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Human Wharton's Jelly Mesenchymal Stem Cells Maintain the Expression of Key Immunomodulatory Molecules When Subjected to Osteogenic, Adipogenic and Chondrogenic Differentiation *In Vitro*: New Perspectives for Cellular Therapy

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Abstract: Rheumatoid arthritis and osteoarthritis are the main diseases that imply an inflammatory process at the joints involving the articular cartilage. Recently, mesenchymal stem cells (MSCs) derived from perinatal tissues were considered good candidates for cellular therapy of musculoskeletal and orthopaedic diseases, since they can differentiate into multiple cell types and are an easily accessible cellular source. Therefore, several protocols exist on the differentiation of mesenchymal stem cells of different origins into osteoblasts and chondrocytes. Another key feature of MSCs is their capacity to modulate the immune system responses *in vitro* and *in vivo*. This may have critical outcomes in diseases of the musculoskeletal system where an inflammatory or autoimmune process is at the basis of the main disease.

In the present paper, after isolation of MSCs from Wharton's Jelly (WJ-MSCs), we performed the three standard differentiation protocols. The acquisition of the differentiated phenotype was demonstrated by the specific histological stains. As the main objective of this work, we determined the expression of immunomodulatory molecules (by immunohistochemistry and qualitative RT-PCR), both in undifferentiated cells and after differentiation. We demonstrated for the first time that immune-related molecules (as B7-H3/CD276 and HLA-E) which have been characterized in undifferentiated MSCs, are also expressed by the differentiated progeny. This strongly suggests that also after the acquisition of a mature phenotype, WJ-MSCs-derived cells may maintain their immune privilege. This evidence, which deserves much work to be confirmed *in vivo* and in other MSCs populations, may provide a formal proof of the good results globally achieved with WJ-MSCs as cellular therapy vehicle.

Keywords: Regenerative medicine, mesenchymal stem cells, umbilical cord, Wharton's jelly, immune modulation, tissue repair, osteogenic differentiation, adipogenic differentiation, chondrogenic differentiation.

INTRODUCTION

As reported by World Health Organization (WHO), the main causes of disability in the world are rheumatic, orthopaedic and musculoskeletal diseases. Rheumatoid arthritis (RA) and osteoarthritis (OA) are two diseases responsible of inflammation of the joints [1].

In OA, inflammatory process represents the result of pathogenetic condition, while in RA it is the main cause [2].

Both diseases, as well as isolated chondral and osteochondral disorders, involve injuries to articular cartilage, a type of hyaline cartilage composed by small chondrocytes and a granular matrix, where is possible to distinguish three different classes of macromolecules: fibrillar and non-fibrillar collagens, collagenous proteins and proteogly [3-4].

It has been demonstrated that an imbalance between catabolic and anabolic functions of the chondrocytes may determine a cartilage injury. In addition, since chondrocytes are fed by diffusion via the synovial fluid, both growth and repair of cartilage are very slow [5].

For these reasons, some research groups investigated alternative methods to promote cartilage formation (such as osteotomy, joint distraction, implants of growth factors or artificial matrices), or to replace the chondrocytes by cell transplantation [6-7].

Chondrogenesis and Chondrocyte Differentiation Process

Chondrogenesis is a dynamic cellular process that involves the formation of hyaline, fibrous and elastic cartilage both during embryogenesis and in adult life. The process of

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chondrogenesis begins with aggregation and condensation of mesenchymal cells with the involvement of bone morphogenetic proteins (BMPs) [8]. In this phase, the condensed mesenchyme expresses some extracellular matrix and cell adhesion molecules such as IIa splice form of type II collagen [Col2a1(IIa)] [9, 10], N-cadherin (Ncad) [11], neural cell adhesion molecule (Ncam1) [12], tenascin C (Tnc) [13], and SRY-box 9 (Sox9), transcription factor involved in the early stages of chondrogenesis [14].

Subsequently, the chondrogenesis process is characterized by early chondrocyte differentiation and development of the cartilage template and by chondrocyte maturation and hypertrophy. If osteogenesis has to follow, during maturation steps, the chondrocytes differentiate, mineralize and undergo apoptosis. When the chondrocytes die, cartilage matrix deposits constitute the basis for mineral deposition and bone formation. In this phase, degraded cartilage will be invaded by blood vessels establishing the bone marrow cavity [15].

Articular Cartilage Tissue Engineering and Factors Involved in Chondrogenic Differentiation

Hyaline cartilage of the limb and trunk skeleton derives from differentiation of mesenchymal stem cells that condense into clusters of cartilage cells (chondrocytes). Physiologically, this cartilage type is susceptible to both normal and pathologic stress [15]. In particular, articular cartilage may undergo degenerative processes involving focal and progressive cartilage loss. The main risk factors are linked to genetic background, obesity, advanced age, ethinicity, osteochondral and chondral lesions and OA.

In the last years, tissue engineering was considered the main approach for repair of articular cartilage lesions. Autologous, heterotypic and allogenic chondrocytes were expanded in culture [16].

The presence in culture media of specific growth factors such as members of TGF-β (transforming growth factor –β) superfamily, insulin like growth factor (IGF), hepatocyte growth factor (HGF) and BMPs can influence the chondrocyte phenotype [17]. BMPs belong to the TGF-β superfamily and can promote ectopic cartilage and bone formation through a direct regulation of the expression of chondrocyte genes [18].

Bone morphogenetic protein-2 (BMP-2) together with TGF-β, can influence the synthesis of type II collagen, the main marker to evaluate acquisition of chondrocyte phenotype. Some authors demonstrated that in costal embryonic mouse chondrocytes, the presence in culture of BMP-2 induced the expression of type IIB and α-10 integrin, while TGF-β1 potentiated the expression of type IIA and α11 integrin [19, 20].

Another work highlighted that the addition of BMP-2 in monolayer cultures of nasal chondrocytes, could reactivate the chondrogenic expression programme, in particular inducing the expression of type II collagen gene by enhancing DNA-binding of the SOX transcription factors [21].

Recently, some research groups suggested the use of adult stem cells to derive chondrocyte-like cells. In particular, mesenchymal stem cells are considered as good candidates for chondrocyte differentiation. In addition, in recent years the interest for perinatal tissues-derived MSCs is increased considering that these cells may be easily sourced [6].

To promote chondrogenic differentiation, MSCs, after expansion in monolayer culture, need a three-dimensional (3D) environment to maintain the chondrocyte phenotype. Different 3D supports were proposed such as polymers, collagen sponges or hydrogels, alginate beads and microspheres [22-25]. Alginate is a linear polysaccaride composed by repeated disaccharides of β-D-mannuronic-acid and α-L-glucuronic acid. In the presence of calcium ions, the alginate can be cross-linked to generate a polymerized hydrogel. In this alginate solution, the cells allocate homogeneously [26]. The porosity of the hydrogel allows the diffusion of large molecules but not passive migration of cells [27].

