, it has been postulated that SIRT1 might mimic the beneficial effects of physical exercise and calorie restriction observed in preclinical models. Moreover, it has been reported that SIRT1 protects cognitive ability and prevents dementia, besides regulating anxiety levels [261].

Under our culture conditions, we have indeed found an increased signal of SIRT1 transcript levels in WJ-MSC-CM human normal treated fibroblasts (SIRT-1 levels were not significant, most probably because of low sample number). Despite the fact that recent studies have shown that SIRT1 activation appears not to affect life span, as opposed to past reports, it has been proved that it counteracts the decline in health that accompanies aging, mainly via the AMPK/SIRT1/PGD-1α/PPAR-γ signaling pathway [261, 272].

Indeed, there is a general consensus in current anti-aging research that the paramount goal of aging is to enjoy a high quality and healthy life, rather than a longer but health-compromised aging.

Although surrounded with controversy, it has recently been shown that SIRT6 might promote DNA repair after oxidative stress and actually increase life span, although it was observed that it caused feminizing effects in male mice, as side effects (which happens to go in accordance with the reported beneficial anti-aging role of estrogens). However, further studies to build more evidence are still needed. What is strongly recognized, notwithstanding, is the fact that aging is characterized by telomere shortening, and SIRT1 and SIRT6 increase telomere length. It has also been reported that sirtuin activity declines with age; therefore, it is conceivable to think that supplementing sirtuins would prevent aging [261, 272].

Accordingly, all these emerging promising SIRT1 properties have pushed pharmacological industries to develop sirtuin mimetics [268] (as SIRT1 effects might probably be shared with the other 5 sirtuins) [261]. The ultimate goal is to fight against age-related diseases and conditions, such as type 2 diabetes, hypertension, artheriosclerosis, dementia, osteoporosis, arthritis, skin aging, and even perhaps cancer. Regarding this latter, however, there is still controversy.

The limitation of this anti-aging study is the small sample size which constrained the statistical power of the experiments. We also did not investigate either telomere length, SOD2, or the complex and still not completely unraveled crosstalks between *SIRT1, p53/PAI-1* and *HIF-1-α*. It has been reported that *SIRT1* and *p53* negatively regulate each other and that *SIRT-1* would deactivate *HIF-1-α*, stimulate *HIF-2-α* and inhibit *NF-kB*. However, hypoxia and all the above mentioned transcription factors and molecules may play specific temporospatial and context-dependent pleiotropic and paradoxical effects, which should be further studied for different degrees of skin aging severity.

Unfortunately, late passage cells and/or aging animal models were not analyzed, but the *in vitro* results that were found, and especially the wound healing promoting effect that was observed with WJ-MSC-CM-treated mice wounds *in vivo*, might prompt to further design new pre-clinical studies to elucidate if it is accompanied by skin anti-aging effects.

Notwithstanding, WJ-MSC paracrine signalling comparison with current skin anti-aging treatments used in the clinic (retinoic acid, CO_2 laser resurfacing and hyaluronic acid) has not been investigated yet.

The novelty of this preliminary work is to present pilot data about WJ-MSCs in skin aging, effects that still remained unreported in the literature. Further research is warranted.

CONCLUDING REMARKS:

In our culture conditions, WJ-MSCs accelerated wound healing in an *in vivo* model and upregulated some anti-aging genes by paracrine signalling. Taking into account the theoretical close relationship between wound healing and anti-aging (aging is accompanied by delayed wound healing), further research is warranted to delineate if the same wound healing promoting *in vivo* effects would also be seen in aged individuals, first at the pre-clinical and then in the clinical setting.

Together, WJ-MSC paracrine signalling emerges as a wound healing and possibly anti-aging promoting therapy which may constitute a new promising non-invasive reconstructive and cosmetic regenerative technology.

