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From Cell Biology to Tissue Engineering

Membranes derived from human umbilical cord Wharton's jelly stem cells as novel bioengineered tissue-like constructs

B.D. Jaimes-Parra[1,2], I. Garzón[1,3], V. Carriel[1,3], D. Durand-Herrera[1], M.A. Martín-Piedra[1,3], J.M. García[1,3], M.C. Sánchez-Quevedo[1,3], M. Alaminos[1,3] and A. Campos[1,3]

[1]Tissue Engineering Group, Department of Histology, [2]PhD programme in Biomedicine, Escuela de Posgrado, University of Granada and [3]Instituto de Investigación Biosanitaria ibs, Granada, Spain

Summary. Cell-derived matrices were recently described as novel biomaterials generated by human cells allowed to grow and synthetize their own extracellular matrix in culture. In the present work, we generated and evaluated a novel tissue-like substitute (WDM) consisting of a membrane derived from cultured human Wharton's jelly stem cells. WDM were evaluated *ex vivo* and *in vivo* by histochemistry and immunohistochemistry for several mesenchymal cell markers and fibrillar and non-fibrillar extracellular matrix components. Results show that WDM were heterogeneous and consisted of dense cell-poor areas surrounded by cell-rich zones with abundant HWJSC. Histological analyses demonstrated that cell-poor areas were very rich in fibrillar and non-fibrillar extracellular matrix components such as collagen and proteoglycans, and cells in the WDM were highly viable and mostly PCNA-positive. HWJSC in the WDM expressed all markers of this cell type, including CD90, CD105, pan cytokeratin and CK8. *In vivo* analysis showed that the WDM was highly biocompatible and grafting this membrane in the muscle of laboratory rats was not associated to increased inflammation, necrosis, tumorigenesis or other side effects, while cells properly integrated at the damage site and showed high proliferation index. These results suggest that the structure and composition of the extracellular matrix of these novel WDM could reproduce the situation of native human tissues and that WDM implanted *in vivo* are highly biocompatible and rapidly integrate in the host tissues. For these reasons, we hypothesize that WDM could be used in regenerative medicine protocols.

Key words: Tissue engineering, Wharton's jelly stem cells, Membranes

Offprint requests to: Dr. I. Garzón, Department of Histology. Faculty of Medicine. University of Granada, Avenida de la Investigación 11, E18016, Granada, Spain. e-mail: igarzon@ugr.es

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Introduction

Tissue engineering has been developing rapidly in recent years due to its potential for the generation of novel medical products with clinical potential to improve, repair or replace tissues affected by different conditions (Griffith and Naughton, 2002; Langer and Vacanti, 2016). One of the goals of current tissue engineering is the construction of human tissue substitutes consisting of living cells with proliferation and differentiation capabilities immersed within a biocompatible biomaterial that should resemble the extracellular matrix (ECM) of native tissues and the topographical appearance of human structures (Koepsell et al., 2011). However, the structural and functional complexity of the human ECM is very difficult to reproduce *ex vivo*, and most bioengineered tissues are devoid of important ECM components and may not replicate the functions and the complex structure of native tissues (Schon et al., 2017).

In general, most bioengineered tissues have been biofabricated by using either a bottom-up modular assembly strategy or, more recently, a tissue decellularization and recellularization approach (Saldin

et al., 2017). The first strategy is based on the combination of different modules or "building blocks" into a functional whole (Schon et al., 2017). In tissue engineering, cultured cells are combined with natural or synthetic biomaterials and growth factors to generate a hydrogel or cellular tissue substitute such as the human cornea (Garzón et al., 2014a; Nakamura et al., 2016), skin (Gomez et al., 2011; Carriel et al., 2012) and nerve (Nune et al., 2016). The second strategy makes use of native tissues that are subjected to different decellularization protocols able to remove all cells and cell debris from the native structure in order to obtain a three-dimensional biologic scaffold material that can be seeded with selected cell populations (Badylak et al., 2011). Although the decellularization and recellularization approach has been efficiently applied to several tissues and organs such as the cornea (Oliveira et al., 2013), heart (Lee et al., 2017), kidney (Poornejad et al., 2016) and lung (Jung et al., 2016), most decellularization protocols may significantly alter the native tissue ECM. Both strategies have the limitation of generating a cellular tissue substitute whose ECM may be devoid of important ECM components and significantly differ from the native ECM (Oliveira et al., 2013).

In this context, cell-derived matrices (CDM) were recently described as novel biomaterials that could contribute to the generation of more biomimetic tissues able to reproduce the molecular complexity and organization of native tissues (Fitzpatrick and McDevitt, 2015). CDM are generated by culturing human cells under different culture conditions and allowing them to synthesize in culture their own ECM containing all the components of the native ECM (Lu et al., 2011a,b). Several cell types have been used for the generation of CDM, including mesenchymal stem cells (Yang et al., 2011), fibroblasts (Kim et al., 2015) and chondrocytes, among others (Lu et al., 2011b). However, none of the bioengineered tissues generated from these CDM has demonstrated to be clinically useful and research in the field is in need.

