

Human Mesenchymal Stem Cells Seeded on Extracellular Matrix-Scaffold: Viability and Osteogenic Potential

LETIZIA PENOLAZZI,¹ STEFANIA MAZZITELLI,¹ RENATA VECCHIATINI,¹
ELENA TORREGGIANI,¹ ELISABETTA LAMBERTINI,¹ SCOTT JOHNSON,²
STEPHEN F. BADYLAK,² ROBERTA PIVA,^{1*} AND CLAUDIO NASTRUZZI³

¹Department of Biochemistry and Molecular Biology, University of Ferrara, Ferrara, Italy

²McGowan Institute for Regenerative Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania

³Department of Pharmaceutical Sciences, University of Ferrara, Ferrara, Italy

The development and the optimization of novel culture systems of mesenchymal osteoprogenitors are some of the most important challenges in the field of bone tissue engineering (TE). A new combination between cells and extracellular matrix (ECM)-scaffold, containing ECM has here been analyzed. As source for osteoprogenitors, mesenchymal stem cells obtained from human umbilical cord Wharton's Jelly (hWJMSCs), were used. As ECM-scaffold, a powder form of isolated and purified porcine urinary bladder matrix (pUBM), was employed. The goals of the current work were: (1) the characterization of the *in vitro* hWJMSCs behavior, in terms of viability, proliferation, and adhesion to ECM-scaffold; (2) the effectiveness of ECM-scaffold to induce/modulate the osteoblastic differentiation; and (3) the proposal for a possible application of cells/ECM-scaffold construct to the field of cell/TE. In this respect, the properties of the pUBM-scaffold in promoting and guiding the *in vitro* adhesion, proliferation, and three-dimensional colonization of hWJMSCs, without altering viability and morphological characteristics of the cells, are here described. Finally, we have also demonstrated that pUBM-scaffolds positively affect the expression of typical osteoblastic markers in hWJMSCs.

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Recently, native allogenic or xenogenic extracellular matrix (ECM) has been proposed for clinical use in tissue engineering (TE) approaches, with the aim to possibly ameliorate tissue regeneration (Wollenweber et al., 2006; Badylak, 2007; Chan and Mooney, 2008). Unfortunately, clinical application of TE technologies has been restricted to a relatively limited number of biomaterials.

Among them, structural proteins such as collagen, gelatine, and fibronectin have been used as vehicles for cell seeding and *in vivo* implantation, since they can approximate the structure and function of ECM (Vinatier et al., 2009; Ode et al., 2010). Nevertheless, it is noteworthy that tissue morphogenesis is heavily influenced by the interaction of cells with the complex architecture and chemical composition of natural ECM (Beattie et al., 2009; Guilak et al., 2009; Iop et al., 2009; Pennesi et al., 2010). Simple polymers provide mechanical support to the seeded/entrapped cells, but do not adequately mimic the multiple interactions between adult stem, progenitor cells, and the ECM, during neo-tissue development. In this respect, exogenous ECM-based biomaterials can provide a native framework for cell adhesion, at the site of a tissue deficit, allowing local cells to migrate into the matrix, adhere, and undergo differentiation.

These effects are mediated both by natural ECM signaling and regulatory functions and soluble signals provided by growth factors and hormones. Furthermore, ECM is characterized by a dynamic 3D-architecture, continuously modified by complex interactions with homing cells (Docheva et al., 2007; Badylak et al., 2009).

The great complexity of ECM and its unique features, has since now, strongly hindered the development of laboratory methods for the complete assembly of a true, native ECM from isolated and purified components. Therefore, decellularized tissues or organs have been proposed as sources of biological

ECM for TE (Badylak et al., 2009; Nam et al., 2010; Soto-Gutierrez et al., 2010; Stabile et al., 2010; Yang et al., 2010).

The relatively high degree of evolutionary conservation of many ECM components has permitted the use of xenogeneic materials. Various acellular matrices have been utilized successfully for TE in animal models and a limited number of xenogeneic products have received regulatory approval for clinical use, including decellularized heart valves, small intestinal submucosa, and urinary bladder matrix (Hodde, 2002; Gabouev et al., 2003; Aitken and Bägli, 2009; Okada et al., 2010; Zhou et al., 2010).

Porcine ECM, derived from urinary bladder used as biomaterials [named throughout the text as porcine urinary bladder matrix (pUBM)-scaffold], can provide the necessary structural support and dynamic exchange signals to local cells leading to tissue infill. The major constituents found in UBM are collagen (types I, III, and IV–VII), glycoproteins (such as fibronectin and laminin), glycosaminoglycans (GAGs) and various growth factors, including transforming growth factor- β , basic fibroblast growth factor and vascular endothelial growth factor (Badylak, 2007; Badylak et al., 2009; Brown et al., 2010; Mazzitelli et al., 2011).

*Correspondence to: Roberta Piva, Department of Biochemistry and Molecular Biology, University of Ferrara, Via Fossato di Mortara, 74, 44121 Ferrara, Italy. E-mail: piv@unife.it

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TE approaches based on the use of ECM-scaffold could represent an alternative strategy (to autologous bone graft) for the treatment of a variety of bone defects caused by trauma, tumor, infection, degenerative joint disease, congenital crippling disorders, periprosthetic bone loss, and for oral and maxillofacial surgery (Datta et al., 2005; Petrovic et al., 2006; Valentin et al., 2006; Park et al., 2007; Dong et al., 2009; Porter et al., 2009).

In this respect the combined use of ECM-scaffold with mesenchymal stem cells (MSCs) could represent an innovative cell-based strategy for both site-specific and systemic bone regeneration (Arthur et al., 2009). In fact, the implantation of MSC-seeded biomaterials has been shown to significantly increase the efficacy to repair skeletal defect over non-seeded constructs (Robey et al., 2007; Tortelli and Cancedda, 2009; Hidalgo-Bastida and Cartmell, 2010; Ode et al., 2010; Zannettino et al., 2010).

Considerable line of evidence from many studies demonstrates that the maintenance of proliferative capacity and osteogenic differentiation of MSCs is positively affected by cultivation of these cells on various ECM substrates (Mauney et al., 2005; Lanfer et al., 2009; Thibault et al., 2010). It is in fact well known the pivotal role of ECM in driving cell behavior through signals controlling cell shape, migration, proliferation, differentiation, morphogenesis, and survival. A significant role in mediating these phenomena is played by the physical interactions between MSCs and certain ECM motifs (Chastain et al., 2006; Kundu et al., 2009; Pennesi et al., 2010).

Previous studies have shown that human MSCs derived from the Wharton's jelly of umbilical cord (hWJMSCs) provide an unique system with unlimited growing potential and ability to differentiate toward osteoblastic lineage (Wang et al., 2004; Hsieh et al., 2010; Nekanti et al., 2010; Majore et al., 2011).

In this study we employed hWJMSCs with the highest degree of osteogenic potential as selected from umbilical cord of the heaviest term newborns (Penolazzi et al., 2009) and pUBM-scaffolds.

