Cell and Tissue Banking, 2010, 11:9144-1

Potential use of the human amniotic membrane as a scaffold in human articular cartilage repair

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Abstract
The human amniotic membrane (HAM) is an abundant and readily obtained tissue that may be an important source of scaffold for transplanted chondrocytes in cartilage regeneration in vivo. To evaluate the potential use of cryopreserved HAMs as a support system for human chondrocytes in human articular cartilage repair. Chondrocytes were isolated from human articular cartilage, cultured and grown on the chorionic basement membrane side of HAMs. HAMs with chondrocytes were then used in 44 in vitro human osteoarthritis cartilage repair trials. Repair was evaluated at 4, 8 and 16 weeks by histological analysis. Chondrocytes cultured on the HAM revealed that cells grew on the chorionic basement membrane layer, but not on the epithelial side. Chondrocytes grown on the chorionic side of the HAM express type II collagen but not type I, indicating that after being in culture for 3–4 weeks they had not de-differentiated into fibroblasts. In vitro repair experiments showed formation on OA cartilage of new tissue expressing type II collagen. Integration of the new tissue with OA cartilage was excellent. The results indicate that cryopreserved HAMs can be used to support chondrocyte proliferation for transplantation therapy to repair OA cartilage.

Keywords
Amniotic membrane Chondrocytes Cartilage Osteoarthritis Cell therapy

Introduction

Osteoarthritis (OA) is a degenerative joint disease characterized by deterioration in the integrity of hyaline cartilage and subcondral bone (Ishiguro et al. *2002*). OA is the most common articular pathology and the most frequent cause of disability. Genetic, metabolic and physical factors interact in the pathogenesis of OA producing cartilage damage. The incidence of OA is directly related to age and is expected to increase along with the median age of the population (Brooks *2002*).

The capacity of articular cartilage to repair is very limited (Steinert et al. *2007*; Mankin *1982*), largely due to its avascular nature. Currently, there are no effective pharmaceutical treatments for OA, although some medications slow its progression (Brandt and Mazzuca *2006*; Steinert et al. *2007*). There are also no surgical approaches to treat OA; however, surgery is an important tool for the repair of cartilage injuries, which if left untreated may result in secondary OA.

To date, most efforts made to repair an articular cartilage injury are intended to overcome the limitations of this tissue for healing by introducing new cells with chondrogenic capacity (Koga et al. *2008*) and facilitating access to the vascular system. Current treatments generate a fibrocartilaginous tissue that is different from hyaline articular cartilage. To avoid the need for prosthetic replacement, different cell treatments have been developed with the aim of forming a repair tissue with structural, biochemical, and functional characteristics equivalent to those of natural articular cartilage.

Cell therapy is a new clinical approach for the repair of damaged tissues. Cell therapy using mesenchymal stem cells (Koga et al. *2008*) or differentiated chondrocytes (autologous chondrocyte implantation, ACI) is one therapeutic option for the repair of focal lesions of articular cartilage, which is most successful in young people producing repair tissue of high quality (Brittberg et al. *1994*; Minas and Chiu *2000*). Aging diminishes the cell density of cartilage and the ability of chondrocytes to proliferate and form cartilage in vivo (Froger-Gaillard et al. *1989*).

ACI has several technical limitations, among which are the effects of gravity causing the chondrocytes to sink to the dependent side of the defect, resulting in an unequal distribution of cells (Jin et al. *2007*) that hampers the homogenous regeneration of the cartilage. To overcome some of the limitations of ACI,

cell delivery supports can be used for cell transplantation. The transplantation of chondrocytes seeded on natural and synthetic scaffolds has been used for cartilage tissue engineering (Kuo et al. *2006*). Scaffolds must readily integrate with host tissues and provide an excellent environment for cell growth and differentiation. Scaffolds must also provide a stable temporary structure while cells seeded within the biodegradable matrix synthesize a new and natural tissue. A number of scaffolds have been developed and investigated, in vitro and in vivo, for potential use in tissue engineering. The human amniotic membrane (HAM) is considered to be an important potential source for scaffolding material (Niknejad et al. *2008*) and has begun to be appreciated for its usefulness in the field of regenerative medicine (Toda et al. *2007*).