Perinatal Mesenchymal Stem Cells: Characteristic Features of WJ-MSCs

The first isolation of multipotent mesenchymal stromal cells from the bone marrow, was made in the 1970 by Friedenstein [28]. Mesenchymal stem cells (MSCs) are mononuclear, multipotent cells featuring a fibroblastoid morphology. They are defined as immature cells with the ability to self-renew and differentiate towards several cellular types such as osteoblasts, adipocytes and chondrocytes. MSCs grow on plastic surface and therefore can be quickly expanded *in vitro* for several passages [29]. Some researchers focused their attention on the use of MSCs derived from other easily accessible tissues with higher cellular yields. For example, bone marrow, is currently the common source of MSCs, even if the number of cells results very low [30] and decreases with donor age [31]. Umbilical cord blood, placenta, amniotic membrane and umbilical cord stroma (Wharton's jelly) are some perinatal sources of MSCs. In literature, several studies demonstrated the differentiation ability of these cells in generating all cellular types derived from the three germ layers [32].

Perinatal MSCs expresses a number of surface molecules such as CD105, CD73, CD106 (vascular cellular adhesion molecule-1), CD54 (intercellular adhesional molecule-1), CD44, CD90, CD29 and STRO-1, some cytokine receptors, molecules involved in immune responses such as canonical type I MHC (major histocompatibility complex) as HLA-A. MSCs do not express HLA-DR, a type II MHC, as well as hematopoietic stem cell markers such as CD45 and CD34. Markers such as CD31 (PECAM-1) and von Willenbrand factor (vWF) are specific for endothelial cells [33, 34] and we and others demonstrated their absence in WJ-MSCs [35].

In the last years, our research group focused on Wharton's jelly-derived mesenchymal stem cells (WJ-MSCs). Wharton's jelly, also known as intervascular stroma, is the main component of the extracellular matrix of human umbilical cord. It is composed by an amorphous substance rich in glycosamminoglycans (GAG) and proteoglycans, which hosts (in a classical view) two cellular types: myofibroblasts and fibroblast-like cells. The latter are commonly identified as the multipotent mesenchymal stem cells [36]. WJ-MSCs express all markers typical of mesenchymal stem cells but

express also CD117 (receptor of the stem cell factor). Moreover, our group demonstrated that WJ-MSCs are positive to the expression of some, endodermal markers such as GATA-4, GATA-5, GATA-6, hepatic nuclear factor-4α (HNF-4α), and mesodermal markers such as vimentin, and α-smooth muscle actin, suggesting that these cells may differentiate in several cellular types derived from all three germ layers [35, 37]. According to the International Society for Cellular Therapy, WJ-MSCs are multipotent because can differenteiate into osteoblasts, adipocytes and chondrocytes. Various *in vitro* and *in vivo* studies demonstrated that WJ-MSCs cultured in a medium supplemented with chondrocyte inducers differentiate into chondrocyte-like cells [1].

Chondrogenic Differentiation by WJ-MSC in Culture and on Scaffolds

Arufe and colleagues, demonstrated that umbilical cord-MSC may differentiate into chondrocyte-like cells when cultured in presence of ascorbic acid, transferrin, dexamethasone, retinoic acid and TGF-β3 for up to 46 days. The expression of type I, II, and X collagens, and some other components of chondrocyte matrix such as decorin, galectin-1 and chitinase confirmed the acquisition of chondrocyte phenotype [38].

A comparative study carried out by Wang *et al*., highlighted the ability to differentiate in chondrocyte-like cells on poly-glycolic acid scaffold (PGA) in presence of chondrogenic medium for 6 week by WJ-MSCs and BM-MSCs. The authors observed that WJ-MSCs produced more GAGs and type I collagen than BM-MSCs [39]. A further study confirmed that WJ-MSCs cultured in a chondrogenic medium on PGA scaffold, for 4 weeks, expressed GAGs and type I and II [40].

Recently, it was suggested that WJ-MSCs may differentiate to chondrocyte-like cells if cultured on nanofibrous substrates with a sequential two-steps culture, and differentiated cells expressed GAGs, hyaluronic acid, and some key genes for chondrocyte differentiation process such as SOX9, COMP (cartilage oligomeric matrix protein), collagen type II [41].

Immunomodulatory Properties and Tolerogenic Activity by MSCs

Several reports described the expression of immunomodulatory molecules by MSCs and in particular by WJ-MSCs. These data boosted the investigations on the possible interactions between MSC and immune response cells.

The mechanisms underlying immunomodulation by MSCs are different: suppression of T cell proliferation and dendritic cell differentiation [42], inhibition of B-cells [43], and natural killer cells (NK) proliferation [44], induction of T cells anergy and stimulation of regulatory T cells (CD4+CD25+FoxP3+ T regs) [45, 46].

MSCs can modulate the immune system response by cytokines, growth factors, as well as the lack of co-stimulatory molecules [47]. Release of soluble factors, such as IL-10 (interleukin-10), HLA-G, tumor necrosis factor alpha (TNF-α), TGF-β, is normally a results of cross-talk between MSCs and T-lymphocytes [48]. In particular, some reports demonstrated that HLA-G and its soluble form (HLA-G5), were expressed in BM-MSCs and WJ-MSCs [35, 47, 49]. Other molecules belonging to non-canonical type I MHC where investigated by our group, on WJ-MSCs: HLA-E and HLA-F. They are involved in the tolerance process between mother and fetus, and in particular HLA-E plays a key role during selective binding with NK cells [50, 51]. In addition, we recently demonstrated the expression of CD68 in WJ-MSCs [52].

A recent report, carried out by Spaggiari and co-workers, showed that MSCs are able to inhibit the NK cells proliferation, cytotoxicity and cytokine production, by down-regulating the expression of activating NK receptors such as NKp30, NKp44, and NKG2D. This study demonstrated that possible mediators released by MSCs and involved in the inhibition of some NK- cells activities could be prostaglandin-E2 (PGE2) and indoleamine 2,3 dioxigenase (IDO) [44]. According to other works, PGE2, released by MSCs of different origin, can also modulate the activation and proliferation of lymphocyte subset [53].

Recently, some researchers suggested that molecules belonging to the B7 family, with a known co-stimulatory role, may have co-inhibitory role in the T-lymphocytes proliferation. Some reports showed that B7-H3 (CD276), a co-stimulatory molecule, inhibits human T cell activation, by decreasing the expression of interleukin-2 (IL-2) [54].

CD80 (B7-1) and CD86 (B7-2) are other two co-stimulatory molecules of T cells proliferation, but Sansom and co-workers highlighted that (in absence of CD86), the CD80 mostly exercises an inhibitory effect through binding of CD152 [55]. In conclusion, all these data supported the idea that MSCs could induce immune tolerance in the host. The main aim of the present work was the investigation of the maintenance of expression of immunomodulatory molecules by WJ-MSCs before and after differentiation.