In order to analyze the ability of the scaffold to support hWJMSCs adhesion, proliferation, and 3D colonization, as well as to determine if pUBM-scaffold is a good source of bone cell differentiation supporting factors, the proliferation, and osteogenic differentiation of hWJMSCs in traditional two-dimensional (2D) cultures and in combination with pUBM-scaffold were compared.

Materials and Methods

Human WJMSCs: Isolation procedure and culture conditions

Human umbilical cords (all from natural deliveries) were collected after mothers' consent and approval of the "Ethical committee of University of Ferrara and S. Anna Hospital." Harvesting procedures of Wharton's Jelly from umbilical cord were conducted in full accordance with the "Declaration of Helsinki" as adopted by the 18th World Medical Assembly in 1964 successively revised in Edinburgh (2000) and the Good Clinical Practice guidelines. Cords were processed within 4 h, and until that moment stored at 4°C in sterile saline. Typically, cord was rinsed several times with sterile PBS before processing and cut into pieces, 2–4 cm in length. Blood and clots were drained from vessels with PBS, to avoid any contamination. Single pieces were dissected, first separating the epithelium of each section along its length, to expose the underlying Wharton's Jelly. Later cord vessels (the two arteries and the vein) were pulled away without opening them. The soft gel tissue was then finely chopped. The same tissue (2–3 mm² pieces) were placed directly into 75-cm² flask for culture expansion in 10% FCS (Euroclone S.p.A., Milan, Italy) DMEM low glucose supplemented with antibiotics (penicillin 100 mg/ml and streptomycin 10 mg/ml) at 37°C in a humidified atmosphere of 5% CO₂. After 5–7 days the culture medium was removed and, thereafter, changed twice a

week. At ~70–80% confluence, cells were scraped off by 0.05% trypsin/EDTA (Gibco, Grand Island, NY; 2 min), washed, counted by hemocytometric analysis, assayed for the viability, and the expression of MSC surface marker molecules (CD-90, CD-105, and CD-29) by FACS analysis, as previously described (Penolazzi et al., 2010). For the determination of viability and osteogenic potential of hWJMSCs seeded on pUBM, cells were cultured in adherent conditions or non-adherent conditions. In the first case, cells, suspended in DMEM low glucose were directly placed into multiwell cell culture dishes, flat bottom with lid tissue culture treated, non-pyrogenic polystyrene (Corning Inc., Corning, NY). Conversely, to prepare parallel non-adherent cultures, the same amount of hWJMSCs was added to identical culture dishes, that were previously coated with a thin agarose gel, obtained by layering the dishes with a 250 µl of 0.9% RNase-free agarose in the serum-free medium, left to gelify for 2 h at 5°C.

pUBM-scaffold preparation

pUBM-scaffolds were prepared with minor modifications of a previously described method (Badyrak et al., 2005). Briefly, porcine urinary bladders were harvested from market weight pigs (approximately 110–130 kg; Whiteshire-Hamroc, Albion, IN). The urinary bladder was trimmed to eliminate external connective tissues, including adipose tissue, finally the residual urine was removed by repeated washes with tap water. Thereafter, the urothelial layer was removed by soaking of the bladder in 1 N NaCl solution. The tunica serosa, tunica muscularis externa, and the tunica submucosa were mechanically delaminated from the remaining bladder tissue. The remaining basement membrane of the tunica mucosa and the subjacent tunica propria, referred as UBM, were then decellularized and disinfected by treatment with 0.1% (v/v) peracetic acid, 4% (v/v) ethanol, and 96% (v/v) sterile water for 2 h. The pUBM was then washed twice for 15 min with PBS (pH 7.4) and twice for 15 min with deionized water. After isolation, the particulate form of pUBM was obtained as follows. The decellularized material was firstly chopped into small sheets for immersion in liquid nitrogen. The snap frozen material was then size reduced by treatment with a blender to obtain particles small enough to be fed into a rotary knife mill. After milling, the obtained powder was sieved to achieve a powder size less than 250 µm. The final pUBM particles were examined for residual cell content after fixation with 10% neutral buffered formalin, embedding in paraffin and staining by DAPI and hematoxylin and eosin. Images of the stained samples were taken using a Nikon (Melville, NY) eclipse E600 microscope.

Morphological analysis of pUBM-scaffold

The analysis of the morphological architecture, the porous structure, and the adhesion/integration of hWJMSCs to pUBM-scaffolds was performed by different microscopic techniques, namely: inverted optical microscopy (Nikon Diafophot, Tokyo, Japan), optical stereomicroscopy (Nikon SMZ 1500 stereo microscope, Tokyo, Japan), scanning electron microscopy (SEM; Cambridge S360 microscope; Cambridge Instruments, Cambridge, UK), and transmission electron microscopy (TEM; ZEISS EM 910 electron microscope; Zeiss, Oberkochen, Germany). SEM analysis in variable pressure (until 100 Pa) were performed without gold coating (EVO 40, Zeiss). X-ray energy dispersive spectroscopy (EDX; Inca Energy 300, Oxford Instruments, Tubney Woods, Abingdon Oxon, UK) was used for a qualitative estimation of crystal deposition and mineralization via the appearance of calcium and phosphorus peaks. Samples for the electron microscopy, were fixed in glutaraldehyde 2.5% buffered solution and osmium tetroxide 2% buffered solution and dehydrated; for SEM analysis, samples were gold coated (Edward Sputter S150), while for TEM analysis, samples were araldite embedded (ACM Fluka Sigma-Aldrich Co., St. Louis, MO) and the ultra-thin sections of a selected area were contrasted with uranyl acetate lead citrate. The analysis

of microphotographs was performed by ImageJ, a public domain, Java-based image processing program developed at the National Institutes of Health.

In vitro assessment of hWJMSCs: Proliferation and viability

Proliferation. The proliferation of hWJMSCs was determined by the AlamarBlue™ assay (Invitrogen Corporation, Carlsbad, CA). The test is based on the metabolic activity of proliferating cells that results in a chemical reduction of AlamarBlue, previously added to the in vitro cultured cells. Briefly, after different length of time, a medium containing 5% AlamarBlue was added to cells cultured into agarose-coated wells, at 37°C and 5% CO₂. After 3 h of incubation, 200 µl samples of culture medium were withdrawn, centrifuged, and subsequently placed on 96-well plates. The visible light absorption of collected samples was determined, at 570 and 620 nm, by a Microplate Absorbance Reader (Sunrise, Tecan, Austria). Final values were calculated as the difference in absorbance units between the reduced and oxidized forms of AlamarBlue.

Viability. Immediately after isolation and after different length and conditions of in vitro cell culture, the viability of hWJMSCs, cultured in the absence or presence of different amounts of pUBM (1–8 mg of pUBM/10⁵ cells), was assessed by staining with propidium iodide (PI; Sigma-Aldrich Co., St. Louis, MO) and Calcein-AM (Sigma), as previously described (Penolazzi et al., 2010). Cells were visualized under fluorescence microscope (Nikon, Optiphot-2, Nikon Corporation, Tokyo, Japan) using the filter block for fluorescein. Dead cells stained in red, while viable cells appeared green.