HAMs develop from extra-embryonic tissue and consist of both a fetal component (the chorionic plate) and a maternal component (the decidua) that are comprised of an epithelial monolayer, a thick basement membrane and an avascular stroma (Niknejad et al. *2008*; Jin et al. *2007*). The amnion is a fetal membrane attached to the chorionic membrane. Both the amnion and chorion form the amniotic sac filled with amniotic fluid, providing and protecting the fetal environment. The outer layer, the chorion, consists of trophoblastic chorionic and mesenchymal tissues. The inner layer, the amnion, consists of a single layer of ectodermally-derived epithelium uniformly arranged on the basement membrane, which is one of the thickest membranes found in any human tissue, and a collagen-rich mesenchymal layer (Wilshaw et al. *2006*). This mesenchymal layer can be subdivided into the compact layer forming the main fibrous skeleton of the HAM, the fibroblast layer and an intermediate layer, which is also called the spongy layer or *zona spongiosa* (Niknejad et al. *2008*). The amnion is a thin (up to 2 mm), elastic, translucent and semi-permeable membrane, which adheres firmly to an exposed surface. These properties enable surgeons to apply the graft on various tissue surfaces without need for suturing or application of secondary dressings. Immediately after grafting, the process of biodegradation begins and the membrane self-dissolves over a period of time from days to 3–4 weeks depending on the characteristics of the wound, the presence or absence of co-existing pathogens, the polarization of the applied graft and the type of graft applied.

The HAM possesses clinical considerable advantages to make it potentially attractive as a biomaterial. It is anti-microbial, anti-fibrosis, anti-angiogenic, anti-tumorigenic and has acceptable mechanical properties. It also reduces pain and inflammation, inhibits scarring, enhances wound healing and epithelialization, and acts as an anatomical and vapor barrier. All these characteristics are not shared by other natural or synthetic polymers, highlighting the clinical advantages of amniotic membrane as a scaffold compared to other biocompatible products. Also, amnios shows little or no inmunogenicity and the immune response against the graft, if there is, is slight and ineffective, so it does not represent transplantation risks. On the contrary, chorion shows high immunogenicity and for this reason it is not used as biomaterial for transplantation purposes. Importantly, HAMs are inexpensive and easily obtained with an availability that is virtually limitless, negating the need for mass tissue banking (Toda et al. *2007*; Niknejad et al. *2008*; Hennerbichler et al. *2007*; Wilshaw et al. *2006*). The extracellular matrix (ECM) components of the HAM include collagens (types I, III, IV, V and VI), fibronectin, nidogen, laminin, proteoglycans and hyaluronan, as well as growth factors (Niknejad et al. *2008*; Rinastiti et al. *2006*; Jin et al. *2007*). The HAM, therefore, has abundant natural cartilage components, which are important in the regulation and maintenance of normal chondrocyte metabolism (Jin et al. *2007*); this suggests that the HAM is an excellent candidate for use as native scaffold for cartilage tissue engineering (Niknejad et al. *2008*).

The aim of this study was to evaluate the potential usefulness of cryopreserved HAMs as human chondrocyte graft support for human articular cartilage repair. For this purpose, we developed an in vitro model to evaluate the capacity of human chondrocytes to grow on a HAM and repair human articular cartilage lesions.

Materials and methods

Harvest and preparation of HAMs

Human placentas were obtained from selected Cesarean-sectioned mothers in Hospital Materno Infantil-Teresa Herrera from La Coruña, Spain. All mothers gave written informed consent prior to collection. This study was approved by the Ethics Committee of Clinical Investigation of Galicia (Spain). Under stringent sterile conditions harvested placentas were placed in 199 medium (Invitrogen S.A., Spain) with antibiotics: cotrimoxazol 50 μg/ml (*Soltrim* ®, Almirall-Prodesfarma S.A., Spain), vancomycin 50 μg/ml (*Vancomicina Hospira* ®, Laboratorio Hospira S.L., Spain), amykacin 50 μg/ml (*Amikacina Normon* ®, Laboratorios Normon S.A., Spain) and B amphotericin 5 μg/ml (*Fungizona* ®, Bristol-Myers Squibb S.L., Spain). The HAM was carefully separated from the chorion of the placenta and the chorion was

discarded. The amnion was then washed 3–5 times with 0.9% NaCl solution to remove blood and mucus. The HAM was then incubated in 199 medium with antibiotic solution: metronidazol 50 μg/ml (*Metronidazol G.E.S.*, G.E.S. Genéricos *Españoles Laboratorio S.A., Spain*), vancomycin 50 μg/ml, amykacin 50 μg/ml and B amphotericin 5 μg/ml for 6–20 h at 4°C and cryopreserved. In some cases, the HAMs were pretreated with 1% trypsin–EDTA (Sigma–Aldrich Química S.A., Spain) for 30 min, as previously described (Ma et al. *2006*), to remove epithelial cells and to facilitate chondrocyte penetration into the porous structure of the denuded HAM.