6. SUMMARY AND FUTURE DIRECTIONS:

The results from this experimental study demonstrated that human WJ-MSCs promoted normal and burn wound healing and anti-aging effects by paracrine signalling, while enhancing a fibrotic phenotype in keloids. However, the direct application of WJ-MSCs had opposite effects on keloid fibroblasts and reversed their pro-fibrotic gene characteristics. Further pre-clinical and clinical research may unravel a promising new therapy in keloid scars, non-healing wounds, anti-aging and burns.

This work is novel because it describes the advantageous effects of a stem cell type on human keloid, burned and normal skin fibroblasts, which has previously not been investigated in detail and that shows promise due to its universal availability and non-invasive isolation. Indeed, between the many potential sources of MSCs, WJ-MSCs emerge as a birth-derived adult or fetal, but not embryonic, young cell source with many advantages: such as a high number of cells (more than the umbilical cord blood and bone marrow [132]), ease of isolation, no relevant ethical concerns, low cost, universal source, wide availability, and a higher degree of stemness and self-renewal compared to BM-MSCs [133] and a more proliferative and less senescent source compared to ADSCs [259].

It has been already reported that human WJ-MSCs prevent fibrosis in rat liver, lung and kidney [160-162]; however, as far as we are concerned, no previous studies have been performed with human-derived WJ-MSC in keloids, or human burned or normal skin.

This study focused mainly on the paracrine signalling effects of WJ-MSCs, which is believed to mediate the majority of MSC action. Theoretically, the application of multipotent cell derived components infer less risks (no direct cell application), while maintaining the key therapeutic advantages of cell-derived-products.

Furthermore, this work translated the *in vitro* results and investigated their action in *in vivo* preclinical models, which provided the underlying nexus of wound healing for the 3 parts of this project (keloid scars or fibrosis, normal wound healing and anti-aging, and burns). Moreover, this study investigated the effects of WJ-MSC paracrine signalling and compared it against direct cell-cell effects.

Wound healing remains a clinical challenge for most surgeons, physicians and researchers, so future work is still needed. Taking into account the results in our culture conditions, further studies on WJ-MSC direct cell-cell effects, especially on human keloid fibroblasts are needed, with an increased sample size and a more extensive experiment design.

Next, the underlying molecular mechanisms or signalling pathways that are involved in the crosstalk between human WJ-MSCs and skin fibroblasts might also be examined. Moreover, testing if the promising *in vivo* normal wound healing effects observed in this study might also be extrapolated into other conditions, such as burned, diabetic and aging wound healing animal pre-clinical models remains unknown. Manipulation of culture conditions, or the application of coadjuvant tissue engineering techniques, might also bring some light to this topic. On the other hand, related studies analyzing the effect of other stem cells (such as burn-derived-MSCs or keloid-derived-MSCs) might open a new avenue to isolate other new stem cell sources and examine their interactions with WJ-MSCs, or even with autologous skin stem cells, or iPSCs. Similarly, comparative studies between WJ-MSCs and BM-MSCs or ADSCs in wound healing have not been performed yet and would be interesting.

One main limitation of this work was a limited sample size for human normal and burned fibroblasts, which prevented reaching statistical significance in some cases, as well as for some non-genetic keloid studies. Albeit, the bare minimum *in vitro* standard of 3 biological samples was achieved throughout the study. Moreover, in light of the difficulty of obtaining human keloid tissue samples, a total of 6 samples were still assayed for the keloid genetic expression studies. The qPCR design of the direct cell-cell contact co-culture system did not include specific cell type sorting (FACS or fluorescence-activated cell sorting) or radiation; in part, to avoid personal risk, high costs and biased results when analyzing fibrosis effects.

To overcome this issue, control samples consisting of WJ-MSCs alone were also analyzed and a valid correction of the Ct values in the treatment group was included. Furthermore, a more clinically relevant normal wound healing model, represented by the red duroc pig, could have been used subsequent to the mice experiments. Similarly, a pre-clinical specific keloid, burned and aging model could have been carried out to study the *in vivo* effects of WJ-MSCs in these particular settings. As a future project, albeit more ambitious, the production of WJ-MSCs under controlled cGMP facilities could be carried out, followed by the design of phase I-II clinical trials.