For the evaluation of the effect of different ratio cells/pUBM on cell viability, 10⁵ hWJMSC were cultured in non-adherent conditions and exposed to four different quantities of pUBM, respectively, 1, 2, 4, and 8 mg. Determination of viable cells was done with MTT colorimetric assay (thiazolyl blue). MTT assay is based on the conversion of the yellow tetrazolium salt MTT to purple formazan crystals in the mitochondria of living cells (Denizot and Lang, 1986). MTT provides a quantitative determination of viable cells. After 72 h of treatments in triplicate, 200 µl of MTT was added to each well of cells, and the plate was incubated for 2 h at 37°C. The medium was removed, and the MTT crystals were solubilized with 50% dimethylformamide (DMF). Spectrophotometric absorbance of each sample was measured at 570 nm.

The level of apoptosis was analyzed, after the deparaffinization, by the DeadEnd colorimetric apoptosis detection system (Promega, Madison, WI) according to the manufacturer's instructions. After TUNEL assay the sections have been hematoxylin counterstained.

In vitro assessment of hWJMSCs: Osteogenic differentiation

hWJMSCs were induced to osteogenic differentiation, by in vitro treatment, for 21 days, in DMEM high glucose (Euroclone S.p.A.) supplemented with 10% FCS, 50 µM ascorbic acid, 1 nM dexamethasone, and 10 mM β-glycerol phosphate (Sigma-Aldrich, St. Louis, MO). Thereafter, samples were paraffin-embedded, cut into 4 µm thick sections and mounted onto glass slides. The specimens were deparaffinized by treatment with xylene and exposure to a graded series of ethanol solutions (100–70%). For immunohistochemistry analysis, the slides were placed in citrate antigen retrieval buffer, washed twice in PBS, treated with 3% H₂O₂ in PBS, and incubated in 2% normal horse serum (S-2000, Vector labs, Burlingame, CA) for 15 min at room temperature. After the incubation in blocking serum, the sections were incubated for 45 min in primary antibody, respectively, diluted anti human Col1A1 (clone H-197) 1:100, OPN (clone LF-123) 1:100, RUNX2 (clone M-70) 1:100 (Santa Cruz Biotech Inc, Santa Cruz, CA). The sections were then incubated in Vectastain ABC (Vector labs) reagent for 30 min and stained with DAB solution (Vector labs). For the alizarin red staining (ARS), samples were stained with 40 mM Alizarin Red S solution (Sigma-Aldrich), pH 4.2 at room temperature for 10 min. Samples were then rinsed five times in distilled water and washed three times in PBS on an orbital shaker at 40 rpm for 5 min each, to reduce non-specific binding. The stained matrixes were microphotographed by optical microscope. The alkaline phosphatase (ALP) activity was quantified by a

colorimetric p-nitrophenylphosphate-based reaction by using Leukocyte Alkaline Phosphatase Kit (Sigma-Aldrich) directly on deparaffinized sections, following the company indications.

For RT-PCR analysis, total RNA was isolated from hWJMSCs using the Total RNA Isolation system (RNeasy Plus Micro Kit, Qiagen S.p.A., Milan, Italy). Two micrograms of total RNA were reverse transcribed with the Improm-II RT System (Promega). mRNA of target genes was quantified by real-time PCR using the ABI Prism 7700 system and TaqMan probes 5'-AACCCAGAAGGCACAGACAGAAGCT-3' for Runx2 (Applied Biosystems, Foster City, CA). PCR was carried out in a final volume of 25 µl. After a 10 min pre-incubation at 95°C (denaturation), 1 min at 60°C (annealing/elongation). The mRNA levels were normalized on the basis of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA levels (reference gene) and normalized to a calibrator sample (control cells) as previously described (Lambertini et al., 2009).

Statistical analysis

Data are presented as the mean ± SE from at least three independent experiments, where indicated. Statistical analysis was performed by one-way analysis of variance followed by the Student's t-test. A P-value <0.05 was considered statistically significant.

Results

Morphological analysis of hWJMSCs seeded on pUBM-scaffold

Firstly, it should be underlined that pUBM possesses the very peculiar property to maintain its hydrated state throughout the entire preparation process (including decellularization and final sterilization). In addition, pUBM can be conveniently comminuted into a fine powder (i.e., particles ranging from 50 to 250 µm in size can be reproducibly manufactured), allowing the easy preparation of fluid cell–pUBM suspensions. More importantly, the pUBM particles present in comminuted powder retain the ultrastructural, 3D surface characteristics of the parent native ECM, therefore, supporting cell adhesion and infiltration.

As model of adult human MSCs, a population isolated from Wharton's jelly umbilical cord (hWJMSCs) was employed. These cells were preliminarily immunophenotypically characterized, employing a panel of surface markers by flow cytometry as previously described (Lambertini et al., 2009; Torreggiani et al., 2011). hWJMSCs were found positive for CD29, CD44 (adhesion markers), CD90, and CD105, (mesenchymal markers) and negative for CD34 and CD45 (hematopoietic cell markers). As further study, the differentiation ability of hWJMSCs was also evaluated, showing that this particular cell population displayed an appreciable differentiation potential toward osteoblasts, chondrocytes, and adipocytes (data not shown).

In order to characterize the possible effects of ECM on morphology, adhesion behavior and colonization, hWJMSCs were cultured, for a limited period of time, in the absence or presence of pUBM in standard plastic (adherent conditions) or agarose coated (non-adherent conditions) multiwells (prepared as described in the Experimental Section). The effects of osteogenic medium on these parameters were also evaluated. The results of these experiments are reported in Figures 1 and 2, which, respectively, report photomicrographs taken after 2 or 24 h from cell plating. The phase contrast photomicrographs taken after 1 h clearly demonstrated that hWJMSCs displayed a spherical shape when they are cultured in standard plastic multiwells (data not shown). After 2 h, cells start to adhere to the plastic surface (Fig. 1B), thereafter, they grow showing an adherent pattern and assuming a spindle-like morphology, that appears completely evident after 24 h (see