Cryopreservation and thawing of HAMs

The HAM was cut in 6 × 6 cm patches and placed on a supportive sterile nitrocellulose filter in 20 ml of medium without antibiotics but with a cryoprotectant, 10% dimethyl sulfoxide (DMSO). Each patch of HAM was cryopreserved following a protocol of controlled freezing using a CM 2000 (Carburos Metálicos, Spain). Freezing rates were −1°C/min to a temperature of −40°C, −2°C/min to −60°C, and −5°C/min to −150°C. All HAMs were stored in the gas phase of liquid nitrogen at −150°C. Thawing was carried out for 5 min at room temperature followed by 37°C until thawing was complete (Fig. 1a–c). To reduce cell damage due to osmotic changes, the DMSO was removed by sequential washing and progressive dilution with 0.9% NaCl at 4°C.

Harvest of human cartilage and isolation of articular chondrocytes

Femoral heads were provided by the Autopsy Service and Orthopaedic Department at Hospital Universitario A Coruña, Spain. Samples comprised 25 donors (15 male and 10 female) with a mean age of 67, 24 years and a range from 25 to 85 years. All samples came from knee donors (13 were diagnosed of osteoarthritis and 12 were healthy). The population of patients included 17 living donors and 8 deceased donors. To obtain chondrocytes, articular cartilage full-thickness slices were used. To develop the in vitro cartilage repair model, 6 mm diameter discs of articular cartilage were used.

Cartilage slices were aseptically removed from femoral heads, sliced full thickness (excluding the mineralized cartilage and subchondral bone), and washed in Dulbecco's modified Eagle's medium (*DMEM*, Sigma–Aldrich Química S.A., Spain) as previously described (Blanco et al. *1998*; Rendal-Vázquez et al. *2001*). Briefly, slices were minced with a scalpel and transferred into a digestion buffer containing DMEM + Glutamax (Sigma–Aldrich Química S.A., Spain), 1% l-glutamine (Sigma–Aldrich Química S.A., Spain), ciprofluoxacin 10 μg/ml (*Ciprofluoxacina*, Laboratorios Vita S.A., Spain), penicillin 100 UI/ml (Invitrogen S.A., Spain) streptomycin 100 μg/ml (Invitrogen S.A., Spain), insulin 100 UI/ml (*Actrapid* ®, Novo Nordisk Pharma S.A., Spain), deoxyribonuclease I (25,000 UI/l) (Sigma–Aldrich Química S.A., Spain), and 1% trypsin–EDTA. The cartilage tissues were then incubated on a shaker at 37°C for 5–10 min until digestion was complete. The supernatant (without chondrocytes) was discarded and the trypsinized cartilage was subjected to a second digestion buffer containing DMEM + Glutamax, 1% l-glutamine, ciprofluoxacin 10 μg/ml, penicillin (100 UI/ml), streptomycin (100 μg/ml), insulin (100 UI/ml), deoxyribonuclease I (25,000 UI/l) and 2 mg/ml clostridial collagenase (Type IV) (Invitrogen S.A., Spain), incubated at 37°C overnight and washed 3 times before being used for culture or cryopreservation. Fresh or thawed primary chondrocytes were grown directly on the basement layer of HAMs prepared as described above.

Chondrocyte proliferation studies on HAMs

For human chondrocyte growth on the chorionic basement layer of the HAM, a suspension containing 5×10^5 primary chondrocytes was deposited on the central part of the amniotic membranes (6×6 cm^2). These chondrocytes on the HAM membrane were grown in DMEM + Glutamax medium containing 20% foetal bovine serum (*FBS*, Invitrogen S.A., Spain), ciprofloxacin 10 μg/ml, penicillin (150 UI/ml), streptomycin (50 mg/ml), insulin (100 UI/ml), deoxyribonuclease I (25,000 UI/l) for 3–4 weeks in a humidified 5% CO_2 atmosphere at 37°C until they reached a confluency of 80–90%. At this time, the membranes were employed to develop an in vitro model for articular cartilage repair (Fig. 1d, f). The number of assays performed in chondrocyte proliferation studies was $n = 59$.