MATERIALS AND METHODS

Cellular Isolation Protocol of Wharton's Jelly Mesenchymal Stem Cells

Isolation protocol was adopted from our previously published data [35, 56]. All umbilical cords were obtained after mother's informed consent according to tenets of the Declaration of Helsinki and local ethical regulation. After normal vaginal or caesarean delivery, after full-term birth, umbilical cords were stored aseptically in cold saline and cellular isolation was started within six hours from partum. The cords were washed in warm HBSS (Gibco), and then were cut in small pieces about 1.5 cm length, sectioned longitudinally so that to exhibit the Wharton's jelly under amniotic epithelium. Different incisions, without vessels removal, were made within matrix with sterile scalpel to increase area exposed to the contact with medium composed by DMEM low glucose (Sigma), supplemented with 10% FBS (fetal bovine serum gold, PAA), 1x NEAA (non-essential aminoacids, Sigma), 1x antibiotics-antimycotics (GIBCO), and 2mM L-glutamine (Sigma). This isolation protocol does not use enzymatic activity to dissociate cells from the embedding matrix but is based on cells migratory ability. Cord pieces were left for 15 days with medium changed every second day.

Table 1. List of antibodies used in the present study.

Antigen	Host	Manufacturer	Diluition
B7-1	Mouse	Santa Cruz	1:50
B7-2	Mouse	Santa Cruz	1:200
B7-H3	Rabbit	Santa Cruz	1:100
Collagen II	Mouse	Merck Millipore	1:50
CD31	Mouse	Santa Cruz	1:50
HLA-ABC	Mouse	Santa Cruz	1:50
HLA-DR	Mouse	Santa Cruz	1:50
HLA-E	Mouse	Santa Cruz	1:50
IDO	Mouse	Santa Cruz	1:50
Vimentin	Mouse	Santa Cruz	1:100

Therefore, the slow degradation of the matrix allowed growth factors and signalling molecules to exit from the cord and continuing provide a positive stimulation to the cultured cells.

After 15 days of culture, cells widely adhered to the plastic surface, and were cultured until confluence.

Cell Culturing and Passaging

After reaching confluence, cells were removed from flasks with Tryple Select (Invitrogen) and were cultured for up to 15 passages. For immunocytochemical analysis, cells were plated in 8-well chamber slides (BD Bioscience) and were used after reaching 90% confluence. For RNA extraction, cells were cultured either in 6-well tissue culture plates or in 25cm^2 tissue culture flasks (Corning).

Immunohistochemistry

De-paraffinized sections, were washed with PBS-$CaCl_2$, followed by addition of 0,3% hydrogen peroxide to inactivate endogenous peroxidases. After 20 minutes in a blocking solution (1% of bovine serum albumin), they were incubated with specific primary antibodies for 1 hour and 30 minutes at room temperature. After another wash with PBS-$CaCl_2$, sections were incubated with species-specific secondary antibodies for 10 minutes. Subsequently, streptavidin-peroxidase (DAKO-Cytomation) was added followed by 3.3'-diaminobenzidine (DAB chromogenic substrate solution, DAKO). At the end, haematoxylin (DAKO) was used to counter stain the nuclei of the cells.

The antibodies used in the present study, with indications of the working conditions used, are listed in Table **1**.

Total RNA Extraction

Total RNA extraction from Wharton's jelly MSCs was made by RNAspin mini RNA isolation Kit (GE Healthcare), following the manufacturer's instructions.

The RNA extract was stored at -20°C until use. The concentration of RNA extracted was determined by spectrophotometer with a wavelength of 260nm. Only samples with A_{260}/A_{280} ratio over 1.7 were considered useful for the following analyses [57].

RT-PCR (Reverse Transcription Polymerase Chain Reaction)

Qualitative RT-PCR was performed using Phusion High-Fidelity RT-PCR kit (Finnzymes). The reaction comprised a reverse transcription step of 50 minutes at 42 C° and an inactivation phase of 5 minute at 92 C°. The an amplification reaction was performed in a five steps: an initial denaturation of 30 seconds at 98C°, followed by another denaturation step of 10 seconds at 98 C°, the annealing phases of 30 seconds an extension step of 30 seconds at 72C°, and final extension for 10 minutes at 72 C°. See Table **2** for the list of primers used in this study.

Osteogenic Differentiation

Differentiation of cells was performed by culturing WJ-MSC at different passages in osteogenic differentiation medium, mainly based on previous reports [35]. Briefly, culture medium was supplemented with 50 μM ascorbate-2-phosphate (Sigma), 10 mM β-glycerophosphate (Sigma), 0.1 SM dexamethasone (Sigma), 10% FCS (PAA), 1x NEAA (non-essential aminoacids) (Sigma) and 1% antibiotic/antimycotic (Sigma). Cells were cultured in six-well tissue culture plates for 3 weeks and medium was replaced every second day. The formation of cell clusters resembling direct ossification was monitored by phase-contrast microscopy along culturing. Controls included WJ-MSCs cultured in normal growth medium for 3 weeks to monitor the eventual spontaneous formation of bone-like nodules.

Adipogenic Differentiation

Differentiation of cells was performed by culturing WJ-MSCs at different passages in adipogenic differentiation medium, mainly based on previous reports [35]: culture me-

Table 2. List of PCR primers used for the present study.

Name	Accession number	Product Size	Forward Primer	Reverse Primer
Actin, beta	NM_001101	350	5'-AAACTGGAACGGTGAAGGTG-3'	5'-TCAAGTTGGGGGACAAAAAG-3'
B7-H1	NM_014143	271	5'-GGTCTGGGACGGTTGGATA-3'	5'-CCCATGGGATCTTTTGAATTT-3'
B7-H3	NM_025240	170	5-CCCTCCCTACAGCTCCTACC-3	5-CAGCAGGATGACTTGGGAAT-3
B7-H4	NM_024626	273	5'-CAGGGAGACACTCCATCACA-3'	5'-TGAGTTGCACGTTTTTCAGC-3'
CD80	NM_005191	259	5'-AGGGCCTCCTTAGATCCCTA-3'	5'-TTAGCTGCCATGAGATGTGC-3'
CD86	NM_175862	250	5'-TCCTGGCTGAGAGAGGAAGA-3'	5'-AGACTGCCCCATCCCTTAGT-3'
HLA-A	NM_002116	262	5'-TGGGACTGAGAGGCAAGAGT-3'	5'-ACAGCTCAGTGCACCATGAA-3'
HLA-DR-B1	NM_002124	349	5'-GCACAGAGCAAGATGCTGAG-3'	5'-AGTTGAAGATGAGGCGCTGT-3'
HLA-E	NM_005516	245	5'-CAAGGGCCTCTGAATCTGTC-3'	5'-CGTGTTAGCCAGGATGGTTT-3'
HLA-G	NM_002127	287	5-GCTCCCACTCCATGAGGTATT-3	5- CTGGAGGGTGTGAGAACTGG-3
HLA-F	NM_001098479	202	5'-TGGAGTTGCTCCGCAGATA-3'	5'-TCCACAAGCTCTGTGTCCTG-3'
TGFβ2	NM_003231	282	5'-TGCGGCCTATTGCTTTAGA-3'	5'-TTGGGTGTTTTGCCAATGT-3'

dium was supplemented with 0.5 mM isobutylmethylxanthine (Sigma),1 μM dexamethasone (Sigma), 10 μM insulin (Sigma), 200 μM indomethacin (Sigma), 10% FBS (PAA), 1x NEAA (non-essential aminoacids) (Sigma) and 1% antibiotic-antimycotic (Sigma).