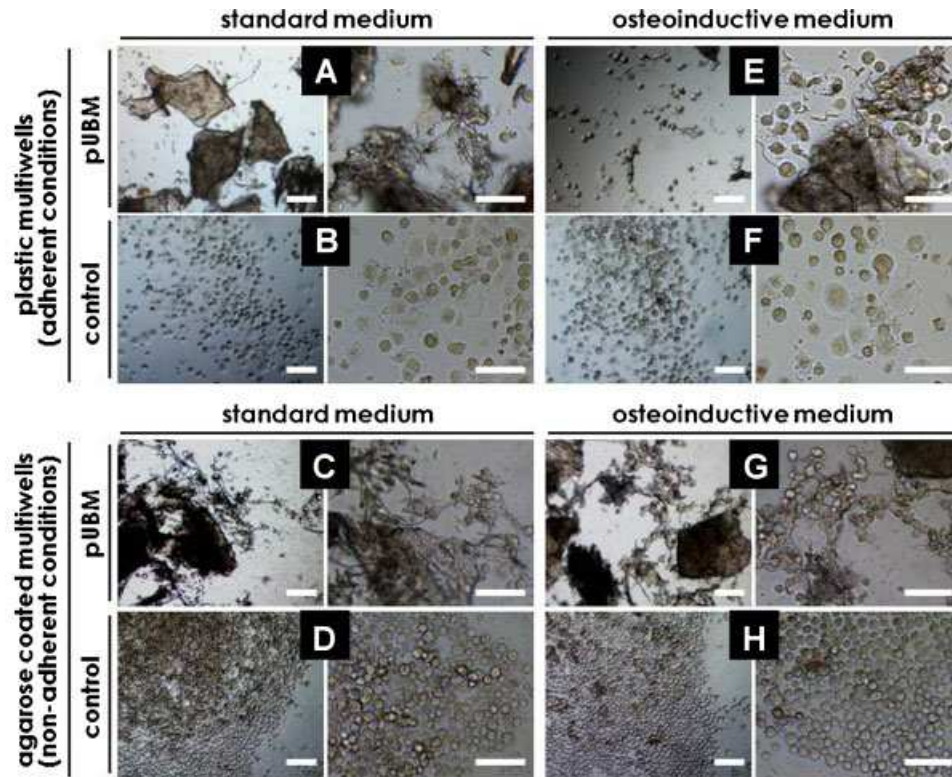


Fig. 1. Optical phase contrast photomicrographs of hWJMSCs cultured, for 2 h, in the indicated conditions: standard plastic (adherent conditions) and agarose coated (non-adherent conditions) multiwells, in the presence of pUBM or not (control). As indicated, cells were grown both in standard and in osteogenic media. Bar corresponds to 120 (first and third lanes) and 80 μm (second and fourth lanes), respectively.

Fig. 2B). On the contrary, when cells are plated onto non-adherent culture dishes (coated with agarose), they maintain, for at least 2 h, a single spherical shape (Fig. 1D). After 24 h, cells aggregate, forming three-dimensional spheroids (Fig. 2D).

Notably, a completely different morphology and growing behavior were observed for cells cultured in the presence of pUBM. As it can be seen in Figure 1A, cells seeded on pUBM show a clear response to the biomaterial in that the appearance of cells that have the tendency to migrate toward the pUBM surface, demonstrating that the cells prefer the pUBM rather than the plastic.

Moreover, at a single cell level (at higher magnification), it is clearly evident a change in morphology related to adhesion and alignment on the surface of pUBM, when compared to cells grown on standard plastic. Moreover, the short time (at 2 h) analysis of cell morphology, showed that cells maintain a single cell growing behavior with a high number of pseudopodial protrusions. After longer growing time (24 h), hWJMSCs start to colonize the surface of the scaffold (Fig. 2A,C), additionally, creating interconnections between different pUBM flakes also when cultured in agarose coated wells. Two and 24 h cell exposure to osteogenic medium did not appreciably affect cell morphology and growing behaviors (Figs. 1 and 2, panels E–H).

In order to further characterize the interactions between cells and pUBM, scanning and transmission electron microscopic analyses were also performed. Figure 3A shows the SEM image of the surface morphology and 3D microstructure of pUBM. This natural biomaterial is characterized by a structure constituted of irregular lamellae separated by interconnected interlamellar spaces that range from several microns up to about 100 μm . The morphology and adhesion behavior of hWJMSCs were also monitored by SEM

analysis, after seeding on pUBM (Fig. 3B); in particular, at high magnification (Fig. 3C), the ability of cells to create interconnections between different lamellae and flakes is clearly evident.

TEM microphotographs, reported in Figure 3D–F, depict the ultrastructure analysis of the pUBM, showing the presence of the typical collagen fibers; in addition the analysis of the cell seeded scaffolds (Fig. 3E,F) confirmed the cell–cell interactions and cell–matrix adhesion regions (see red arrows in panel F). These results are particularly important since the intrinsic nature of the surface where cells grow has been recognized of crucial importance with respect to its influence on cell adhesion and the phenotypic expression (Chastain et al., 2006; Kundu et al., 2009; Chen, 2010; Hidalgo-Bastida and Cartmell, 2010; Pennesi et al., 2010; Bandiera, 2012).

In vitro performances of hWJMSCs seeded on pUBM-scaffold: Proliferation and viability

The viability and proliferation of hWJMSCs cultured on pUBM-scaffold were evaluated by using the Alamar Blue assay. This analysis provides a quantitative evaluation of the number of viable cells when cultured in the presence or absence of pUBM. In Figure 4A the cell proliferation over the culture time is reported (average $\pm$ standard error of three independent experiments run in triplicate). It is noteworthy to observe that a statistically significant difference ($P < 0.01$) was determined in the proliferation profile of cells growing in standard plastic (adherent conditions, red closed squares) and those growing on agarose coated multiwells (non-adherent conditions, open circles). On the contrary, no statistically significant difference was observed for cells cultured in the presence (closed circles)

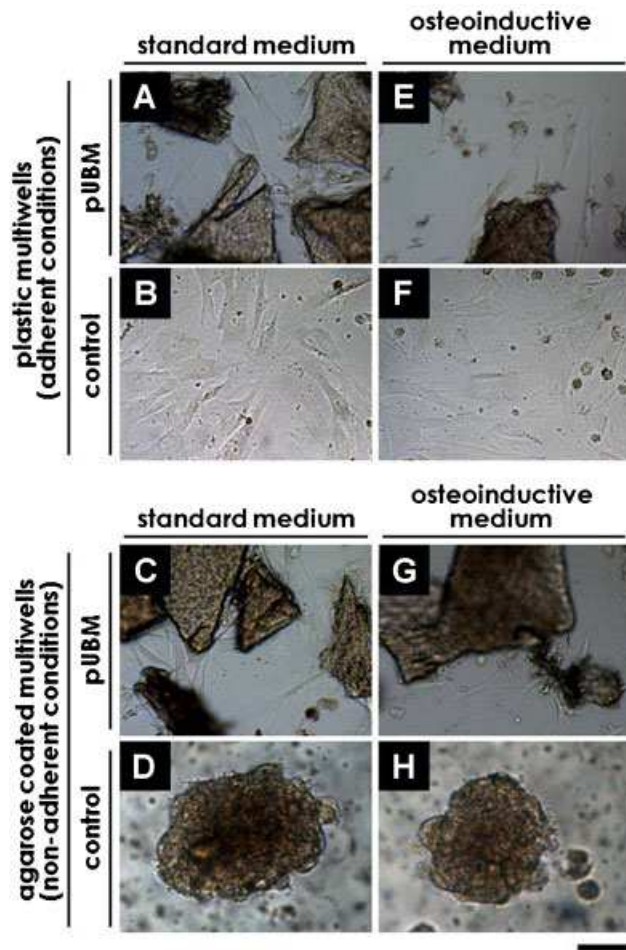


Fig. 2. Optical phase contrast photomicrographs of hWJMSCs cultured, for 24 h, in the indicated conditions: standard plastic (adherent conditions) and agarose coated (non-adherent conditions) multiwells, in the presence of pUBM or not (control). As indicated, cells were grown both in standard and in osteogenic media. Bar corresponds to 35 μm .

or in the absence of pUBM (open circles). The *in vitro* performances of hWJMSCs seeded on pUBM were also evaluated by the live/dead cell double staining and fluorescence microscope observations, to visualize the metabolically active and dead cells. The photomicrograph of the viability test (reported in the inset of Fig. 4A) confirmed the Alamar Blue assay data, showing that, after 21 days of culture, viable cells are homogeneously distributed on the entire scaffold. Moreover, the seeded cells display an almost complete colonization of the entire surface; few red dead cells are, on the contrary, sparsely present on the scaffold.