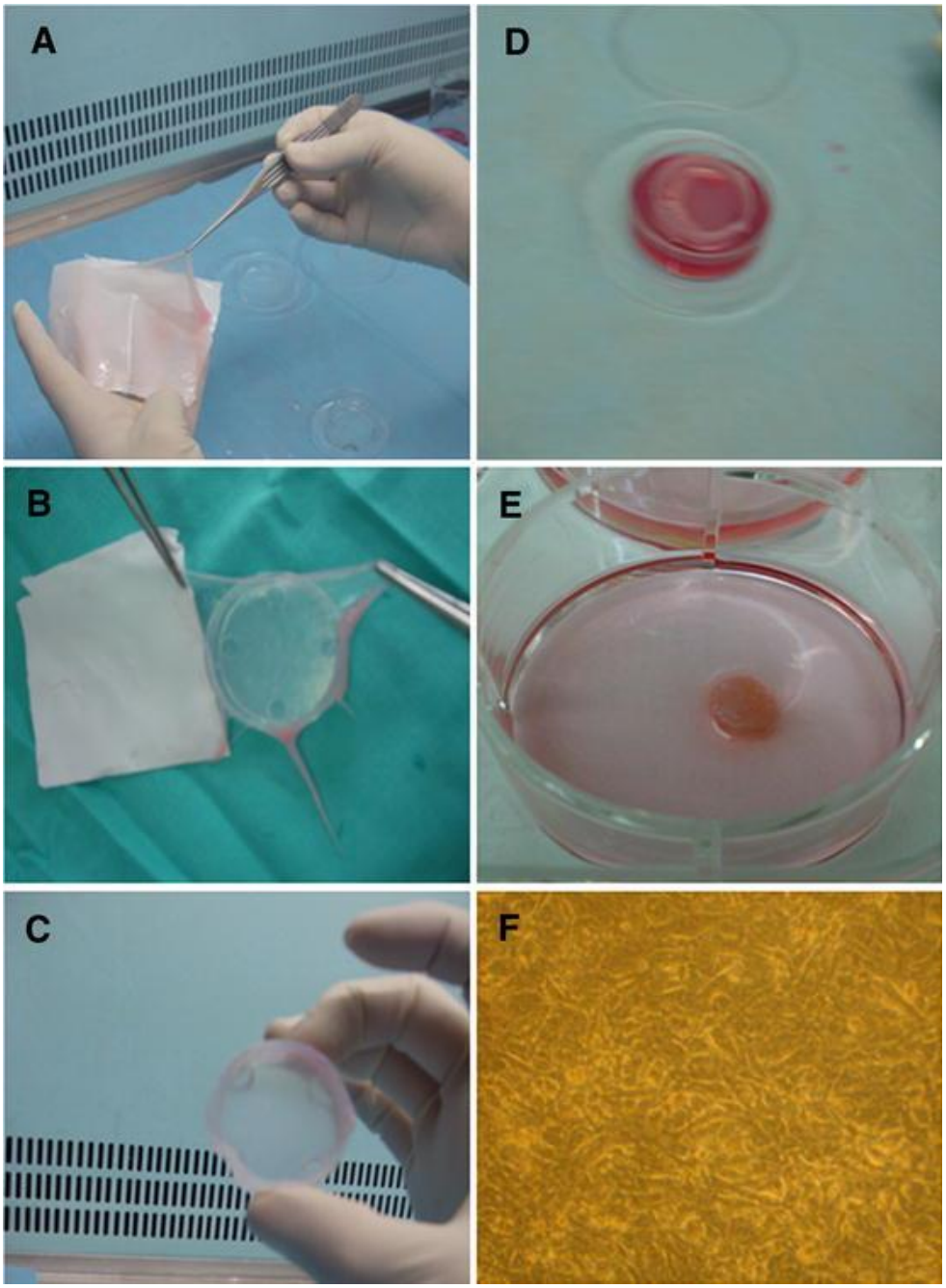

Fig. 1 Illustrations of the developed methodology for articular cartilage repair. A cryopreserved human amniotic membrane (HAM) was thawed (**a**) and placed over ring-shaped support (**b**, **c**) that was then placed in a petri dish containing growth medium. Human articular chondrocytes were seeded (5×10^5) on the HAM (**d**). After chondrocyte proliferation the HAM with chondrocyes was used for in vitro cartilage repair (**e**). Human chondrocytes grown on the chorionic basement membrane layer of the HAM (10X) (**f**)

Development of an in vitro model for articular cartilage repair

Each 6 × 6 cm patch of HAM was cut into three fragments of 0.7 × 0.7 cm and placed on the superficial surface of three different 6 mm OA cartilage discs such that the basement layer of the HAM, on which the chondrocytes were grown, was in direct contact with the superficial surface of the cartilage. The three cartilage discs layered with the chondrocyte-cultured HAM were placed in six-well culture plates (Costar®, USA) (Fig. 1e). In each well, 2 ml of culture medium containing DMEM supplemented with penicillin (100 UI/ml), streptomycin (100 μg/ml) and 10% fetal bovine serum was placed. The culture plates were incubated in humidified 5% CO_2 atmosphere at 37°C. The culture medium was replaced twice weekly. All procedures were performed under stringent sterile conditions. After 4 weeks, the first

cartilage disk was retrieved from the culture plate, fixed in 4% paraformaldehyde, dehydrated and embedded in paraffin. The same procedure was followed at 8 and 16 weeks with the second and third discs. The resulting blocks were cut into 4 μm-thick sections using a microtome that were mounted on poly-l-lysine-coated glass slides for histological and immunohistochemical analyses. The number of in vitro models for articular cartilage repair developed was $n = 44$. Also, one group of controls, with amniotic membrane but without chondrocytes, were included ($n = 4$).