Human umbilical cord Wharton's jelly stem cells (HWJSC) have been extensively used in tissue engineering and regenerative medicine due to their accessibility, proliferation capability without being tumorigenic (Joerger-Messerli et al., 2016) and their differentiation potential to multiple cell types, including connective-type tissues such as bone, cartilage and adipose tissue (Troyer and Weiss, 2008), but also epithelial-type tissues such as the human cornea (Garzón et al., 2014a), skin and oral mucosa epithelia (Garzon et al., 2013). In addition, these cells offer the possibility of autologous application when cryopreserved (Joerger-Messerli et al., 2016) and are considered to be immunoprivileged for allogeneic use due to their low expression of major histocompatibility complex class I and II markers (Witkowska-Zimny and Wrobel, 2011). Although these cells have been previously studied and characterized in culture, CDM generated from these cells have not been described to date.

In the present work, we used cultured HWJSC in order to generate a novel CDM that we called HWJSC-derived CDM (WDM). Histological, histochemical and immunohistochemical analyses were carried out both *ex vivo* and *in vivo* to determine the potential usefulness of this novel tissue-like structure.

Materials and methods

Generation of a WDM

WDM were generated in the laboratory by using cultured human umbilical cord Wharton's jelly stem cells (HWJSC). First, HWJSC were isolated from healthy human umbilical cords as previously described (Garzón et al., 2012, 2013, 2014a). Briefly, umbilical cord Wharton's jelly biopsies were washed with phosphate-buffered saline (PBS) and enzymatically digested with type I collagenase from *Clostridium hystoliticum* (Thermo Fisher Scientific-Gibco, Waltham, Massachusetts, USA) for 4-6h at 37°C. Once tissues were digested, HWJSC were harvested by centrifugation and used for the generation of primary cultures using Amniomax culture medium (Thermo Fisher Scientific-Gibco) in cell culture flasks of 25 cm^2 of surface. The presence of relevant mesenchymal stem cell markers CD90 and CD105 was assessed by flow cytometry of cultured HWJSC. Briefly, 10^6 cells were incubated with allophycocyanin-conjugated CD90 and phycoerythrin-conjugated CD45 antibodies (R&D Systems Inc., Minneapolis, MN, USA), Fc receptors were blocked and samples were analyzed with each antibody or each corresponding isotype control antibody at a concentration of 1:100 using a FACSCalibur flow cytometer (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). Human samples were obtained from the Biobank of the Public Health System of Andalusia after obtaining the approval of the ethics committee for research with human cells and tissues.

To obtain a WDM, primary cell cultures were allowed to grow and expand to 100% confluence. At this level of confluence, cells began to produce natural extracellular matrix (ECM) components among the cells and spontaneously formed the WDM membrane containing the HWJSC around the 14^{th} day of confluent culture. At this period of time, these WDM membranes tended to spontaneously detach from the culture flask and were harvested for *ex vivo* or *in vivo* analysis.

Ex vivo histological analysis of WDM

For the analysis of cell viability of the HWJSC included in the WDM, we used calcein/AM-ethidium homodimer-1 Viability/Cytotoxicity assay kits (LIVE/DEAD, Life Technologies, Carlsbad, CA). Briefly, WDM membranes were washed three times in PBS, incubated in calcein/AM-ethidium homodimer-1 mixture for 15 min, washed again in PBS (three times)

and placed on a glass slide for analysis of live (green) and dead cells (red) in a Nikon Eclipse 90i fluorescent microscope.

For histological analysis using hematoxylin-eosin staining and for histochemistry and immunohistochemistry, WDM membranes were washed in PBS, fixed in 10% neutral buffered formalin for 24 h at room temperature, dehydrated in increased concentrations of ethanol, cleared in xylene and embedded in paraffin. Then, 5 μm sections were obtained, dewaxed and rehydrated and processed accordingly. ECM proteoglycans were identified by the alcian blue histochemical method at pH 2.5, whilst fibrillar collagen was stained by using picrosirius method as previously described (Carriel et al., 2012).

Routine immunohistochemistry methods were used for the identification of immature collagen synthesis, cytokeratin 8 and proliferating cells using anti-type I collagen (R1038, Acris, Herford, Germany; dilution 1:200), anti-CK8 (005095QD, Master Diagnostica, Granada, Spain; prediluted) and anti-PCNA (P8825, Sigma-Aldrich, St. Louise, MO, USA; dilution 1:1000) primary antibodies. For immunofluorescence, WDM were washed in PBS, fixed for 15 min in 70% ethanol, rinsed three times in PBS and processed by following standard methods with casein antigen retrieval. Anti-pancytokeratin (C2931, Sigma-Aldrich; dilution 1:100), anti-CD90 (EPR-2926, Novus Biological, Littleton, CO, USA; dilution 1:500) and anti-CD105 (VP-C371, Vector Laboratories, Burlingame, CA, USA; dilution 1:50) primary antibodies were used.