In order to evaluate the possible effect of different quantity/concentration of pUBM on hWJMSCs viability, 10^5 cells were cultured in the presence of 1, 2, 4, and 8 mg of scaffold and cell viability was evaluated by MTT assay after 72 h. As shown in Figure 4B, increasing amount of pUBM did not significantly affect cell survival of the cells cultured in non-adherent conditions (closed circles). On the contrary, the cells cultured in adherent conditions slightly suffered from the presence of increasing amount of pUBM (open circles). These results were furthered by the analysis of the apoptosis carried out in the same experimental conditions. As shown in Figure 4C, TUNEL test revealed the absolute absence of apoptosis in hWJMSC

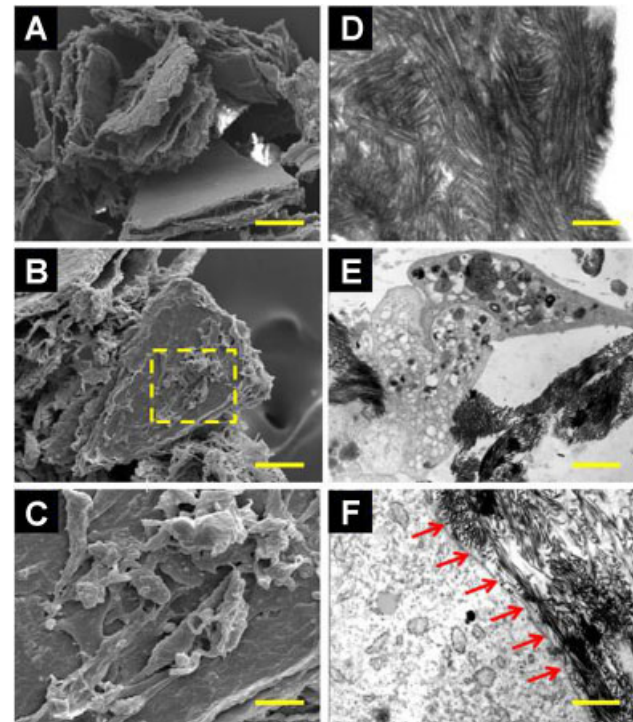


Fig. 3. SEM (A–C) and TEM (D–F) photomicrographs of pUBM before (A,D) and after seeding with hWJMSCs (B–C and E–F). Pictures of cell-seeded scaffolds were taken after 10 days of *in vitro* cell culture in agarose-coated multiwells. Bar corresponds to 22.0 μm (A, B), 12.0 μm (C), 0.4 μm (D), 2.5 μm (E), and 1.3 μm (F). The red arrows, in panel F, indicate the adhesion areas between hWJMSCs and pUBM.

cultured in agarose coated wells in the presence of increasing amount of pUBM.

Effect of pUBM-scaffold on Cyclin D1, MMP13, and β -catenin expression

As preliminary test, hWJMSCs cultured on pUBM-scaffold were assayed by TUNEL, after 48 h of cell culture from seeding, in order to determine that they were not apoptotic (Fig. 5A). To test whether pUBM could play a role in modulating the gene expression of hWJMSCs, mRNA levels of Cyclin D1, matrix metalloproteinase 13 (MMP13), and β -catenin genes were then determined (Fig. 5B). Cyclin D1 is a key regulator of cell proliferation (Musgrove, 2006), while MMP13 has been shown to contribute to the degradation of various ECM proteins, promoting the migration of cells through matrix barriers (Nagase and Woessner, 1999); both genes are direct target of β -catenin (Neth et al., 2006; Yun et al., 2009), an integral component of Wnt signaling pathway. Interestingly, as shown by the bar plot reported in Figure 5B, the pUBM upregulated Cyclin D1 (3.47 ± 1.14 folds increase), strongly decreased MMP13 (8.83 ± 0.8 folds decrease), but did not significantly affect β -catenin mRNA levels. In order to tentatively interpret the obtained results, a scheme of putative interactions among the three analyzed genes is reported in Figure 5C that will be specifically considered in the Discussion Section.

In vitro performances of hWJMSCs seeded on pUBM-scaffold: Osteogenic differentiation

Further experiments were devoted to assess the possible ability of pUBM to induce/modulate the osteogenic differentiation of