Histological and immunohistochemical analyses

For general histological analyses, 4 μm-thick paraffin sections were deparaffinized in xylol, rehydrated in a graded series of ethanol, and stained with hematoxylin and eosin (H–E), Masson's trichrome or Safranin O staining for proteoglycans.

Paraffin sections (4 μm-thick), which had been deparaffinized and hydrated as described above, were incubated with monoclonal antibodies to detect the presence of collagen types I (Abcam, Spain) and II (BioNova Científica, Spain), Ki-67 (Novocastra, UK), integrin β-1 subunit (Abcam, Spain) and glycosaminoglycans: chondroitin-4-sulphate (ICN Biomedicals Inc, Spain), chondroitin-6-sulphate (ICN Biomedicals Inc, Spain) and keratan sulphate (Seikagu America Inc, Rockville, MD). To facilitate the exposure of epitopes, sections stained for collagens were pretreated with hyaluronidase (Sigma–Aldrich Química S.A., Spain), and those stained for glycosaminoglycans were pretreated with chondroitinase ABC (Sigma–Aldrich Química S.A., Spain). The peroxidase/DAB ChemMateTM DAKO EnVisionTM detection kit (Dako Citomation, USA) was used to determine antigen-antibody interaction. Negative staining controls were achieved by omitting the primary monoclonal antibody or the secondary detector antibody. Samples were visualized using an optical microscope.

Results

Selection of the appropriate side of the HAM

As a first approach to the study of the potential usefulness of the HAM as a support for the cultivation of human chondrocytes, we cultured chondrocytes on the HAM. In this study, the chorion and amnion were carefully separated from the human placenta to assess only the amnion as a human chondrocyte delivery support for human articular cartilage repair. To determine which side of the amnion would be the most appropriate, we first tested the growth of human chondrocytes on both the epithelial side, the single monolayer of epithelial cells from the extra-embryonic ectoderm ($n = 9$), and the chorionic thick basement membrane side ($n = 8$). The amnion also consists of a delicate avascular mesenchymal layer, the extra-embryonic remainder of the mesoderm, located under the thick basement membrane. Preparations that are shown in this study do not include this mesenchymal layer.

Primary human chondrocytes were grown on the chorionic and epithelial sides of the HAM. When chondrocytes reached confluency at approximately 3–4 weeks (Fig. 1f), they were stained with H–E and Masson's trichrome. As shown in Figs. 2 and 3, the distribution of chondrocytes on both the chorionic and epithelial sides of the HAM showed a characteristic monolayer pattern of cell growth. However, on the epithelial side the chondrocyte monolayer was not attached to the extra-embryonic ectoderm. Despite the fact that the epithelial cells of the extra-embryonic ectoderm of HAM showed good preservation, the cultured chondrocytes were not in contact with the epithelial cells, indicating a possible competition between these cell types. As a result of this incompatibility, we observed apparent cell death of both chondrocytes and epithelial cells and, in fact, epithelial cells from the HAM caused the release of chondrocytes cultured upon it. In several areas the layer of cultured chondrocytes is lifted, detached and fragmentized. This cellular competition produced eosinophilia and massive necrosis in the epithelium in addition to the detachment of the chondrocyte monolayer cultured upon it.