For scanning electron microscopy (SEM), WDM samples were fixed in 2.5% buffered glutaraldehyde, dehydrated, dried by the critical point method, mounted on aluminum stubs, sputter-coated with gold and examined in a FEI Quanta 200 scanning electron microscope (FEI, Eindhoven, The Netherlands). Transmission electron microscope (TEM) analysis was performed by fixing WDM samples in 2.5% buffered glutaraldehyde, postfixing in 1% osmium tetroxide, and dehydration in graded acetone series, embedding in Spurr's resin, and staining with aqueous uranyl-acetate and lead citrate. Ultrathin sections were examined using a EM902 microscope (Carl Zeiss Medi-tec, Oberkochen, Germany).

In vivo biocompatibility analysis of WDM

Twenty-four 12-week-old male Wistar rats weighing 250-300 g maintained at the Experimental Unit of the University Hospital Virgen de las Nieves of Granada (Spain) were used. Animals were kept in a temperature-controlled room (21±1°C), with a 12 h light/dark cycle with *ad libitum* access to water and standard rat chow. For all surgical procedures, animals were deeply anesthetized by intraperitoneal injection of acepromazine (Calmo-Neosan®, 0.001 mg/g of body weight), ketamine (Imalgene 1000®, 0.15 mg/g of body weight) and atropine (0.05 µg/g of body weight). For tissue harvesting at the end of the experiment, animals were euthanatized by anesthesia overdose. This project was approved by the ethical committee of Granada.

To evaluate the biocompatibility of the WDM generated in the study, these membranes were grafted in the skeletal musculature of the laboratory rats by following a myectomy model previously described for the study of skeletal muscle regeneration (Pereira et al., 2014). First, a small incision was made in the skin of the left leg of the animals and the gluteus maximus muscle was exposed. Then, a circular defect of 5-mm of diameter was created in the left gluteus maximus muscle by using a sterile punch, which was filled with the WDM tissues in the study group or was left unfilled in the control group. In the study group, a 5x5 mm piece of the WDM was inserted at the injury site and covered with the surrounding muscles. Skin was sutured and analgesia was used in the drinking water for 48h. In each animal, the right gluteus maximus was left as control. Rats were euthanatized 4 or 8 weeks after the surgical procedure.

Tissue samples were obtained from the right and left gluteus maximus muscle of each animal for histological studies using hematoxylin-eosin, picrosirius histochemistry and CK8 and PCNA immunohistochemistry as described above.

Results

Generation and ex vivo analysis of WDM

First, our flow cytometry analyses showed that most cultured HWJSC expressed CD90 and CD105 mesenchymal stem cell surface markers (94.46% and 99.43% positive cells, respectively). Then, bioengineered WDM were obtained after 14±2 days of culture under confluent conditions of the HWJSC. Previous to WDM formation, cells showed a typical spindle-shape morphology. Then, cells formed a thin membrane attached to the culture flask that tended to detach from the surface. Macroscopic evaluation of the WDM showed a rectangular shape structure of approximately 2x1.5 cm (Fig. 1).

Ex vivo characterization of WDM by H&E staining revealed a high amount of cells immersed within the structure. In addition to the cells, numerous areas with very scarce cells mainly consisting of ECM (cell-poor tissue patches) were found randomly distributed all along the entire WDM, with cells surrounding these patches (Fig. 1B,C). Secondly, the analysis of the presence of proteoglycans showed positive staining predominantly in the cell-poor tissue patches (Fig. 1D). Similarly, the analysis of collagen fibers using picrosirius staining showed that collagen was present in all the WDM, but it was more abundant in the cell-poor areas (Fig. 1E). This was in agreement with the type I-collagen immunostaining, which was highly positive in

all the WDM, especially in the cell-poor tissue areas (Fig. 1F). Moreover, cell proliferation analysis as determined by PCNA staining showed that most cells were able to express PCNA antigen (Fig. 1G). In addition, the cell viability assay revealed that 99% of cells were viable (green cells) (Fig. 1H). Regarding cytokeratin expression, CK8 was positive in less than 50% of the cell population immersed in the WDM (Fig. 1I), whereas more than 50% of the cell population was positive for pancytokeratin (Fig. 1J). The analysis of CD90 and CD105 antigens showed that most cells were highly positive in the WDM (1K-L).