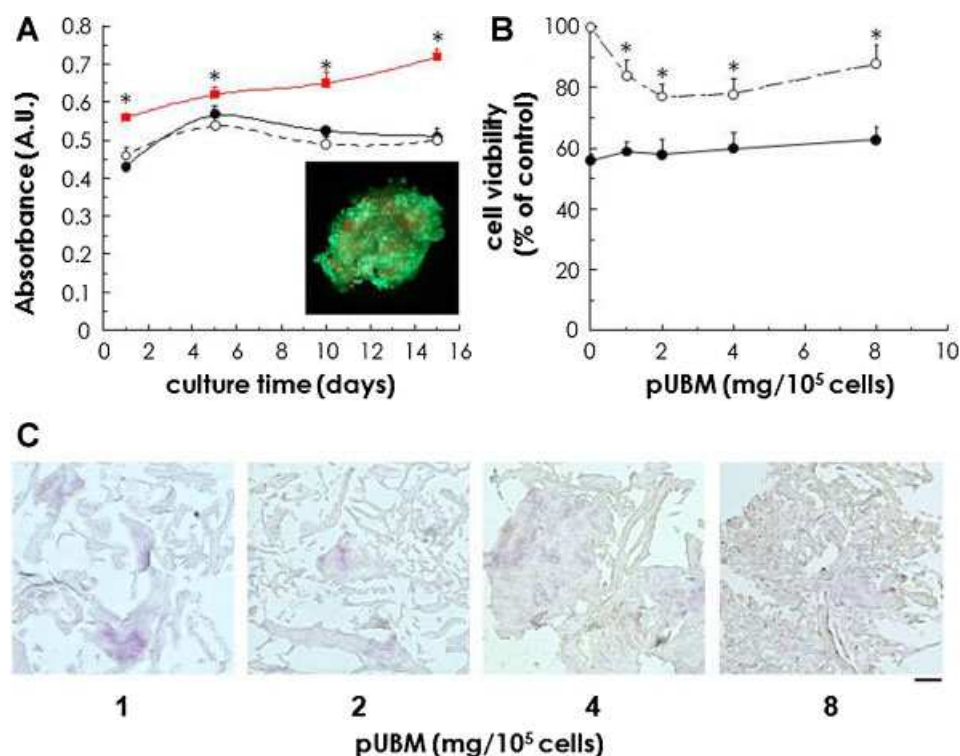


Fig. 4. Effect of pUBM on proliferation rate, viability, and apoptosis of hWJMSCs. **A:** hWJMSCs were cultured for the indicated length of time in standard plastic (adherent conditions; red squares) or in agarose coated (non-adherent conditions) multiwells, in the absence (open circles, dashed line) and the presence of pUBM-scaffolds (closed circles, plain line). In the inset, cell viability of hWJMSCs seeded on 2 mg of pUBM cultured for 21 days in agarose coated (non-adherent conditions) multiwells is shown. Viability was determined by double staining assay with Calcein-AM and PI. **B:** hWJMSCs were cultured for 3 days in standard plastic (adherent conditions; open circles) or in agarose coated (non-adherent conditions) multiwells (closed circles), in the presence of the indicated amounts of pUBM. Cell viability was determined by MTT colorimetric assay. Results are expressed as the percentage of surviving cells and are the average of three independent experiments run in triplicate $\pm$ SEM; stars indicate $P < 0.01$. **C:** Apoptosis of hWJMSCs, determined by TUNEL assay; cells were cultured for 3 days in agarose coated (non-adherent conditions) multiwells, in the presence of the indicated amounts of pUBM. The absence of brown color reaction indicates that the cells did not undergo apoptosis. Bar corresponds to 100 μ m.

hWJMSCs. To this aim, cells growing in the absence or the presence of pUBM, were cultured in the presence or in the absence of osteogenic supplements, such as dexamethasone, β -glycerol phosphate, and ascorbic acid.

After 21 days of in vitro culture, the effects of osteogenic differentiation inducers on hWJMSCs were evaluated both in term of morphological characteristics (by SEM, EDX, and TEM analyses) and gene expression modulation.

The SEM photomicrographs, recorded in variable pressure conditions (VPSEM), reported in Figure 6 show that, after 3 weeks of in vitro culture, a significant increase in cell–biomaterial interaction appears evident. The observations at low magnification, both in control and osteogenic conditions (Fig. 6A,D) show that hWJMSCs completely enveloped pUBM particles resulting in the formation of smooth spheroids, displaying an ECM network covering the surface. The spheroid sections observed at high magnification (Fig. 6B,E) reveal a compact structure, with cells that often share contacts among them and the biomaterials. Interestingly, the internal structure of spheroid cultured in osteogenic conditions presents some restricted regions with crystals (probably apatite), indicating the presence of mineralized area. In order to obtain a quantitative evaluation of calcium deposition, the EDX analysis, on spheroid sections, was performed. The spectra reported in Figure 6C,F indicate that only the spheroids grown in osteogenic medium show the typical calcium and phosphorous peaks (see arrows in panel F).

The cell–cell and cell–biomaterial interactions revealed in the spheroid section by SEM are even more evident when analyzed by TEM; Figure 7 highlights the presence of focal contacts between cells and pUBM-scaffold.

Finally, the osteogenic differentiation has been evaluated after 21 days of culture by analyzing the expression of osteogenic markers including: (a) the expression of osteogenic related genes such as Runt-related transcription factor-2 (Runx2), Col1A1, and osteopontin (OPN), (b) the ALP activity, and (c) the deposition of mineralized matrix (Fig. 8).

As shown by histograms of quantitative real-time PCR (Fig. 8A) and photomicrographs of immunohistochemical analyses (Fig. 8B), Runx2 expression is strictly correlated with the presence of osteogenic medium, and pUBM alone does not affect Runx2 transcriptional levels (Fig. 8A). When hWJMSCs seeded on pUBM-scaffold were cultured in osteogenic medium, Runx2 expression was not prevented by the presence of pUBM, indeed specific areas with uniform nuclear staining were revealed by immunohistochemical analysis (see the arrows in the relative panel). This finding suggests that pUBM may positively affect the accumulation of Runx2 protein at comparable levels in the cells, facilitating the development of more homogeneous and committed cell population.

The analysis of other typical osteoblastic markers demonstrated that hWJMSCs seeded on pUBM-scaffold were able to produce Col1A1 and OPN after 21 days in osteogenic medium (Fig. 8B). In the same conditions the cells showed

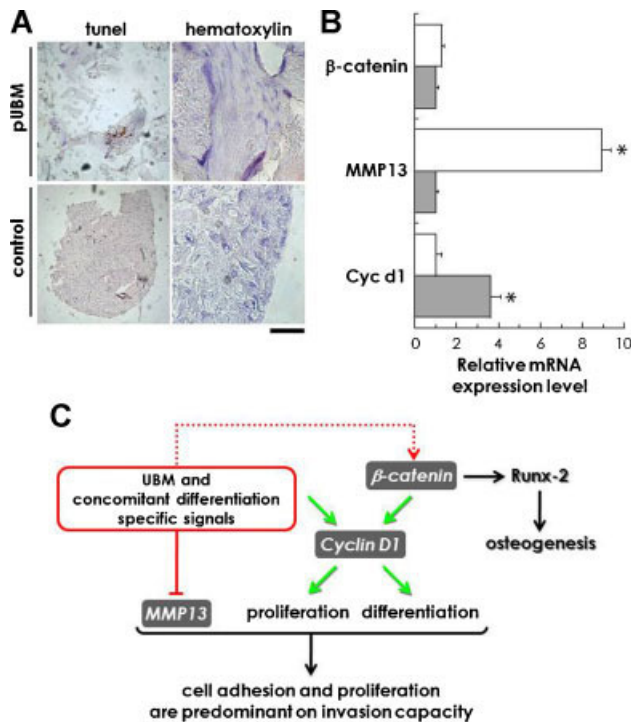


Fig. 5. Effect of pUBM-scaffolds on the apoptosis and gene expression of hWJMSCs cultured for 48 h, in the presence of pUBM (4 mg of pUBM/ 10^5 cells) or not (control). **A:** TUNEL assay and hematoxylin counterstaining show the absence of brown color indicating that cells did not undergo apoptosis. Bar corresponds to 100 and 40 μ m for TUNEL assay and hematoxylin staining, respectively. **B:** Cyclin D1, MMP13, and β -catenin mRNA expression levels in cells seeded on pUBM (closed bars) or not (open bars). Expression levels were determined by real-time quantitative RT-PCR analysis and calculated using the $\Delta\Delta$ Ct method, GAPDH as housekeeping gene, and the sample with the highest Δ Ct as the calibrator for each gene analysis. Standard error of the mean ($\pm$ SEM) was calculated. *, $P < 0.01$. **C:** Schematic representation of the possible pUBM effects on hWJMSCs in vitro functions.

substantial ALP activity and ability to deposit mineralized matrix in specific nodules highlighted by ARS (Fig. 8B). In particular, among the analyzed markers, we found that the OPN expression levels were much higher in the cells grown on pUBM-scaffold in osteogenic medium than in the cells grown only in osteogenic medium, as demonstrated by the area of OPN-expressing cells after immunohistochemical analysis.