Notwithstanding, the central aim of this three-part project has been accomplished: the thorough investigation of the effect of human WJ-MSCs on skin wound healing and some of the most current and challenging peripheral related topics, such as keloid scars, burns, and anti-aging/cosmesis. This study contributed to provide new knowledge about an innovative, promising and universal stem cell source.

If the reported immunoprivileged and safe role of WJ-MSCs observed in previous experimental and first clinical models is further scientifically proven, WJ-MSCs might represent a feasible, universal and off-the shelf technology to enhance wound healing and improve patient survival and quality of life.

Together, the purpose of this work was to offer new insights in the regenerative medicine and tissue engineering field, focusing on WJ-MSCs and skin. This three—part dissertation opened a new research and clinical arena by contributing to increase scientific knowledge and evidence to potentially offer a promising ethically sound, efficient, cost-effective and high-quality clinical alternative to heal and help patients worldwide.

7. CONCLUSIONS:

7. 1. Skin fibrosis (keloid scars):

In our culture conditions, human WJ-MSC paracrine signalling (via conditioned media or a microporous membrane) enhanced the pro-fibrotic phenotype of keloid fibroblasts.

In our culture conditions, the direct (i.e. cell-cell contact) application of human WJ-MSCs to keloid fibroblasts was demonstrated to revert their fibrotic genotype.

7. 2. Burn wound healing and burns:

Under our culture conditions, human WJ-MSCs may promote burn wound healing and repair by paracrine signalling mechanisms. Some pro-wound healing gene expression factors were upregulated, and burned skin fibroblast migration and wound closure was enhanced by human WJ-MSC-CM *in vitro*.

In our culture conditions, cell-cell direct WJ-MSC therapy might aid in controlling the fibroproliferative phase of wound healing, and promote scarless or minimal scar wound healing. Direct application of WJ-MSCs downregulated the expression of wound healing and pro-fibrotic genes in human burned skin fibroblasts.

7. 3. Normal wound healing and anti-aging:

The results of this study suggested that WJ-MSC may enhance wound healing, normal wound repair and exert anti-aging effects by paracrine signalling mechanisms. Under our culture conditions, human WJ-MSC-CM upregulated the gene expression of wound healing and anti-aging related factors in normal skin fibroblasts, and promoted normal fibroblast proliferation and migration to coapt wound borders *in vitro*.

Accordingly, WJ-MSC-CM accelerated the reepithelialization rate and promoted wound repair *in vivo* in a full-thickness skin excisional mice wound healing model.

In our culture conditions, the direct cell-cell contact effect of human WJ-MSC on normal skin fibroblasts had opposite genetic effects.

8. ACKNOWLEDGEMENTS:

8. 1. DISCLOSURE STATEMENT:

None of the authors has any potential conflict of interest whatsoever in any of the techniques or instruments mentioned in this manuscript.

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8. 2. PERSONAL ACKNOWLEDGEMENTS:

It is the moment. Now. The dissertation is completed. The goal has been achieved; or at least, the work has been finished. The expert Committee has yet to decide… NOW. Many and many books defend the "power of now". Now you should be enjoying life, you should focus on "now", the path, and not the final goal. If you just focus on getting the goal you might indeed put your aim even further away making it that much more difficult to reach. Anxiety might increase and be the underlying responsible mechanism.

We've been so many years listening to this word: "Mechanism"… as well as other which NOW have a more meaningful power, like "mesenchymal stem cells", "umbilical cord", "Wharton's jelly", "paracrine signalling"… "cell-cell" (indeed, this study has been a experiment in person-person work; a team and especially a personal effort. Dedication and perseverance constitute the success of this work)….