Cells were cultured in six-well tissue culture plates for 3 weeks, and medium was replaced every second day. The formation of cytoplasmic lipid vacuoles was monitored by phase-contrast microscopy along culturing. Controls included W-MSCs cultured in standard growth medium for 3 weeks to monitor the spontaneous formation of lipid vacuoles.

Chondrogenic Differentiation

Differentiation of cells was performed by seeding WJ-MSCs into alginate beads, using slight modifications of previously published protocols [58, 59]. Briefly, WJ-MSCs were resuspended in sodium alginate (Sigma-Aldrich) (4×10^6 cells/ml at a final concentration of 1,2% sodium alginate in sterile physiologic solution). Beads were formed by slowly dispensing droplets of the alginate cell suspension from a 22-gauge needle syringe into a 100 mM CaCl2 solution. After washes with 0.15 M NaCl, the beads were rinsed with DMEM. Then, beads were cultured either in standard growth medium (controls) or chondrogenic medium, prepared using published protocols with slight modifications (DMEM supplemented with 1% FBS, 6.25 μg/ml insulin, 10 ng/ml TGFβ1, 50nM ascorbate-2-phosphate, 1% antibiotic/antimycotic, 1x NEAA) [36]. Beads were maintained in culture for three weeks, with medium changes every second day. For fixation and paraffin embedding, beads were processed as previously described [58, 59]. The beads were fixed in 4% paraformaldehyde, 0.1 M cacodylate buffer, pH 7.4, with 10 mM $CaCl_2$ for 4 hrs at 20°C. and then washed overnight at 4°C in 0.1 M cacodylate buffer (pH 7.4) containing 50 mM $BaCl_2$. The beads were standard dehydrated through alcohols and xylene and embedded in paraffin. Sections (6μm) were processed for histology (Alcian Blue and nuclear fast red staining) and IHC.

Histochemical Staining

To demonstrate the acquisition of the osteogenic phenotype, the Alizarin Red S staining was performed. Briefly, cells were fixed in 4% paraformaldehyde and stained with 1% solution of Alizarin Red S (Sigma). Stained cells were rinsed with water for three times to remove excess stain, and then photographed at the photomicroscope.

To demonstrate the adipogenic differentiation, cells were stained with Oil Red O (Sigma), and photographed at the photomicroscope. After medium aspiration, a brief wash was performed with PBS. Cells were fixed with 10% formalin (Sigma) for 30 min at room temperature, followed by subsequent washes with distilled water and 60% isopropanol. Oil Red O working solution was added to the cells for 5 min, followed by four washes (5 min each) with distilled water. The wells were viewed and photographed using an inverted phase-contrast microscope. Following a further step of counterstaining (Meyer's haematoxylin, 1 minute), lipid vacuoles appeared red and nuclei appeared blue. To demonstrate the acquisition of the chondrogenic phenotype, the alcian blue with nuclear fast red staining was performed. Cells cultured in alginate beads were formalin-fixed and paraffin embedded using standard protocols. Sections were de-paraffinized and hydrated to distilled water, then Alcian Blue (Sigma-Aldrich) solution (1% Alcian Blue 8GX in 3% acetic acid, pH 2.5) was added for 30 minutes. After washing, nuclei were counterstained with nuclear fast red (Sigma-Aldrich)

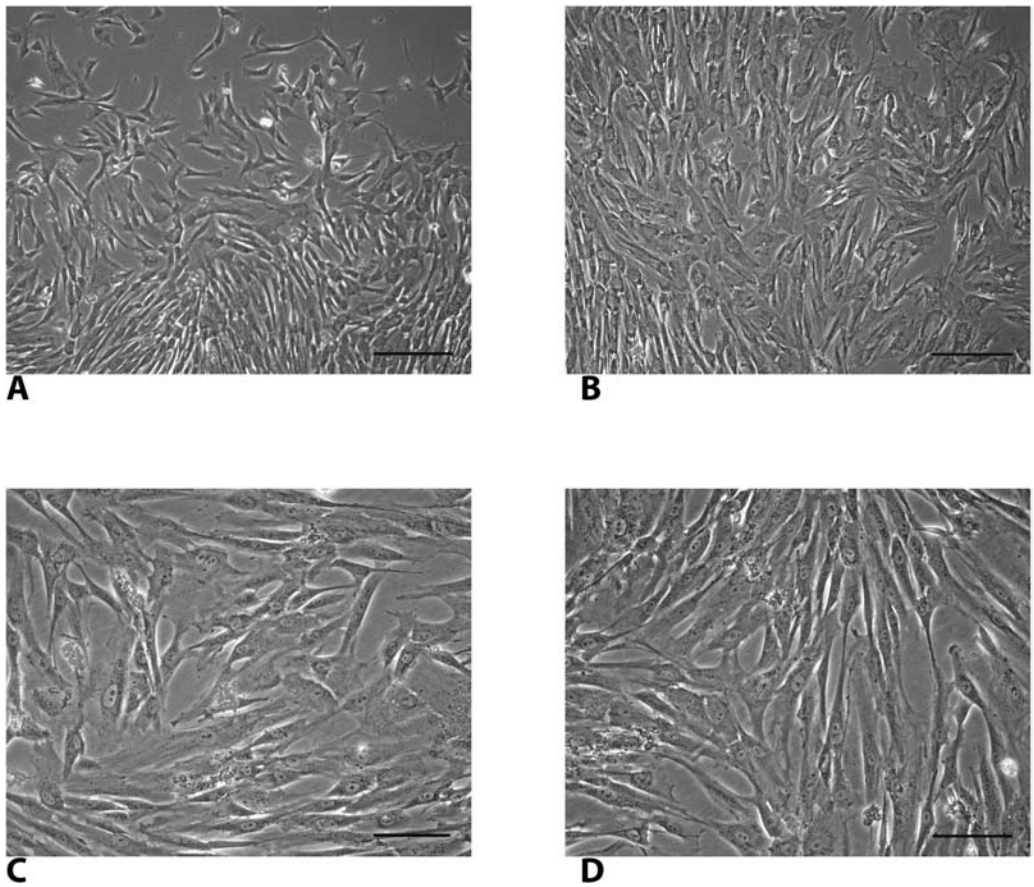

Fig. (1). Light microscopic micrographs of different WJ-MSCs populations in monolayer culture. Cultured cells assumed a polymorphic, fibrobroblast-like morphology, which was maintained throughout the passaging process. The panels show: cells at passage 1, prior to reach confluence (**A**); confluent cells at passage 1 (**B**); confluent monolayer at passage 1 (**C, D**). Magnification x20. Bar: 100µ.

solution (0.1%). Sections were then dehydrated and mounted for observation (not shown).

RESULTS

Cellular Isolation of WJ-MSCs

The isolation protocol of WJ-MSCs which we developed allows to reproducibly isolate and expand plastic-adherent cells starting from tissue explants. During 15 days, WJ-MSCs exit from samples and migrate to adhere to the substrate, therefore the slow degradation of the matrix determine a continue stimulation for cells by growth factor and signalling molecules action. WJ-MSCs cultured in standard growth medium showed fibroblastoid morphology at different confluence states. As visible in Fig. (**1**) the typical mesenchymal morphology of WJ-MSCs isolated from different samples is maintained at different passages.