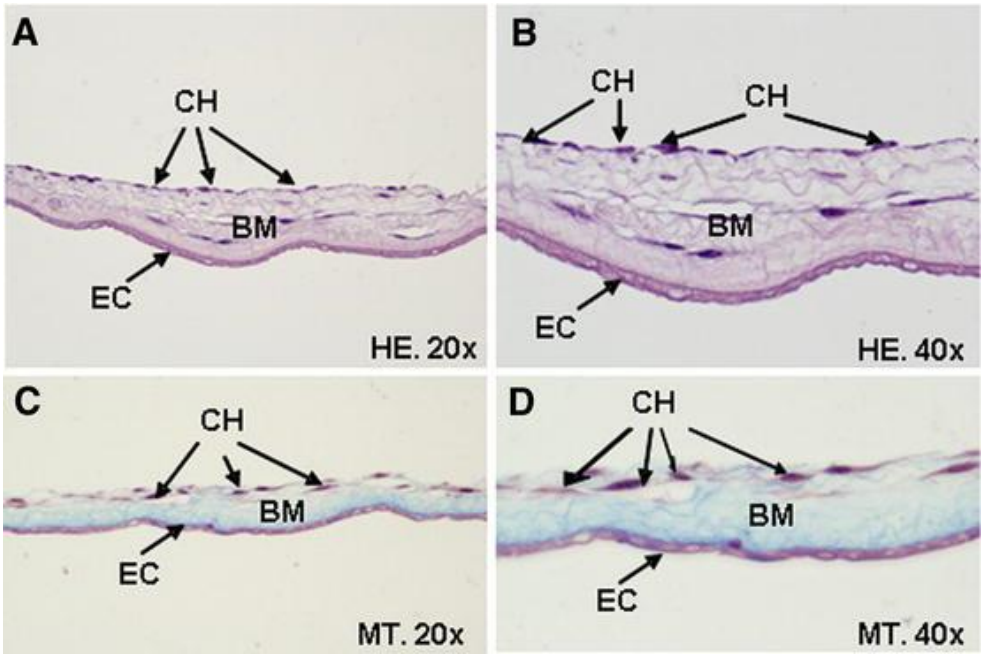

Fig. 2 Distribution of human articular chondrocytes cultured on the basement membrane of human amniotic membranes (HAMs). H–E (hematoxylin–eosin) (**a**, **b**) and M–T (Masson's Trichrome) (**c**, **d**) staining. CH, monolayer of cultured cells (chondrocytes) on the thick basement membrane of the HAM; *BM* basement membrane; *EC* epithelial cells from the extra-embryonic ectoderm

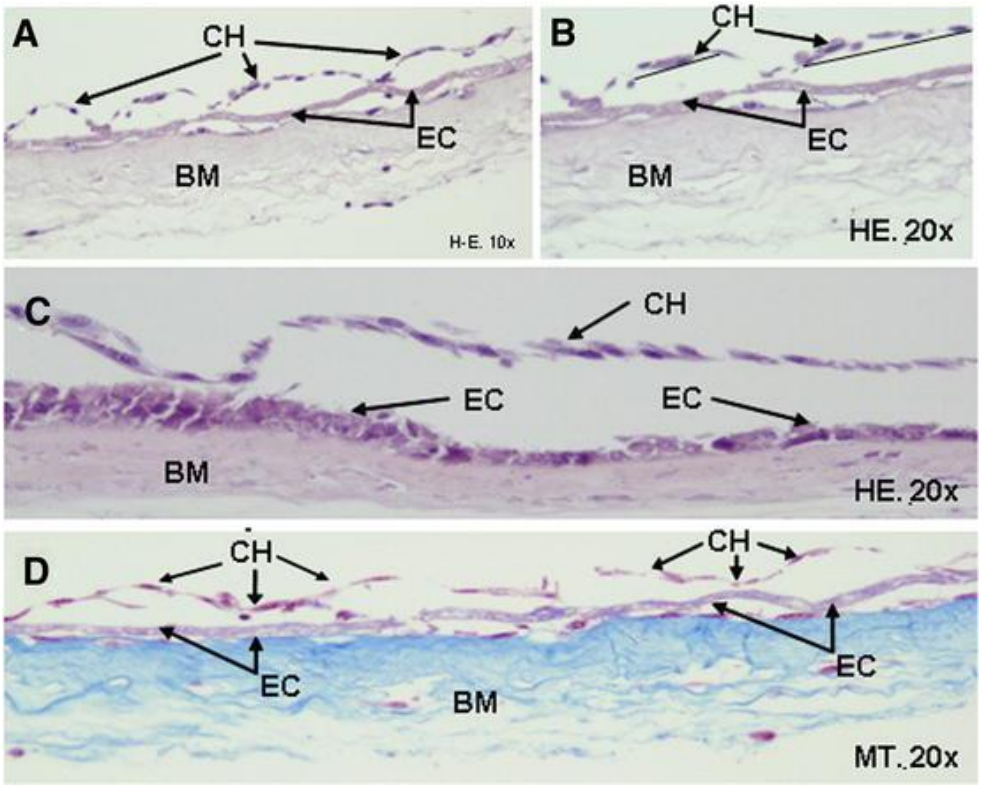

Fig. 3 Distribution of human articular chondrocytes cultured on the epithelial cells from the extra-embryonic ectoderm of human amniotic membranes (HAMs). H–E (hematoxylin–eosin) (**a**, **b**, **c**) and M–T (Masson's Trichrome) (**d**) staining. The monolayer of chondrocytes is separated or raised from the epithelial cells of the extra-embryonic ectoderm. CH, monolayer of cultured cells (chondrocytes) on the epithelial cells from the extra-embryonic ectoderm of HAMs; *BM* basement membrane; *EC* epithelial cells from the extra-embryonic ectoderm