Either "Direct"ly or "indirect"ly, if we want to name all the people who contributed to help or inspire us in the path of building this multi branch project, we surely know we would fill such an un-ending list… even the self-help book authors whom help us along the way of achieving this work; they taught us to just keep moving, taught us to get back up, and overcome all types of challenges… Research is an attitude, a work and a life-style with a double-edged sword: It can be extremely interesting and rewarding, but it has often hidden draw-backs and failures that might touch your brain, your body and your soul. Any idea might be initially tested. Open your mind to the world and nature. All is possible. Limits are mostly set by you. Don't let others stop you. Don't let your worst enemy disturb you. Consider that sometimes your worst enemy could even be you.

Family and friends usually appear in the acknowledgement section of any dissertation. We don't personally like to state a list of all the specific names like serial numbers... A list of written names make us recall cemeteries. Names are just ways of referring to things; we prefer to keep people in a more spiritual and abstract form.

You really know we love you, and you have been and are in our mind and heart at all moments. Even under the snow, stormy weather or burning sun…. Everything has a start and an end. We will all be born and die (taking into account the current physiological conditions, of course). However, we can keep a thought for more or less time. Sometimes it might turn to be so short, that the next step is going to write a list of some of the individuals who we would like to thank and we are so happy to have crossed along our journey: Dr Marc G Jeschke, for his "welcome on board" attitude, mentorship, generosity, and unending support. Dr Juan P Barret, for offering us the opportunity of further knowledge and work as a model surgeon to follow and challenge. Dr Manuel Armengol, for his guidance and direction. Dr Cartotto, for his professionalism and great personality.

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Especial and the most natural feelings of gratitude and love to my parents, Ana María and Fernando. You created this; you designed and built our body and mind, which developed independently with the major dependencies from the umbilical cord. Inspiring phrase… We really love you, immensely appreciate and thank you to be as you are. My siblings, Gemma, Mònica and Ferran, for always being there. For your love. For your kind words from your soul. Lourdes, Marta, Anna, Sonia…My former medicine classmates and ever close friends. José Antonio: thank you for your invaluable advice throughout my PhD. Lars, mein Schatz, only the future knows how far this loving living path will go and last. Thank you for being you. Umbilical cord inspiration resumes.

We hope this research opens a new pre-clinical and clinical arena for a new strategy for regenerative medicine. Maybe we were wrong, but even this fact may lead to further scientific progress, like many examples from the old past. Or perhaps, we might have been right and our preliminary data might turn into more ambicious clinical trials and even promising real world therapies.
Anyway, the now is here. Another realistic goal has to be set now.

Shall we remember that happiness is not bought by accomplishing goals, although it might be easier and “cheaper” to get it, then. True happiness is to self re-build your personal thoughts and look at the positives in all you see and also not have right in front of your eyes, understanding and appreciating the irreversible “expiry date” as a final magic process of nature. Enjoy the nurturing, mysterious and exciting growing path with no fear.

Thank you so much for all your support, guidance, help, and especially love.

Thank YOU!

9. APPENDIX:

Parts of this work have been accepted for publication in the journal "Stem Cell Transl Med" as: Arno AI, Amini-Nik S, Blit PH, Al-Shehab M, Belo C, Herer E, Jeschke MG. Effect of human Wharton's jelly mesenchymal stem cell paracrine signaling on keloid fibroblasts. Accepted to Stem Cell Transl Med on October 30th, 2013 (SCTM-13-0120.R1)*. Other parts are currently in the peer review process.

• "EFFECT OF HUMAN WHARTON'S JELLY MESENCHYMAL STEM CELL PARACRINE SIGNALING ON KELOID FIBROBLASTS" - SCTM-13-0120.R1

Dear Dr. Arno:

We are pleased to inform you that your manuscript has been accepted for publication in STEM CELLS Translational Medicine. The final version of the manuscript is now ready for approval in the submitting author's account on Manuscript Central.