Confluent cells were routinely splitted and maintained in culture up to 15^{th} passage, with a mean population doubling time of 7.5 days. To confirm the long-term storability and the ability to survive deep freezing, the cultured cells were freezed and stored in liquid nitrogen at different passages and subsequently were defrosted and cultured (data not shown). Standard immunocytochemical and molecular analyses were routinely performed on freshly isolated cells to assess the expression of typical mesenchymal markers and the absence of haematopoietic/endothelial markers (data not shown).

Tri-Lineage Differentiation of WJ-MSCs

According to the general consensus, MSCs are considered multiopotent stem cells since they can be differentiated into almost three different cell types: osteocytes, adipocytes and chondroblasts.

For osteogenic differentiation, WJ-MSCs were cultured in a maturation medium for three weeks. For the same time, control cells were cultured in standard growth medium. The process of osteoblast differentiation was assessed by Alizarin Red S staining: as visible in Fig. (**2A**, **B**), control cells were negative to the staining, while the differentiated cells showed a red staining, indicating of the accumulation of extracellular calcium-containing deposits. Moreover, the control cells, cultured in a standard growth medium, maintained the fibroblastoid morphology, while the cells induced to osteogenic differentiation, showed the formation of cellular aggregates, similar to bone nodules.

To demonstrate that WJ-MSCs may differentate towards adipocyte-like cells, we cultured our cells in a medium supplemented with adipogenic inducers and in a control medium for three weeks. By phase-contrast microscopy, it possible to observe in Fig. (**2C**, **D**) the presence of multiple intracellular

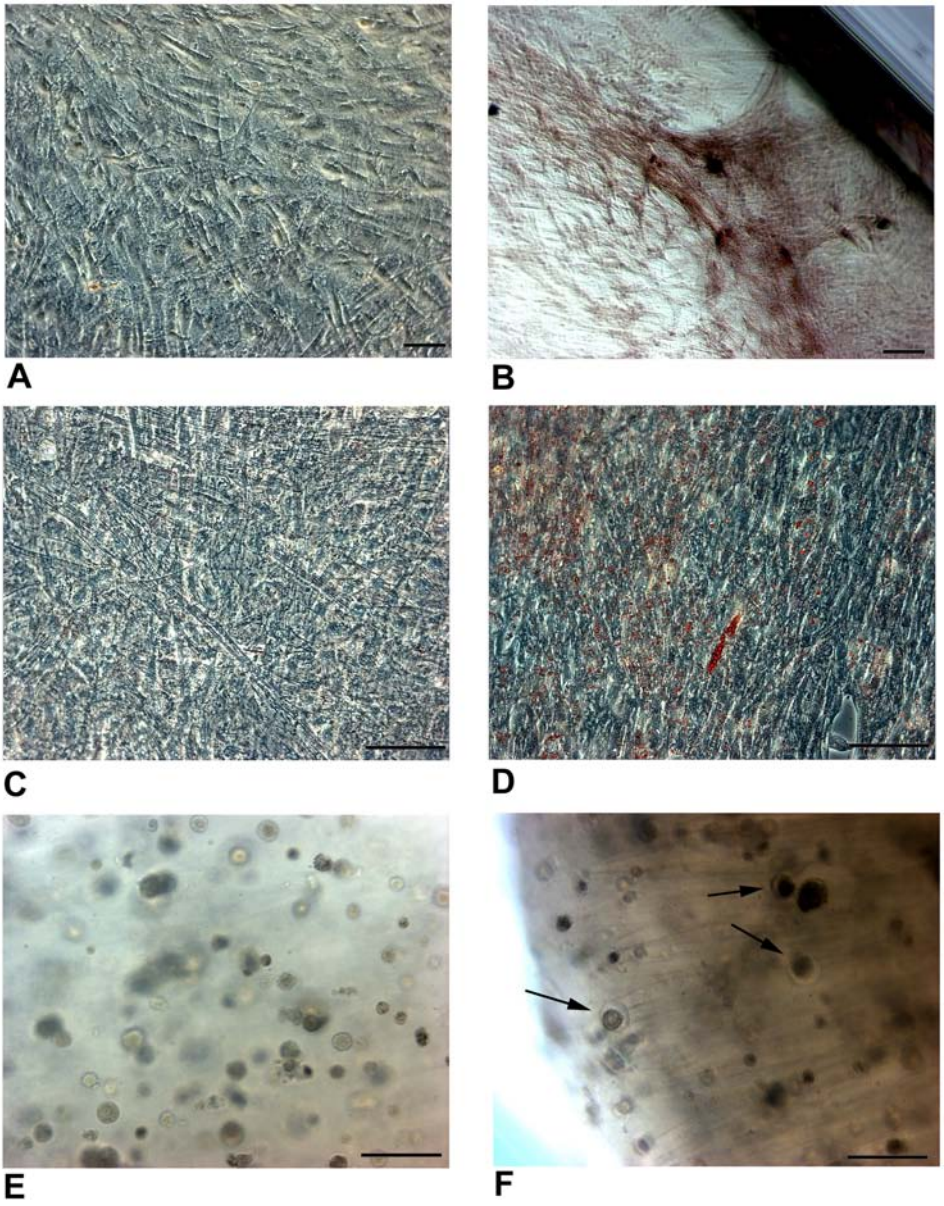

Fig. (2). Light microscopic demonstration of bone tissue formation by WJ-MSCs following osteogenic differentiation. WJ-MSCs cultured in osteogenic medium assume a cuboidal shape with formation and deposit of a mineralized matrix assessed by Alizarin Red S staining (**B**), with respect to fibroblastoid, staining-negative control cells (**A**). Adipogenic-like cells feature the presence of neutral lipid vacuoles, evidenced by Oil red staining (**D**), which are absent in control cells **(C)**. WJ-MSCs cultured for 3 weeks in chondrogenic medium, in alginate matrix showed morphological variations (appearance of rounded lacunae, indicated by arrows) (**F**) with respect to control cells (**E**). Magnification: x10 (**A**, **B**), x20 (C-F). Bar: 100μ.

vacuoles, only in differentiated cells, as demonstrated by Oil Red O staining, which allowed to highlight the presence of multiple neutral lipid vacuoles of different sizes in adipocyte-like cells, resembling multivacuolar adipocytes of brown fat. As expected, control cells were negative to this staining.

For chondrogenic differentiation, we cultured cells, after seeding in alginate matrix, in presence of a medium supplemented with chondrogenic inducers and, for controls, in a standard growth medium for three weeks, as we reported previously [59]. As visible in Fig. (**2F**), only differentiated cells featured the expected formation of lacunae, while control cells do not (Fig. **2E**). The acquisition of a chondrogenic phenotype was also confirmed by immunocytochemical analysis (see below), by evaluating the expression of type II collagen, which is characteristically expressed in cartilage.