Scanning electron microscopy (SEM) of WDM samples showed that the surface of the WDM consisted of a mixture of ECM fibers that covered the surface of the cells (Fig. 2A,B), with fibers randomly scattered in different orientations. Porosity of the WDM was high, with 40.5±11.4% of the surface corresponding to porous areas. Transmission electron microscope (TEM) showed the presence of an abundant ECM material surrounding the cells immersed within (Fig. 2). The extracellular material mostly consisted of non-fibrillar material, with some pseudofibrillar areas, but mature ECM fibers were not identified (Fig. 2C,D,G,H). Cells had euchromatic nuclei with evident central nucleoli and most of the cells showed the presence of well-developed synthesis organelles, including rough endoplasmic reticulum, smooth endoplasmic reticulum, associated and non-associated ribosomes, mitochondria, and exocytosis vesicles (Fig. 2D-F).

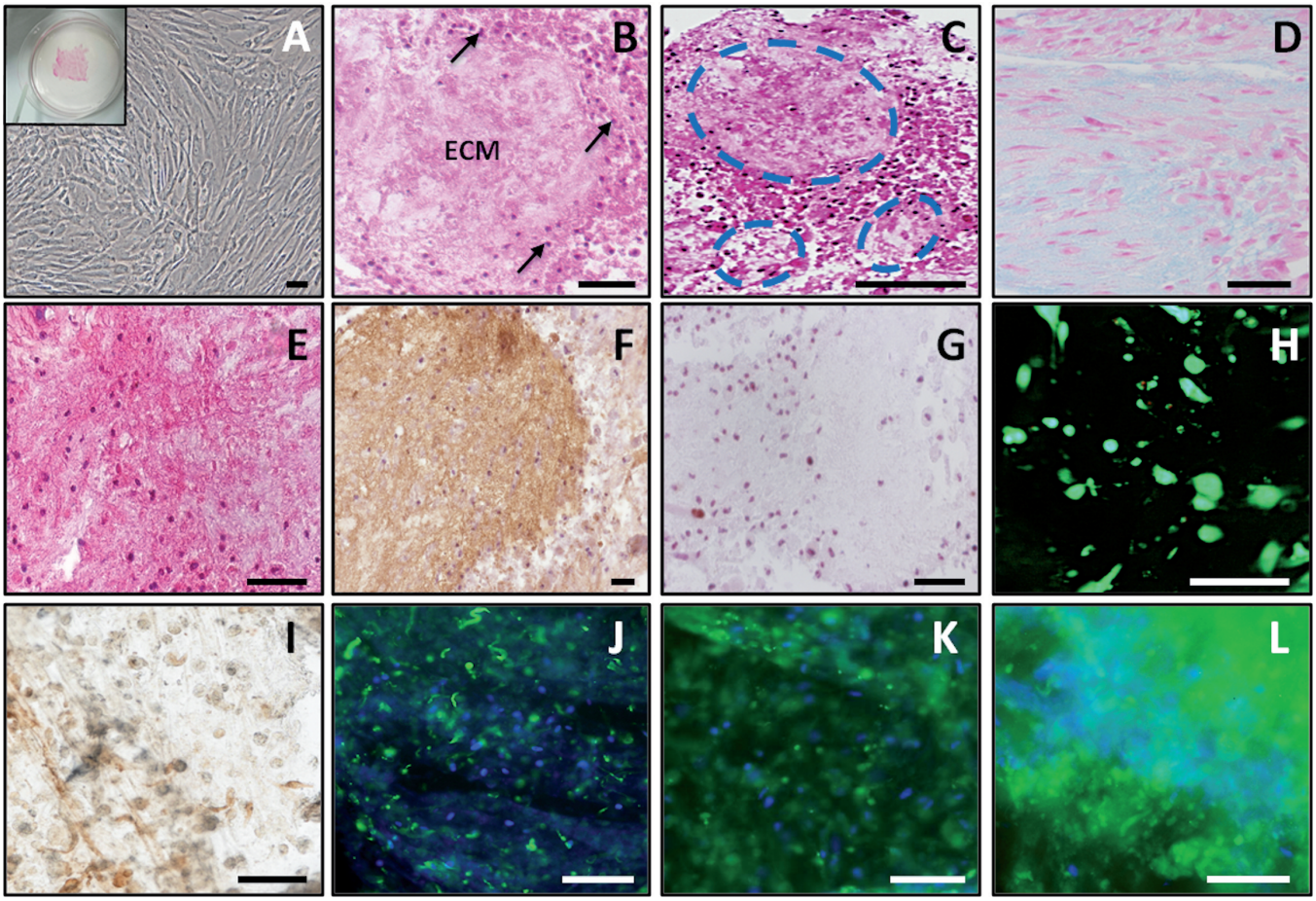

Fig. 1. *Ex vivo* analysis of the HWJSC-derived matrices (WDM) generated in the laboratory. **A.** Phase contrast microscopy image of confluent HWJSC; insert shows the macroscopic appearance of the detached WDM. **B.** Histological analysis with hematoxylin-eosin showing the cell-poor areas (ECM), with arrows showing some of the cells at the limit with the cell-rich areas. **C.