The author who submitted the manuscript now needs to log on to the site at http://mc.manuscriptcentral.com/stemcellstm and go to the "Manuscripts Accepted for First Look" folder in his or her account to complete the necessary steps before the Editorial Office can schedule the manuscript for publication.

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Sincerely,

Dr. Anthony Atala
Editor
STEM CELLS Translational Medicine

9. 1. WJ-MSC *IN VIVO* DELIVERY WITH SCAFFOLDS:

Complementing the work in this dissertation, we have also contributed by taking part into a related *in vivo* tissue engineering project using the BALB-c mouse splint wound healing model. WJ-MSCs were delivered alone and with different types of polysaccharide based scaffolds, designed by Dr Blit, using different routes of administration. They included pullulan-gelatin scaffolds, among others. The main aim was to test the wound healing effects of the direct application of WJ-MSCs to wounds *in vivo*, and comparing against co-delivery of the cell seeded scaffolds.

Other dissertation-related projects we have been working on are mentioned in the next sections.

9. 2. EFFECTS OF CARBOXY-METHYL-CHITOSAN ON HUMAN SKIN FIBROBLASTS AND WJ-MSC WOUND HEALING AND FIBROSIS GENE EXPRESSION

9.3. PATHOLOGICAL SKIN CONDITION-DERIVED- MESENCHYMAL STEM CELLS

9. 3. 1. KELOID-DERIVED-MSC: (Ongoing project with research proposal written by Arno A, whose corresponding author was Jeschke MG. This research proposal won the AB-Award 2012).

Title of Project:

STEM CELLS IN HYPERTROPHIC SCARRING AND KELOIDS AFTER BURNS: ARE THEY A DOUBLE EDGED SWORD?

9. 3. 2. BURNED SKIN-DERIVED MSC-like cells.

10. LIST OF TABLES:

11. LIST OF FIGURES AND FIGURE LEGENDS:

Figure 1: The composition and layers of normal skin.

Figure 2: Phases of wound healing.

Wound healing has three phases: Inflammation (A,B), proliferation (C) and remodelling (D). The inflammatory phase begins with hemostasis or fibrin clot formation (A). Platelets (small red dots), red blood cells (bilobular red structures), polymorphonuclear neutrophils (purple rounded structures), macrophages (represented in green colour), fibroblasts and myofibroblasts (fusiform nuclear structures, in brown) are the main cell types involved.

Figure 3: The composition of the umbilical cord.

Schematic representation of the umbilical cord, which contains 2 arteries and one vein. The outer cord layer gives rise to the cord lining epithelial stem cells, whereas the Wharton's jelly is a gelatinous inner part from which mesenchymal stem cells are derived. Cord blood-derived stem cells are isolated from the cord venous blood.

Figure 4: Human skin fibroblast isolation.

Figure 5: Human umbilical cord dissection.

Figure 6: Umbilical cord-derived Wharton's jelly MSCs isolation.

Figure 7: Umbilical cord-derived Wharton's jelly MSCs attached to plastic surfaces.

Figure 8: WJ-MSC characterization.

Flow-cytometry markers expressed by human WJ-MSC harvested from umbilical cords (a-d), and further grown on plastic plates. Cells were able to differentiate into 3 mesenchymal cell lineages: osteocytes (e), chondrocytes (f) and adipocytes (g). Images shown after oil red (e), safranin O (f) and alizarin red (g) stainings, respectively.

Figure 9: Human WJ-MSC one-way paracrine signalling effects on human keloid skin fibroblast gene expression.

Figure 10: Human WJ-MSC two-way paracrine signalling effects on human keloid skin fibroblast gene expression.

Figure 11: Human WJ-MSC direct cell-cell contact effects on human keloid skin fibroblast gene expression.

Figure 20: Human burned fibroblasts remain viable and proliferate with WJ-MSC-CM.

Figure 21: WJ-MSC-CM promotes burned skin fibroblast migration and wound closure *in vitro*.