Expression of Immunomodulatory Molecules in Control and Chondrogenic-Like Cells, Cultured in Alginate Matrix

Since one of the main features of MSCs is the immune modulation of lymphocyte activity *in vitro* and *in vivo*, the maintenance of these characteristics by differentiated cells clearly deserves further investigation. Few papers are present

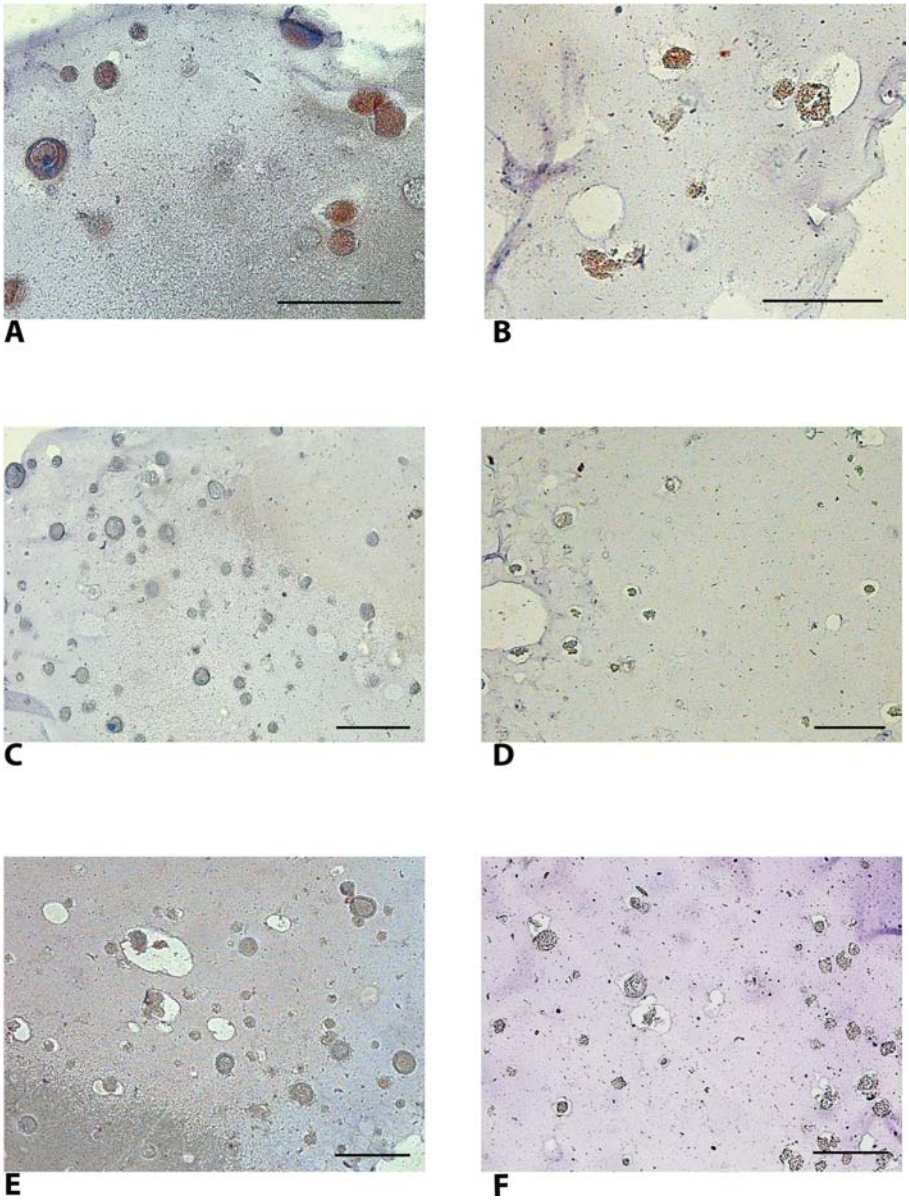

Fig. (3). Representative panels of immunohistochemistry detection of immune-related molecules by WJ-MSCs cultured in alginate matrix and induced to differentiate in presence of chondrogenic medium. Control cells were cultured in a standard growth medium. HLA-A/B/C was expressed in both control (**A**) and chondrogenic-like cells (**B**), while HLA-DR was not detectable in any condition (**C**, **D**). Control cells showed positivity for B7-H3 (CD276) (**E**), with the light staining which was also present in differentiated cells (**F**). Magnification x 40 (**A**, **B**); x20 (**C**-**F**). Bar: 100µ.

in literature dealing with the immune features of differentiated cells, with contrasting results between the osteogenic and chondrogenic model. In addition, the number of immune-related molecules investigated remains low, often limited to the MHC molecules and classical B7 costimulators (CD80 and CD86).

Therefore, to extend the previous reports and try to elucidate whether the expression of immunomodulatory molecules may be a feature only of undifferentiated WJ-MSCs, we evaluated the expression of different immune molecules also in differentiated cells and in particular in chondrogenic-like cells. Immunohistochemistry analysis on alginate-cultured cells confirmed the expression of type I MHC such (HLA-A-B-C) and the lack of HLA-DR, a type II MHC, both in undifferentiated and differentiated cells Fig. (**3**), panels **A**-**D**). According to recent data in literature [56], also WJ-MSCs expressed HLA-E, a non-classical type I MHC molecule involved during tolerance-induction processes and in particular in the selective binding with NK cells. Even if WJ-MSCs do express HLA-E when cultured in standard conditions, as shown below for the osteogenic and adipogenic differentiation experiments, chondrogenic differentiation shows that neither alginate-seeded undifferentiated cells, nor differentiated ones, did express this marker at the protein level. We may hypothesize that the three-dimensional alginate matrix influences the cellular behaviour so that the expression of some factors is lost even in undifferentiated cells.