** Lower augmentation images of the WDM stained with hematoxylin-eosin showing some of the cell-poor areas surrounded by discontinuous lines. **D.** Histochemical detection of proteoglycans using alcian blue staining. **E.** Histochemical detection of fibrillar collagen using picrosirius staining. **F.** Analysis of type-I collagen as determined by immunohistochemistry. **G.** Analysis of proliferating cells as determined by PCNA immunohistochemistry. **H.** Analysis of cell viability as determined by LIVE/DEAD Viability/Cytotoxicity assay kit. **I.** Analysis of CK8-positive cells as determined by immunohistochemistry. **J.** Analysis of pancytokeratin-positive cells as determined by immunofluorescence. **K.** CD90 analysis as determined by immunofluorescence. **L.** CD105 analysis as determined by immunofluorescence. Scale bars: 50 μm.

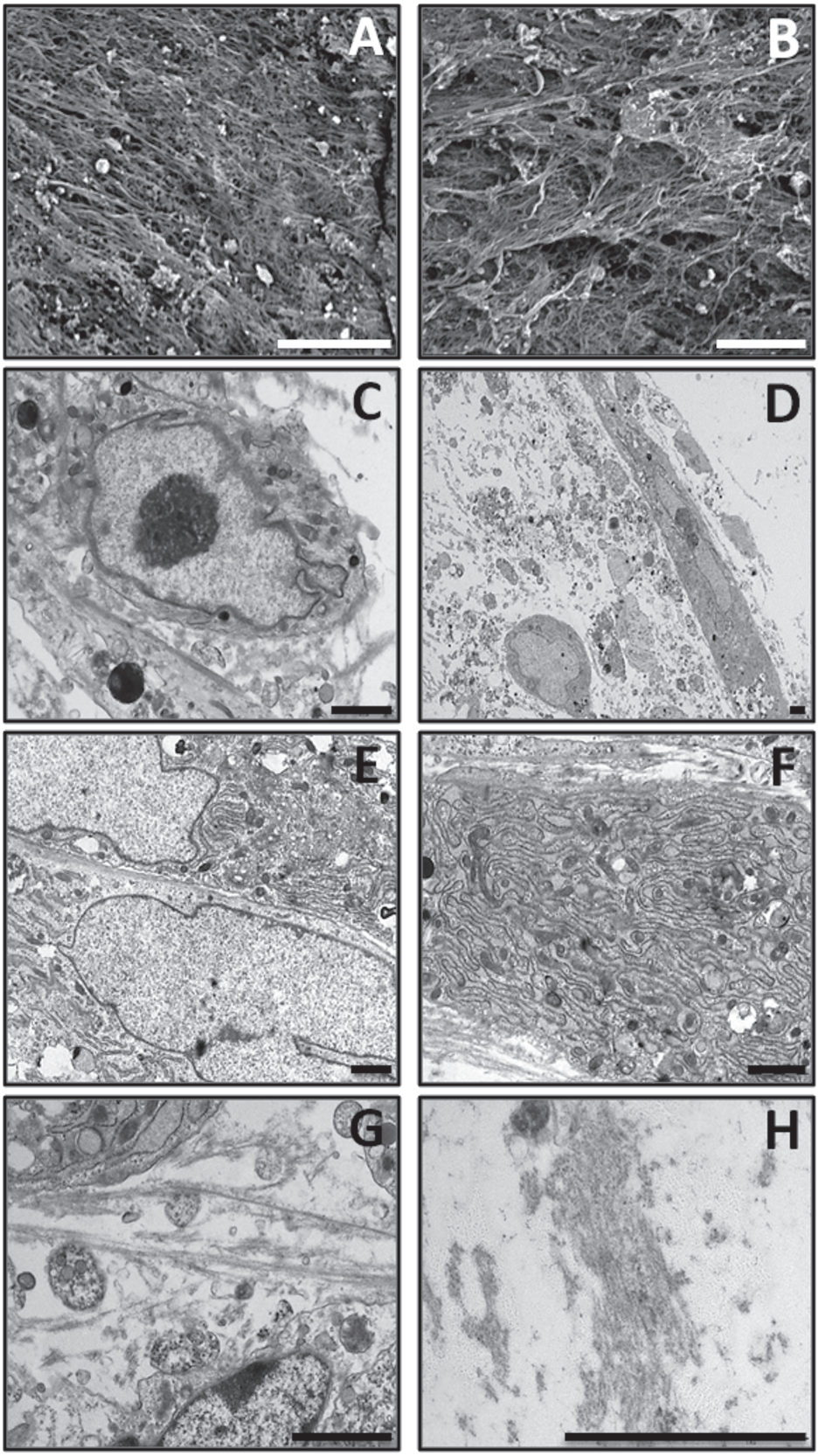

Fig. 2. Electron microscope analysis of the HWJSC-derived matrices (WDM) generated in the laboratory. **A, B.** Scanning electron microscopy analysis of the WDM showing abundant ECM fibers at the surface of the WDM. Scale bars: 20 μm. **C-H.** Transmission electron microscopy analysis of the WDM. Scale bars: 2 μm.