Figure 22: Human WJ-MSC one-way paracrine signalling effects on human normal skin fibroblast gene expression.

mRNA transcript expression relative to 18S after 7 days of culture of human normal skin fibroblasts with WJ-MSC-CM (treatment group) or non-conditioned medium (control group) from 5 different patients (but 4 in *FGF-2* and *Sirt-1*, and 3 in *collagen I, collagen III* and *decorin*). Overall, WJ-MSC-CM enhanced a wound healing promoting phenotype in human normal skin fibroblasts in our culture conditions.

- = $p \leq 0.05$.

Figure 23: Human WJ-MSC two-way paracrine signalling effects on human normal skin fibroblast gene expression.

Figure 24: Human WJ-MSC direct cell-cell contact effects on human normal skin fibroblast gene expression.

Figure 25: WJ-MSC-CM enhances human normal skin fibroblast proliferation and not apoptosis.

Cell proliferation was examined using Ki67 staining. WJ-MSC-CM-treated normal fibroblasts showed enhanced proliferative rates compared to the control group (A, quantified in B). TUNEL staining of WJ-MSC-CM-treated human normal skin fibroblasts versus control (non-WJ-MSC-CM treated) normal skin fibroblasts showed no significant difference in induction of apoptosis (C, quantified in E). Note that the total number of viable cells was significantly higher in the WJ-MSC-CM-treated cells comparing to the non-WJ-MSC-CM treated cells (D, $p \leq 0.001$). (n=3 samples each group).

*** $= p \leq 0.001$.

Figure 26: WJ-MSC-CM enhances human normal skin fibroblast proliferation.

Figure 27: Human normal fibroblasts remain viable and proliferate with WJ-MSC-CM.

Figure 28: WJ-MSC-CM accelerates wound closure *in vitro*.

A, B) Scratch wound assay was performed to examine migration properties of WJ-MSC-CM-treated and untreated normal skin fibroblasts. The treated group showed significantly enhanced migration rates and coapted wound borders faster than the control group. * $p \leq 0.05$.

Figure 29: WJ-MSC-CM enhances wound healing in an *in vivo* mouse model.

A mice wound healing model was used, and animals were wounded and treated, as previously described. Histological sections of wounds and satellite donut area (10X) from BALB-c mice, after 1 week of full-thickness excisional skin wounding and reconstruction with WJ-MSC-CM and vehicle (matrigel ®) (B), or vehicle alone (A).

Photomicrographs were taken after Mason's Trichrome staining. Increased and complete re-epithelialization, higher cellularity in newly formed granulation tissue, and less random and more organized extracellular matrix were observed in the WJ-MSC-CM-treated wounds, suggesting that WJ-MSC-CM promoted wound healing and repair *in vivo* in mice.

* $p \leq 0.05$; ** $p \leq 0.01$. Error bars represent 95% confidence interval.

Figure 30: WJ-MSC-CM promotes cell proliferation in an *in vivo* mouse wound healing model.

A BALB-c mouse wound healing model was used and animals were wounded and treated, as previously described. Animals received one dose of BrdU intraperitoneally 24h before harvesting of wounds. Four animals were included in each group, and 4 wounds of 4 mm diameter each were performed per animal (total of 16 wounds in each group). Cutaneous tissue specimens were stained for BrdU in both groups, control (A) and treatment (B).

Enhanced magnification (40X) of the above microscopic images were included for non-conditioned medium treated (C) and WJ-MSC-CM-treated normal skin fibroblasts (D) to examine in further detail the increase in cell number or stained nuclei (black arrows, BrdU positive cells) in the WJ-MSC-CM-treated wounds, compared to controls. This denoted that WJ-MSC-CM stimulated cell proliferation in vivo (F, ** $p \leq 0.01$). Together, these results suggested that WJ-MSC promoted wound healing and repair by one-way paracrine signalling in an *in vivo* preclinical model. * $p \leq 0.05$ and ** $p \leq 0.01$. Error bars represent 95% confidence interval. Arrows show BrdU+ nuclei, while arrowheads indicate BrdU- ones.