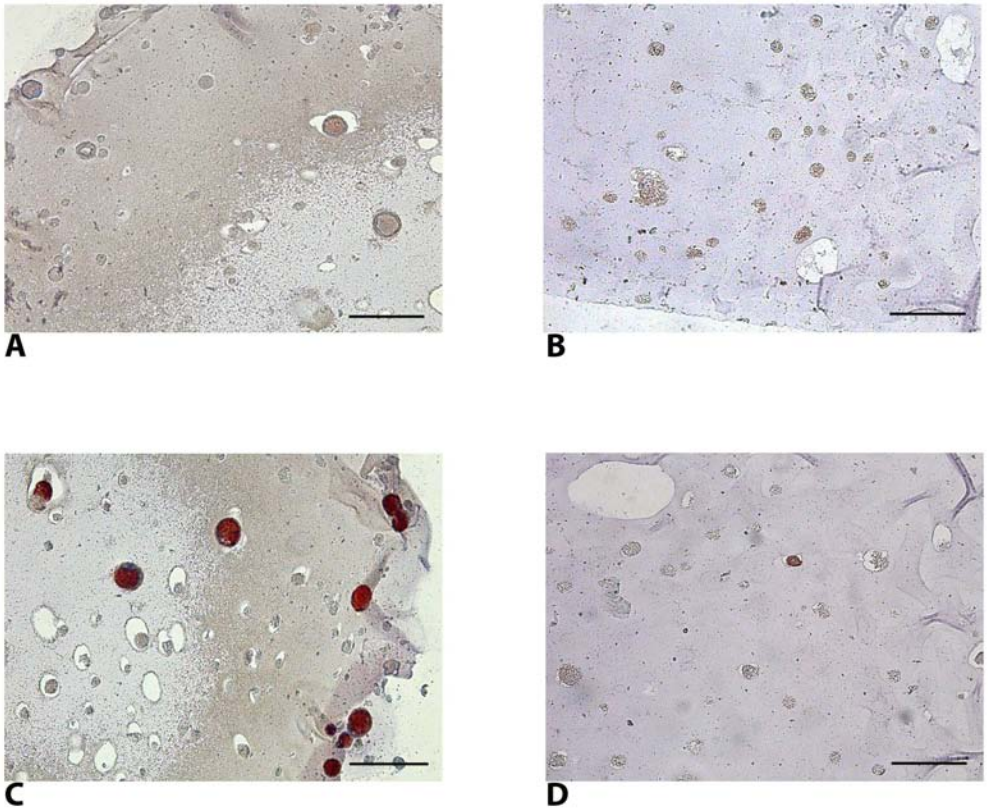

Fig. (4). Immunohistochemical demonstration of collagen type II expression by chondrogenic-differentiated WJ-MSCs. Cells cultured in alginate matrix with standard growth medium did not form lacunae around cells, and did express both type II collagen (**A**) and vimentin (**C**). Alginate-seeded cells grown in differentiation medium showed the appearance of pericellular lacunae, and maintained the expression of both type II collagen (**B**) and, at a lesser extent, vimentin (**D**). Magnification: x20. Bar: 100μ.

In our opinion, further research is needed to better clarify this issue in the chondrogenic differentiation model. Another immunomodulatory molecule which has been demonstrated to be expressed by MSCs is IDO, an enzyme involved in the tryptophan metabolism, an essential aminoacid for lymphocyte proliferation (see Table **3**). Also for this molecule, its expression in WJ-MSCs was demonstrated in our laboratory (Anzalone *et al.*, manuscript in preparation). However, as observed for HLA-E, alginate embedding seems to block the expression of IDO at the protein level in both undifferentiated and differentiated cells.

The expression of molecules belonging to the B7 superfamily is of critical importance for their role as costimulators in immune processes. MSCs are mainly negative for classical members of this family, as B7-1 (CD80) and B7-2 (CD86). As shown in Table **3**, both molecules were absent in both control and chondrogenic cells, therefore confirming the maintenance of the immune asset of these molecules after differentiation. In addition, for the first time in WJ-MSC, we demonstrated in both undifferentiated and chondrogenic-like cells the presence of B7-H3 (CD276), (Fig. **3E**, **F**). For this co-stimulatory molecule also co-inhibitory roles have been suggested [49]. Immunohistochemical detection allowed to confirm the mesenchymal phenotype by these cells, highlighting the expression of vimentin, an intermediate filament found in cells of mesenchymal origin (Fig. **4C**, **D**). To confirm the acquisition of chondrogenic phenotype by WJ-MSCs, we evaluated the expression of a cartilage-specific protein, type II collagen, as shown in Fig. (**4A**,**B**). According general consensus MSCs population do not express haematopoietic stem cells markers, and here we showed the lack expression of CD31 both in undifferentiated and differentiated WJ-MSCs. (see Table **3**).

Expression of Immunomodulatory Molecules in Osteogenic and Adipogenic Control and Differentiated Cells

A further comparative analysis was carried out in parallel between WJ-MSCs and differentiated adipogenic and osteogenic-like cells by qualitative RT-PCR.

As shown in Table **4**, WJ-MSCs differentiated towards osteogenic and adipogenic lineages do maintain the expression of some immune-related molecules compared to control cells. In fact, as also shown in Fig. (**5**), the non-classical type I MHC molecules HLA-E, HLA-F and HLA-G, are expressed in both control and differentiated cells. This constitutes, to the best of our knowledge, the first evidence in MSC-derived differentiated cells. Both undifferentiated and differentiated cells the lacked the expression of HLA-DR as shown in Table **4** and in Fig. (**5**). HLA-F and HLA-G are two non canonical class Ib MHC molecules involved during the establishment of tolerance processes between mother and fetus. Molecular biology analysis further confirmed the expression of B7-H3 in both undifferentiated and differentiate cells. (see Table **4**). Since other reports highlighted the pres-

Table 3. Immunohistochemistry results of markers expression by undifferentiated and chondrogenic-like cells. Results of the immunohistochemistry analysis are represented semiquantitatively.

Antigen	Control	Differentiated
B7-H3	++	++
B7-1	-	-
B7-2	-	-
CD31	-	-
Collagen II	+	++
HLA-ABC	++	+
HLA-DR	-	-
HLA-E	-	-
IDO	-	-
Vimentin	++	+

ence in BM-MSCs of other molecules belonging to B7 family, known to have also a co-inhibitory role, such as B7-H1 and B7-H4, we wanted to assess their expression in WJ-MSCs. As detailed in table **4**, both undifferentiated and differentiated cells do not express these molecules.

The presented data collectively demonstrate that WJ-MSCs maintain the expression of immune-related molecules in both undifferentiated and differentiated state, therefore suggesting that these cells may be considered good candidates for clinical applications.

DISCUSSION

In the last years, musculoskeletal and arthritic disorders are increasingly viewed as one of the main causes of disability in the world. OA and RA are now recognized, by World Health Organization, as the musculoskeletal conditions that induce major health troubles [60].

Articular cartilage is the main target of these disorders which heavily affect its structure. Chondrocytes represent the only cellular type of cartilage. They are plunged in the matrix and are fed thanks to diffusion processes due to the avascularity of this tissue. For this reason, the cartilage regeneration and repair processes are very slow. Thanks to the differentiative capacity of mesenchymal stem cells, there is accumulating evidence for their use in cartilage tissue engineering and musculoskeletal tissue regeneration [2]. Articular cartilage is avascular while bone is very well vascularized: thanks to the presence of some capillaries in subchondral bone at the cartilagine-bone interface, chondrocytes receive nutrients and can perform gas exchange. A recent work suggested the use of a vascularized tissue engineered construct obtained by presence of tissue progenitor and vascular progenitor cells into biocompatible scaffold [61]. Particular interest was demonstrated in the use of specific media to support proliferation by MSCs and their transdifferentiation process. Some studies showed that hypoxia can induce chondrogenesis and chondrogenic differentiation of MSCs [62-64]. Further reports highlighted the use of a bioreactor with co-culture of MSCs and other stem cells to study the culture and mechanical stimulation [65]. Emerging data showed that MSCs could modulate innate and adaptive immunity by several mechanisms, therefore reinforcing the concept of their use for cellular therapy. MSCs inhibit T-

Table 4. RT-PCR results of immune molecules expression by control and differentiated cells (adipogenic-like cells and osteogenic-like cells). Results of molecular analysis are represented qualitatively.