Human chondrocytes cultured on HAMs maintain their phenotype

Histological techniques demonstrated that type II but not type I collagen was expressed by the human chondrocytes cultured on the basement membrane layer of the HAM (n = 10) (Fig. 4). This confirms that human chondrocytes grown on HAMs for 3–4 weeks until confluent did not de-differentiate into fibroblasts or another cell type, but maintained the characteristic phenotype of human chondrocytes.

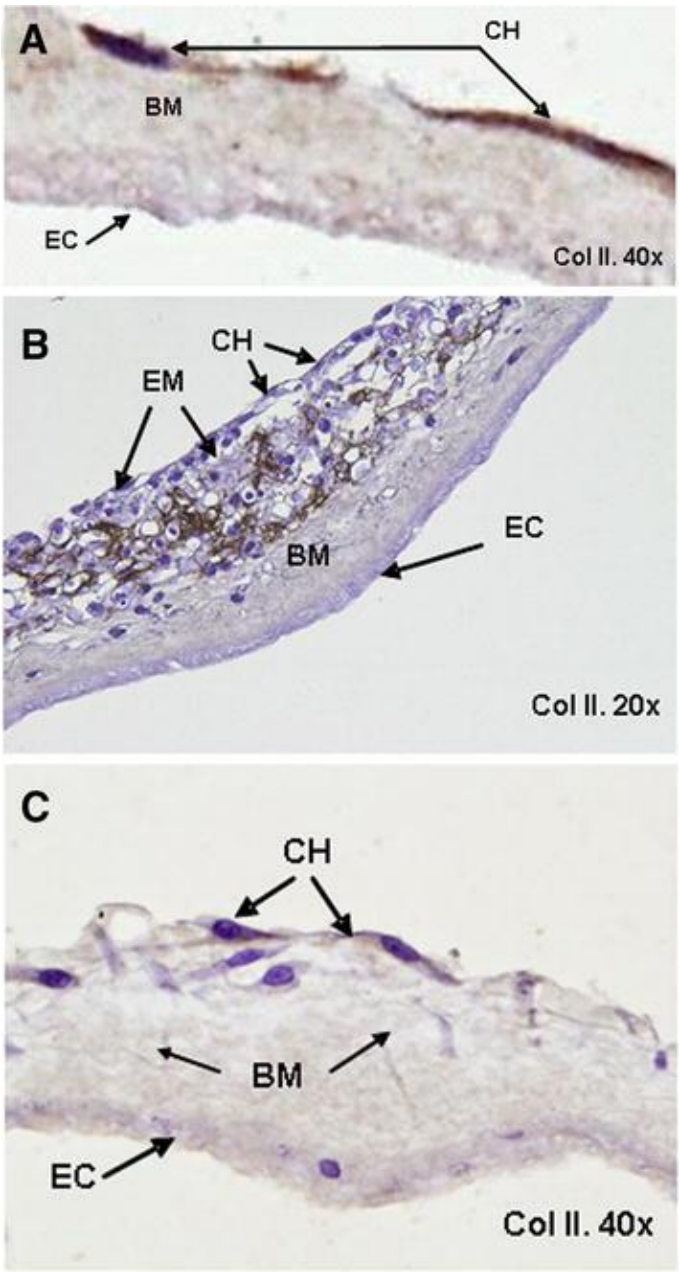

Fig. 4 Immunohistochemistry of the monolayer of human articular chondrocytes grown on the basement membrane of human amniotic membranes (HAMs) showed positive staining for type II collagen (Col II) (**a**, **b**, **c**). The thick basement membrane of the HAM also expresses Col II. CH, monolayer of human articular chondrocytes cultured on the basement membrane HAMs; *BM* basement membrane; *EC* epithelial cells from the extra-embryonic ectoderm. *ECM* extracellular matrix formed by cultured cells

The effect of trypsin on chondrocyte growth on HAMs

To remove epithelial cells from HAMs and facilitate chondrocyte penetration into the porous structure of the denuded HAM, we treated HAMs with various trypsin concentrations ($n = 7$). As a result of trypsin treatment we observed a massive necrosis of epithelial cells from the extra-embryonic ectoderm of HAMs. Chondrocytes cultured on trypsin-treated HAMs had disintegrated cytoplasmic membranes, and many of the chondrocytes were necrotic. Chondrocytes were found in very few areas, sometimes appearing in clusters, and they were separated from the basement membrane. We did, however, observe areas with well-conserved chondrocyte monolayers attached to the basement membrane of the generally fragmented HAM (Fig. 5). For this reason we only performed 3 in vitro repair models using trypsin-treated HAMs.