Discussion

As demonstrated by many pre-clinical and clinical studies, human adult MSCs represent promising candidates in regenerative medicine involving cell-based approaches for the therapy of bone defects. Recent investigations have demonstrated that further improvements of tissue-engineering for specific bone regeneration can be achieved by the combined use of MSCs with ECM based-scaffolds. ECM plays, indeed, an important role in driving cell behavior through signals, controlling cell shape, migration, proliferation, differentiation, morphogenesis, and survival.

The purpose of the present study was to investigate the possible use of a natural ECM biomaterial, derived from porcine urinary bladder (named pUBM-scaffold) as matrix for the growth and differentiation of a selected population of MSCs

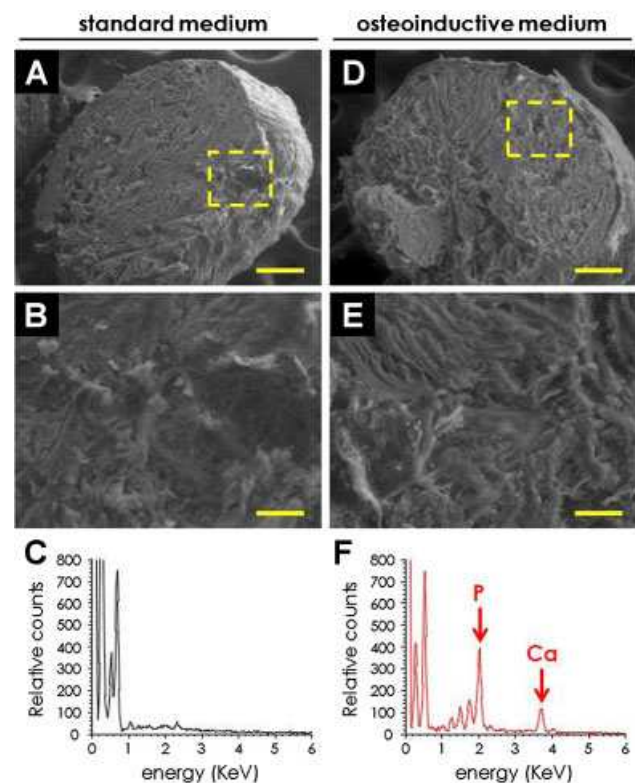


Fig. 6. Effect of pUBM on osteogenic differentiation of hWJMSCs. VPSEM analysis was performed on hWJMSCs seeded on pUBM and cultured for 3 weeks in non-adherent condition, in the absence (A–C) or in the presence of osteoinductive medium (D–F). Bars: 100 μ m (A,D) and 20 μ m (B,E). In panels C and F, the relative spectra from EDX analysis on spheroid sections are reported. The arrows reported in panel F indicate the typical calcium and phosphorus peaks.

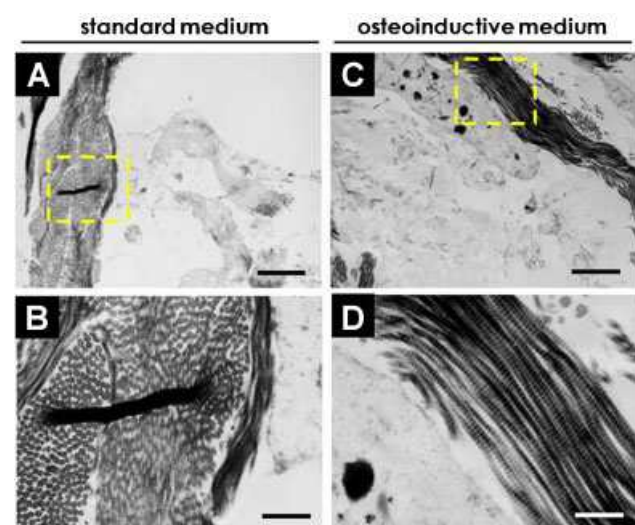


Fig. 7. Effect of pUBM on osteogenic differentiation of hWJMSCs. TEM analysis was performed on hWJMSCs seeded on pUBM cultured for 3 weeks in non-adherent condition, in the absence (A,B) or presence (C,D) of osteoinductive medium. Bars: 2.5 μ m (A,C) and 1.3 μ m (B,D).