In vivo biocompatibility of WDM in a model of muscle regeneration

First, the analysis of control animal tissues in which a defect was created in the gluteus maximus muscle showed that the area of the defect was filled with a connective tissue with abundant cells, ECM and blood vessels 4 and 8 weeks after the surgical procedure (Fig. 3). When picrosirius staining was used to identify collagen fibers, we found very low presence of these fibers in native control muscle, but the regeneration site in animals with a muscle defect was collagen-rich, especially after 8 weeks. Histological analysis of animals with the WDM grafted in the damaged muscle revealed that the area of the surgical defect was comparable to control animals. In short, the connective tissue formed at the damage area was rich in cells and ECM and contained abundant blood vessels in both groups. No signs of tumorigenesis, necrosis or rejection were detected in any of the animals by macroscopic ocular inspection or by histology, and the WDM membrane implanted in the muscle was not identifiable. Analysis of type I collagen fibers showed that the amount of this ECM component was high at the regeneration area at 4 and 8 weeks.

Then, we identified the grafted HWJSC by CK8 immunohistochemistry. As shown in Fig. 3, native muscle and control animals with a created defect had negative expression of CK8 at both study periods. In contrast, animals with grafted WDM had positive expression restricted to the lesion site after 4 weeks, whereas at 8 weeks animals had slightly lower signal and positive cells were scattered along the regeneration tissue. Regarding PCNA expression, this marker was