Immune Molecules	Control Cells	Osteogenic-Like Cells	Control Cells	Adipocyte-Like Cells
B7-H1	-	-	-	-
B7-H3	+	+	+	+
B7-H4	-	-	-	-
CD80	-	-	-	-
CD86	-	-	-	-
HLA-A	+	+	+	+
HLA-DR	-	-	-	-
HLA-E	+	+	+	+
HLA-F	+	+	+	+
HLA-G	+	+	+	+
TGFβ2	+	+	+	+

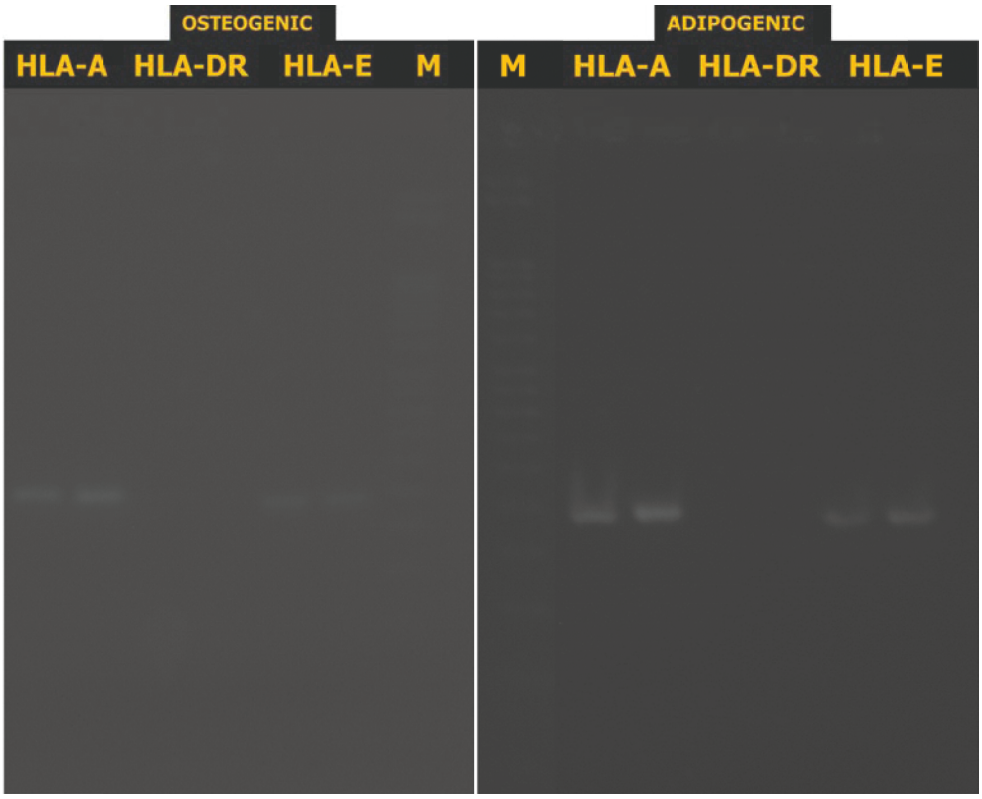

Fig. (5). Representative panels of RT-PCR analysis of immune molecules expression in WJ-MSC subjected to adipogenic or osteogenic differentiation. WJ-MSC cultured for three weeks in presence of osteogenic and adipogenic medium showed the expression of HLA-A and HLA-E, while HLA-DR was not detectable. Control WJ-MSC cultured for three weeks in a standard growth medium show the same pattern of immune molecules expression. M: 50 bp ladder. Left lanes are referred to controls and right lane sto differentiated cells, for any molecole considered.

lymphocyte proliferation and activation by soluble factors such as TGF-β PGE2, HGF and IDO [66]. In particular, MSCs could decrease IFN-γ production and could increase the IL-4 expression, determining a switch from pro-inflammatory state to anti-inflammatory one [67].

In the present paper, we aimed to determine the expression, both in undifferentiated and differentiated cells, of some of the major immune-related molecules which have been suggested as responsible of MSCs immune privilege.

T- cell anergy is mechanism by which MSCs induce immunosuppression: some authors demonstrated that the lack expression of two co-stimulatory molecules such as CD80 (B7-1) and CD86 (B7-2) by MSCs could induce an anergy state in T cells [68]. In our report, we showed by immunohistochemistry analysis that both undifferentiated and differentiated WJ-MSCs do not express the two B7 molecules. Recent reports indicated that other members of the B7-family, namely B7-H1, B7-H3, B7-H4 may have promising co-inhibitory roles in addition to known co-stimulatory roles. Previously, the expression of B7-H1 and B7-H4 has been demonstrated in BM-MSCs [50]. In contrast, our results showed the lack expression of these two molecules in both undifferentiated and differentiated WJ-MSCs. On the contrary, we demonstrated that our cells do express B7-H3 (CD276) at both the protein and RNA level. The expression of such marker seems to be unaffected by the differentiation protocol applied, and recalls what we just observed in human heart-derived MSCs, which are the first class of adult MSCs in which CD276 expression has been characterized [59].

Classical and non-classical class I MHC molecules are increasingly being considered as promising molecules for the global immune privilege of mesenchymal stem cells. In literature, some reports highlighted the capacity by MSCs to determine a tolerance process by inducing the proliferation of regulatory T cells (T regs) by release of the HLA-G isoform HLA-G5. In our experiments, RT-PCR analysis allowed to highlight the expression of HLA-G mRNA in control cells and osteogenic and adipogenic differentiated cells. Other class Ib MHC molecules are HLA-E and HLA-F, produced by trophoblast cells together with EPF (early pregnancy factor) [69]. All these molecules have been involved in the tolerance process between mother and fetus. The expression of these molecules in MSCs has been highlighted in heart-derived MSCs [59], and we wanted to better detail their presence also in WJ-MSCs. We demonstrated by RT-PCR analysis that WJ-MSCs express both these class Ib MHC, in both undifferentiated and differentiated state. This is the first report highlighting the expression of HLA-F in WJ-MSCs.

Collectively, these data may provide a further point in the characterization of differentiated cells not only on the basis of the expression of desired markers of the mature cytotype,

but also for the maintenance of the immunomodulatory properties of naïve cells, which may further promote the reparative action of these cells if used in regenerative medicine applications. A number of diseases which in their final stages require organ transplant or cellular therapy, derive from or are accompanied by, an unbalance in the organ inflammatory or immune state. To this regard, the use of a cellular therapy vehicle which may provide both organ recellularization and restoration of a physiological microenvironment, may be a further benefit for patients. In addition, as MSCs are globally recognized for their immune privilege, which allows to evade the host immune response also in allogeneic settings, the possibility that also differentiated cells may maintain this feature deserves further research and *in vivo* applications for the increasing potential beneficial outcomes it can reserve. Literature reports did suggest that the chondrogenic differentiation process often fails in maintaining the immunomodulatory features of undifferentiated cells, when compared to the adipogenic or osteogenic differentiations [70-72]. Our present data suggest that for some antigens (as HLA-E), the alginate-embedding protocol may result in a phenotypycal switch also in the control cells. However, the positive correlation observed for the other MHC molecules, and the B7 costimulators monitored, strongly point to the maintenance of immune-related molecules as a global feature of differentiated WJ-MSCs.

REFERENCE

Our data strongly support the concept of WJ-MSCs as a reliable and promising option for cellular therapy, in the regenerative and reparative medicine field.

CONFLICT OF INTEREST

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