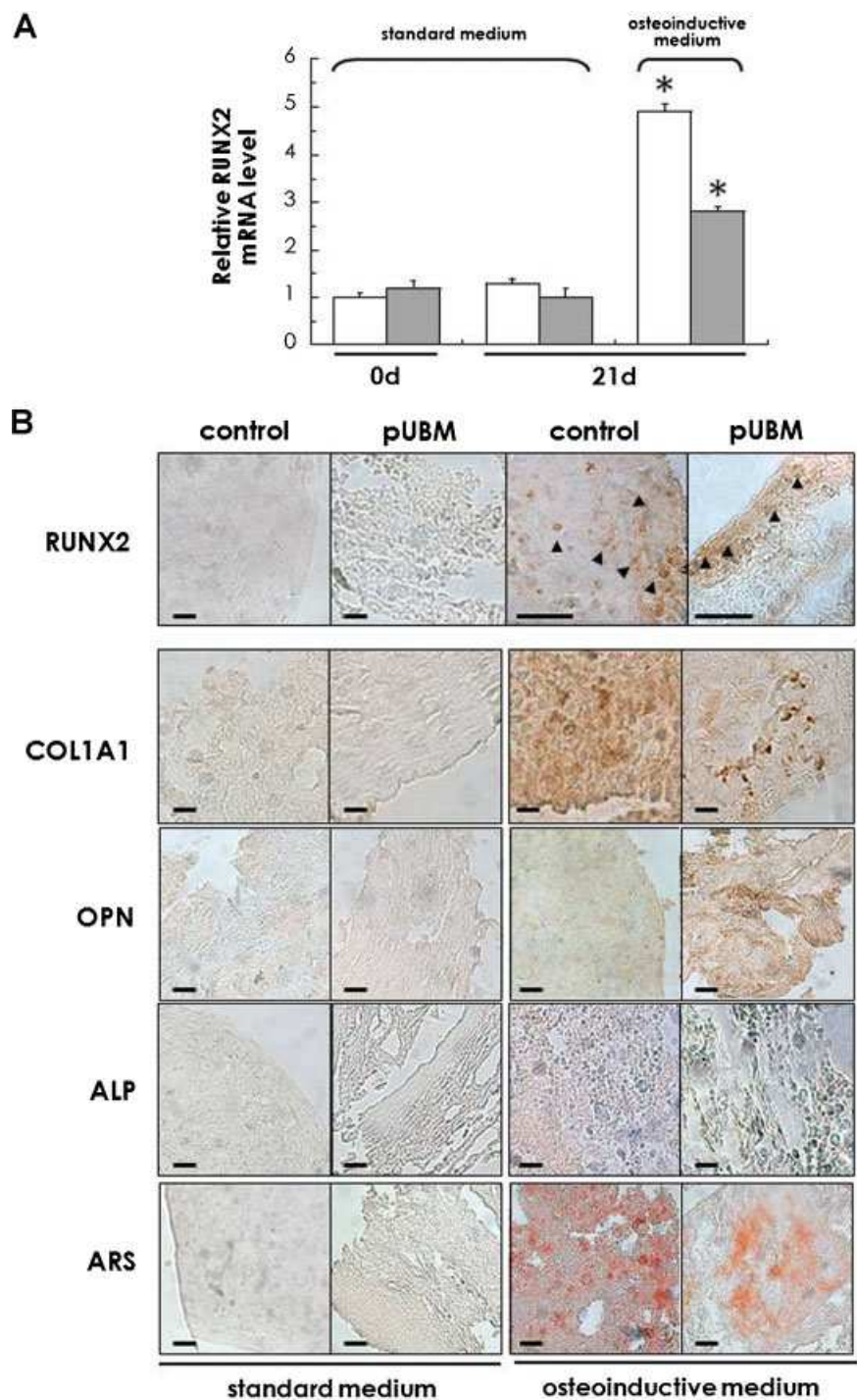


Fig. 8. Effect of pUBM-scaffolds on osteogenic differentiation of hWJMSCs as determined by gene expression analysis of specific osteogenic markers. **A:** The RUNX2 expression has been valuated at mRNA level in hWJMSCs cultured in the presence of pUBM (closed bars) or not (open bars) in standard and in osteoinductive medium. The reported results were normalized on the basis of GAPDH expression and represent the relative mRNA expression levels over the control at 0 day. $\Delta\Delta CT$ method was used to compare gene expression data; standard error of the mean ($\pm SEM$) was calculated. *, $P < 0.01$. **B:** Analysis of the indicated specific osteogenic markers after 21 days of in vitro culture. RUNX2, collagen type I (Col1A1), and osteopontin (OPN) were analyzed by immunohistochemical analysis (the presence of RUNX2 in the nuclei is indicated by arrows); alkaline phosphatase activity (ALP) was measured by colorimetric *p*-nitrophenylphosphate based reaction; matrix mineralization was analyzed by alizarin red staining (ARS). Bars: 10 μm .

isolated from human umbilical cord (hWJMSCs). Such cells, previously characterized in our laboratory (Penolazzi et al., 2009) represent a non-controversial source for human MSCs.

The obtained results demonstrated that hWJMSCs, when seeded on the biomaterial, were able to interact, adhere, remain viable, and then proliferate on pUBM-scaffold. Notably, despite the attachment and spreading of the cells on the

biomaterial, the cell proliferation rate appears to be slower with respect to that of the same cell population growing on standard control plastic multiwells.

These findings, together with the evidence that cell–cell interactions and specific matrix adhesion regions are detectable in the cell-seeded scaffold, represent important prerequisites for the potential use of pUBM-scaffold as guide for cell phenotypic expression. Therefore, the possibility that the pUBM has intrinsic properties that can induce or improve osteogenic differentiation without or with the presence of osteogenic supplements, such as dexamethasone, β -glycerol phosphate, and ascorbic acid has been explored. A first set of experiments demonstrated that the expression of two direct target genes of β -catenin, cyclin D1 and MMP13 (Neth et al., 2006; Yun et al., 2009), is differently affected by pUBM; in particular, pUBM upregulated cyclin D1, strongly decreased MMP13, and did not significantly affect β -catenin mRNA levels. These data suggest that cyclin D1 and MMP13 expression levels may be controlled, independently on β -catenin, by pUBM and concomitant differentiation specific signals that remain to be investigated. Basically, β -catenin regulates cell functions not only as transcription factor co-activator mediating Wnt signaling (Soltanoff et al., 2009), a pathway with a central role in osteogenesis, but also as cell adhesion molecule to promote both proliferation and differentiation (Perez-Moreno and Fuchs, 2006). It will be interesting understand if pUBM affects β -catenin protein accumulation, and/or its subcellular localization, and how other Wnt signaling mediators may be modulated in the cells seeded on pUBM-scaffold. Interestingly, the decrease of MMP13 in presence of pUBM supports the hypothesis that cell adhesion and proliferation are predominant events on invasion capacity of the hWJMSCs combined with pUBM, as the scheme reported in Figure 5C summarizes. This would be consistent with the electron microscopic analyses (see Fig. 3) showing that the cells do not appear to migrate into the matrix.

The capability to produce proteins specific for bone formation was shown by Runx2, OPN, Col1, and ALP immunocytochemical analysis that particularly revealed an high expression of Runx2 and OPN in cells seeded on pUBM-scaffold, in presence of differentiation medium. It is important to underline that Runx2 is the master regulator of osteogenic differentiation and is involved in the expression of bone-specific genes, binding to their promoter regions: for example, osterix (Osx), collagen type I alpha-1 (Col1a1), osteocalcin (OC), and bone sialoprotein (BSP) (Ducy et al., 1997; Rossert and Crombrughe, 2002; Lian et al., 2006). At the same time, OPN, expressed later in the differentiation process, is one of the major BSPs which is essential for the process of mineralization (Sodek et al., 2000), and promotes general attachment of osteoblasts to bone. Also the mineralization process, that is generally employed as a late stage marker and is correlated with complete osteogenic differentiation, was positively affected by pUBM-scaffold.

All these findings are a crucial prerequisite for bone formation, and demonstrate that some aspects of hWJMSCs osteogenic potential, including the expression of specific osteogenic markers correlated also with adhesion process, such as OPN, are improved by pUBM-scaffold.

Although, as a whole, these experiments suggested the ability of the pUBM to support the adhesion, spreading, and proliferation of hWJMSC, and to modulate gene expression, details of such mechanisms regulated in the microenvironment here described remain to be elucidated and further investigations of our system is needed.

To our knowledge this is the first report about the combination between hWJMSCs and pUBM-scaffold, and confirms the promise of potential applications of natural and biocompatible ECM-based biomaterials for cell culture of MSCs. Moreover, these scaffolds may also be considered as

potential materials for making composite constructs. For instance, combining natural ECM with biocompatible polysaccharides (e.g., alginate, agarose, and chitosan) may capture the advantages of both types of materials: (a) the bioactivity, which is provided by the ECM and (b) the mechanical and material properties, which can be manipulated with the “on demand” by the gelling behavior of polysaccharides. This preliminary investigation highlights the interest of these new materials in the very challenging area concerning the development of artificial extracellular matrices for TE.

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