12. LIST OF ABBREVIATIONS:

ADSCs	Adipose-Derived Stem Cells
α-SMA	Alpha-smooth muscle actin
AMPK	5' adenosine monophosphate-activated protein kinase
ANGPT2	Angiopoietin-2
Ang- II	Angiotensin-II
ANOVA	Analysis of Variance
ASCs	Adult Stem Cells
BM	Bone Marrow
BrdU	Bromodeoxyridine
BSA	Bovine Serum Albumin
cDNA	Complementary DeoxyriboNucleic Acid
CEA	Cultured Epithelial Autografts
cGMPs	Current Good Manufacturing Practices
CIHR	Canadian Institutes of Health Research
CM	Conditioned Medium
CMRL	Connaught Medical Research Laboratories (medium)
Ct	Cycle Threshold
CTGF	Connective Tissue Growth Factor
CO_2	Carbon Dioxide

DAB	Diaminobenzidine
DAPI	4',6-Diamidino-2-Phenylindole, Dilactate
DI	Deionized
DMEM	Dulbecco's Modified Eagle Medium
ECM	Extracellular matrix
EMT	Epithelial-to-Mesenchymal-Transition
Erk	Extracellular signal-regulated kinase
ESCs	Embryonic Stem Cells
FACS	Fluorescence-Activated Cell Sorting
FBS	Fetal Bovine Serum
FDA	U.S. Food and Drug Administration
FGF-2	Fibroblast Growth Factor-2
FITC	Fluorescein isothiocyanate
HCl	HydroChloric Acid
HIF-1-α	Hypoxia Inducible Factor-1-α
HLA	Human Leukocyte Antigen
hMSCs	human mesenchymal stem cells
HPF	High Power Field
HRP	Horseradish Peroxidase
HSCs	Hematopoietic Stem Cells
HSF-1	Heat Shock Factor-1
HTS	Hypertrophic Scar
IGF-I	Insulin Growth Factor-I
IL-6	Interleukin-6

IL-8	Interleukin-8
iPSCs	Induced Pluripotent Stem Cells
KMLSCs	Keloid-derived Mesenchymal-Like Stem Cells
LSM	Laser Scanning Microscope
MSCs	Mesenchymal Stem Cells
MMPs	Metalloproteinases
mRNA	Messenger Ribonucleic Acid
NaCl	Sodium Chloride
NF-kB	Nuclear factor-kappaB
NIH	National Institutes of Health
P	Passage
PAI-1	Plasminogen activator inhibitor- I
PBS	Phosphate Buffered Saline
PBST	Phospahte-Buffered Saline/TweenX20 or TritonX100
PCR	Polymerase Chain Reaction
PDGF	Platelet Derived Growth Factor
PFA	Paraformaldehyde
PGD-1α	Prostaglandin D-1α
PLGF	Placental Growth Factor
POMC	Proopiomelanocortin
PPAR-γ	Peroxisome Proliferator-Activated Receptor- γ
ROS	Reactive Oxygen Species
RT-PCR	Real Time-Polymerase Chain Reaction
SCC	Squamous Cell Carcinomas

SCs	Stem Cells
SHEDs	Stem cells from Human Exfoliated Deciduous teeth
SIRT-1	Sirtuin-1
SIRTs	Sirtuins
SOD2	Superoxide dismutase 2
TAHSN	Toronto Academic Health Sciences Network
TGF-β	Transforming growth factor- β
Th2	T helper 2 (cells)
TIMP	Tissue Inhibitor of Metalloproteinase
TUNEL	Terminal Transferase TdT-mediated dUTP biotin End-Labeling
UC	Umbilical Cord
UV	Ultraviolet
VEGF	Vascular Endothelial Growth Factor
WJ-MSC	Wharton's Jelly derived-mesenchymal stem cells

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