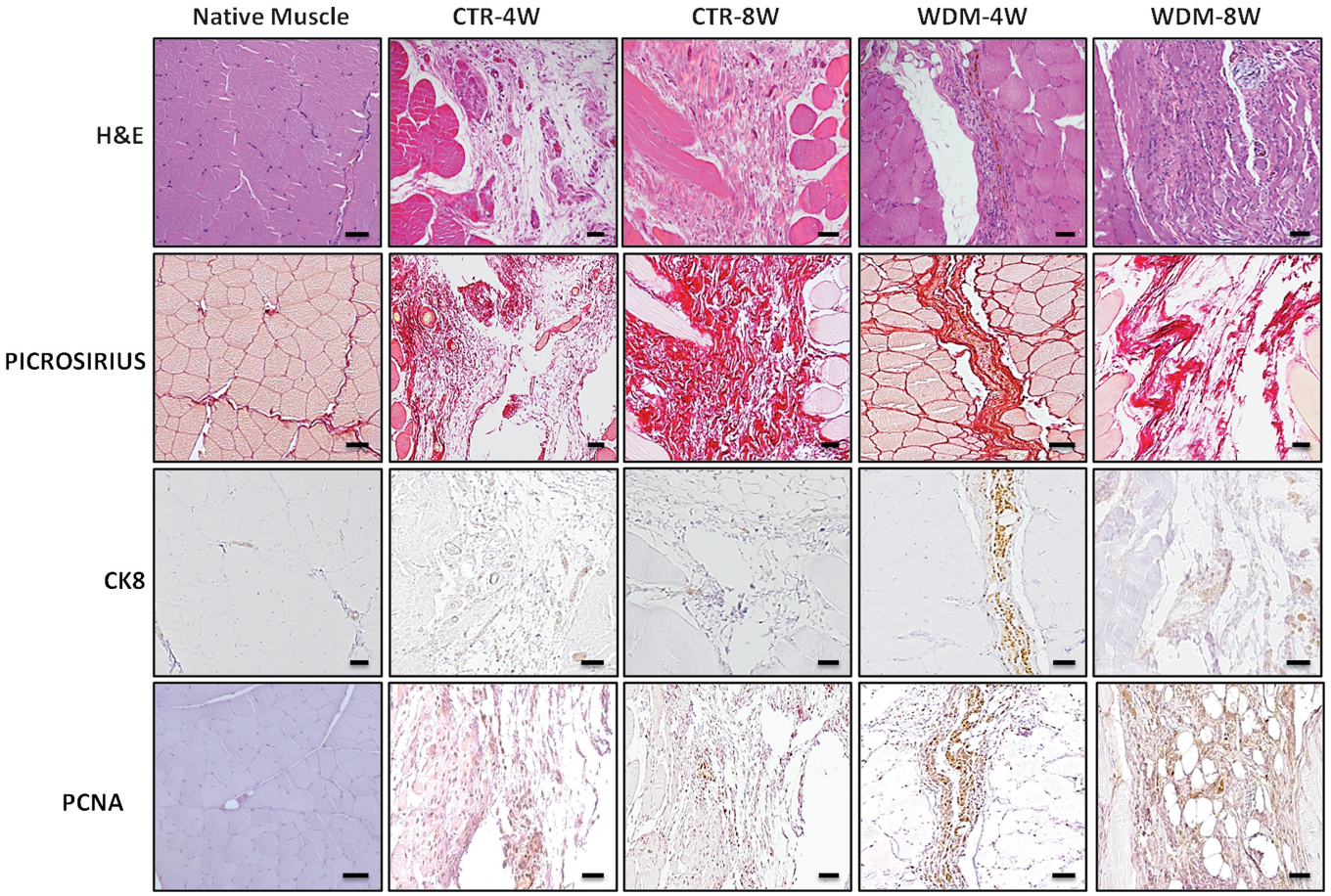

Fig. 3. *In vivo* histological and immunohistochemical analysis of the HWJSC-derived matrices (WDM) in a rat model of muscle damage. H&E: histological analysis using hematoxylin-eosin staining; Picrosirius: analysis of collagen fibers by picrosirius histochemistry; CK8: detection of CK8-positive cells by immunohistochemistry; PCNA: analysis of proliferating cells by PCNA immunohistochemistry; Native muscle: Native rat muscle used as control; CTR-4W: control animals with a muscle lesion evaluated at week 4 of the procedure; CTR-8W: control animals with a muscle lesion evaluated at week 8 of the procedure; WDM-4W: animals with a muscle lesion filled with the WDM and evaluated at week 4 of the procedure; WDM-8W: animals with a muscle lesion filled with the WDM and evaluated at week 8 of the procedure. Scale bars: 50 μm.

negative in control native muscle and positive in the cells found at the regeneration site of animals with a muscle defect with no grafted WDM. However, expression was very high in animals with implanted WDM (Fig. 3).

Discussion

Several scaffolds and biomaterials have been used in tissue engineering (Griffith and Naughton, 2002; Augst et al., 2006; Gomez et al., 2011; Ionescu et al., 2011; Koepsell et al., 2011; Carriel et al., 2012; Khan and Khan, 2013; Garzón et al., 2014b; Nakamura et al., 2016; Nune et al., 2016; Saldin et al., 2017), but the ideal substitute for the human native ECM has not been found to date, and most described biomaterials are not able to reproduce the refined structure and composition of native tissues (Koepsell et al., 2011). For this reason, in the present work we have developed a fully natural tissue-like structure generated by cultured human cells that contains HWJSC and a dense extracellular material that could mimic the native ECM.

Our results first show that generation of WDM is a simple and straightforward technique as compared to more complex biomaterials (Chan and Leong, 2008). The biofabrication method described in the present manuscript is inexpensive and does not require any special equipment, since cells are able to generate the WDM membrane in culture flasks and to detach from these flasks by themselves. Membranes can be generated with different sizes and handled with forceps for future use. Although the putative usefulness of these structures must be evaluated in the future, previous reports on other kinds of scaffold-free cell sheets fabricated with other cell types suggest that this technology is promising and may be clinically useful (Matsuura et al., 2014).

Once generated, we analyzed the histological structure and composition of the WDM and we found out that cells tended to grow in clusters into the membrane, with the formation of cell-rich and cell-poor areas in each WDM. Our histochemical and immunohistochemical analyses demonstrated that cell-poor areas were rich in all major fibrillar and non-fibrillar components of the ECM, although cell-rich areas were not devoid of these components. This finding suggests that WDM are histologically heterogeneous but contain the main cellular and non-cellular components of human tissues. Probably, the abundant ECM components of the cell-poor areas were synthetized by the HWJSC as demonstrated by the presence of abundant synthesis organelles in these cells as determined by TEM, and HWJSC must have migrated from cell-poor areas to cell-rich areas once the ECM components were synthetized. The fact that the ECM was synthetized by the human cultured cells themselves and the abundance of fibrillar and non-fibrillar ECM components that are present in native tissues suggest that this ECM could be basically analogue to the ECM of some native human tissues (Oliveira et al., 2013). Indeed, human skin, oral mucosa, cornea, intestine and other tissues are rich in collagen and proteoglycans (Oliveira et al., 2013; Viñuela-Prieto et al., 2015) and, since the composition of the bioengineered WDM is highly biomimetic and may reproduce the composition and physiological properties of the native ECM, WDM might eventually be used for the treatment or replacement of these structures. In fact, previous works suggest that the use of cell-derived matrices may have numerous applications in regenerative medicine, including cartilage and bone replacement (Pham et al., 2008), regeneration of damaged cardiovascular structures (Weber et al., 2013; Shimizu, 2014) or their use as models of stem cell niches and *ex vivo* tissue-mimetic microenvironments (Fitzpatrick and McDevitt, 2015), for example. We hypothesize that WDM could also be used in cornea and oral mucosa tissue engineering. Future clinical experiments should confirm this statement.

Apart from the ECM present in the WDM, one of the advantages of these novel tissue-like substitutes is the presence of live HWJSC. These mesenchymal cells have been extensively studied in tissue engineering due to their unique properties and capabilities, including their proliferation and multi-lineage differentiation abilities (Troyer and Weiss 2008; Garzón et al., 2013, 2014b), high accessibility and immunomodulatory effects (Witkowska-Zimny and Wrobel 2011; Kim et al., 2013) as well as their capability to synthetize ECM components (Garzón et al., 2014a,b). Despite their clear potential in tissue engineering, CDM membranes generated from HWJSC had not been described before.

In this regard, we have demonstrated that HWJSC are able to form a WDM with abundant cells and extracellular material that may be of interest in tissue engineering. Although we cannot exclude the possibility that cells immersed in the WDM might become inactive and undergo senescence after reaching contact inhibition in culture, our results suggest that most cells in the membrane were alive and metabolically active. This suggests that the generation and harvesting of the WDM does not affect cell viability and that cell-poor areas are probably not devoid of cells due to a process of cell death, and supports the hypothesis that cells may have migrated from the cell-poor areas to the cell-rich areas. The fact that most cells were positive for the cell-proliferation marker PCNA is in agreement with this statement and, along with the abundance of synthesis organelles, implies that cells in the WDM are capable of growing and proliferating. Regarding their potential usefulness in regenerative medicine, we demonstrated that HWJSC immersed in the WDM kept their original phenotype. First, we verified that these cells expressed pancytokeratin and CK8, markers that are very positive in HWJSC allocated at the umbilical cord and in primary cultures of these cells (Garzón et al., 2014b). Then, we demonstrated that most cells were positive for the mesenchymal stem cell markers CD90 and CD105.

These findings allow us to state that HWJSC immersed within the dense ECM of the WDM retained their original undifferentiated phenotype (Tantrawatpan et al., 2013) and could therefore keep their native mesenchymal multi-lineage differentiation potential. Although these histological analyses contributed to characterize the WDM, future works should confirm these results using additional approaches and methods, including genetic, epigenetic and biochemical analyses.

To further characterize the novel WDM described in this work, we carried out *in vivo* biocompatibility tests in laboratory animals. Our results showed that WDM were well tolerated by the host animals. As compared to controls, grafting these membranes in the muscle tissue of laboratory rats was not associated to necrosis, increased inflammation or other side effects. Although these results were obtained by macroscopic inspection and hematoxylin-eosin histological analysis, and should be confirmed by immunohistochemistry and other complementary methods, they suggest that WDM grafting was safe for the host and did not generate any important complications. Instead, the WDM tended to properly integrate at the defect site, and grafted cells were clearly detectable after 4 weeks, with a decrease at week 8. Interestingly, cell proliferation was higher at the defect area of animals with an implanted WDM, which may imply that the cells grafted here were able to proliferate and participate in the regeneration process. Although our histological analysis did not detect an increase in inflammatory cells in these groups of animals, we cannot exclude the possibility that these PCNA-positive cells are inflammatory cells recruited at the damage site. No differences in the connective tissue formation and collagen synthesis were found with control animals, suggesting that WDM grafting is not associated to fibrosis or other alterations of the healing process. Even though further research is necessary to determine the biocompatibility of WDM in other models of tissue damage, these results support the biocompatibility of these novel tissue-like products. In addition, our work did not allow us to determine if the WDM may contribute to tissue regeneration. Future works should be carried out to analyze its regeneration potential and clinical usefulness and to better characterize the biological effects of the WDM using complementary histological and biochemical analyses.

As with other types of CDM, WDM might have several advantages in comparison with other tissue substitutes generated by other biofabrication protocols in terms of cell screening and pathogen control, greater preservation of the structure and protein composition of the extracellular matrix, and the possibility of producing and mixing different matrices derived from cells of different cell types (Lu et al., 2011a; Shimizu, 2014; Fitzpatrick and McDevitt, 2015). In addition, WDM are able to provide a desired geometry and shape without the limitation of poor cell penetration that normally occurs in repopulation of decellularized tissues. Moreover, WDM can be prepared from autologous cells and autologous ECM can be generated from a specific patient avoiding host responses derived from the use of allogeneic or xenogeneic biomaterials (Lu et al., 2011a,b). For these reasons, the novel WDM described here could potentially be used for the treatment of conditions affecting the human cornea, skin and oral mucosa without the need for generating a tissue-engineered product based on the combination of cells and biomaterials as previously described (Gómez et al., 2011; Ionescu et al., 2011; Carriel et al., 2012; Oliveira et al., 2013; Garzón et al., 2014a). Future works should analyze the cells immersed in the WDM to exclude the possibility of senescence or genetic alterations due to the culture conditions used in this experiment in order to determine the *in vivo* usefulness and efficacy of the WDM in these particular applications.

In summary, we have developed a novel biocompatible model of WDM by using a straightforward method. These bioengineered tissues demonstrated to have a highly biomimetic ECM supporting HWJSC growth and metabolism. All these results suggest that these novel tissue-like constructs could have potential usefulness in tissue engineering and regenerative medicine.

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