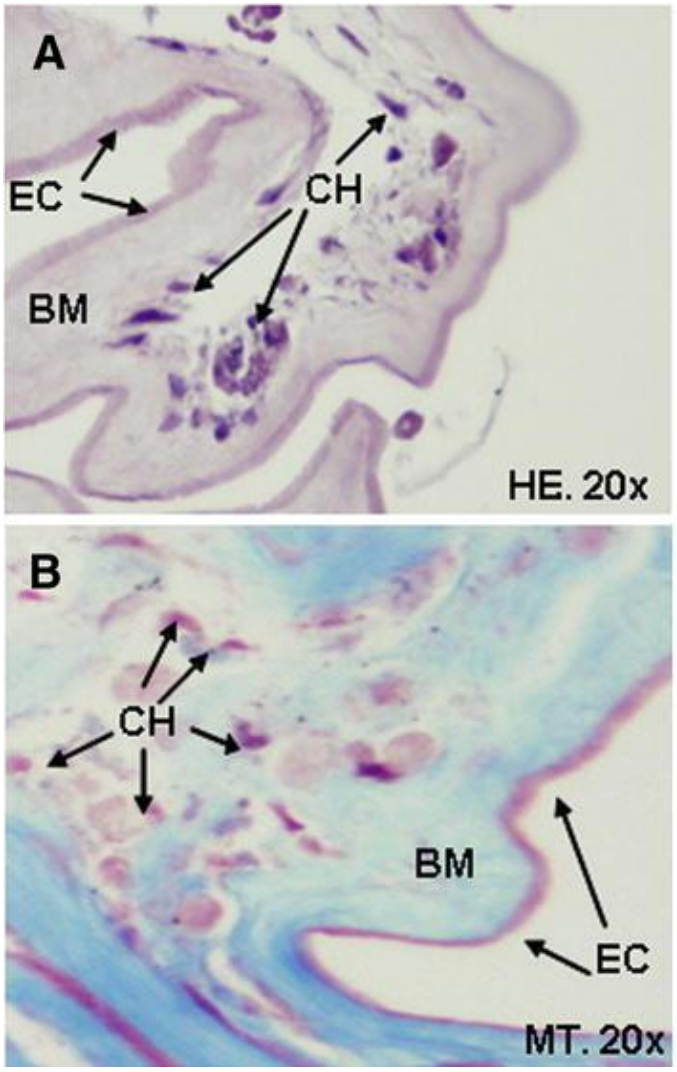

Fig. 5 Treatment of human amniotic membranes (HAMs) with trypsin 0.25% (**a**, **b**). Human articular chondrocytes grown on the thick basement membrane of HAMs have disintegrated cytoplasmic membranes, most cells are necrotic, and some are separated from the HAM. Epithelial cells from the extra-embryonic ectoderm of the HAM suffered a massive necrosis. CH, monolayer of human articular chondrocytes cultured on the basement membrane of HAMs; *BM* thick basement membrane; *EC* epithelial cells from the extra-embryonic ectoderm. H–E (hematoxylin–eosin) staining (**a**) and Masson's Trichrome staining (**b**)

In summary, these results demonstrate that chondrocytes grew when cultured on the chorionic basement membrane layer of the HAM, but did not proliferate well when grown on the epithelial side. For this reason the articular cartilage repair experiments were carried out using the chorionic basement membrane layer of the HAM.

In vitro cartilage regeneration

The surface of OA articular cartilage is irregular with numerous fissures and small cavities distributed along the edge of the lesion. The HAM with chondrocytes grown on the cartilage biopsy provided a more regular surface with the new tissue able to fill the fissures and cavities of the OA cartilage. The newly-formed tissue formed from the HAM with chondrocytes showed a tendency towards a linear way, providing a superficial cell cover that decreased the irregularities of the damaged OA cartilage surface. Generally, this newly-formed tissue showed good integration with the OA cartilage. Some newly-formed tissue had cells in monolayers while others had bi-layers or even multiple layers when there were deep cracks in the OA cartilage (Fig. 6a). The cells had rounded morphology with the characteristics of chondrocytes (Fig. 6a).

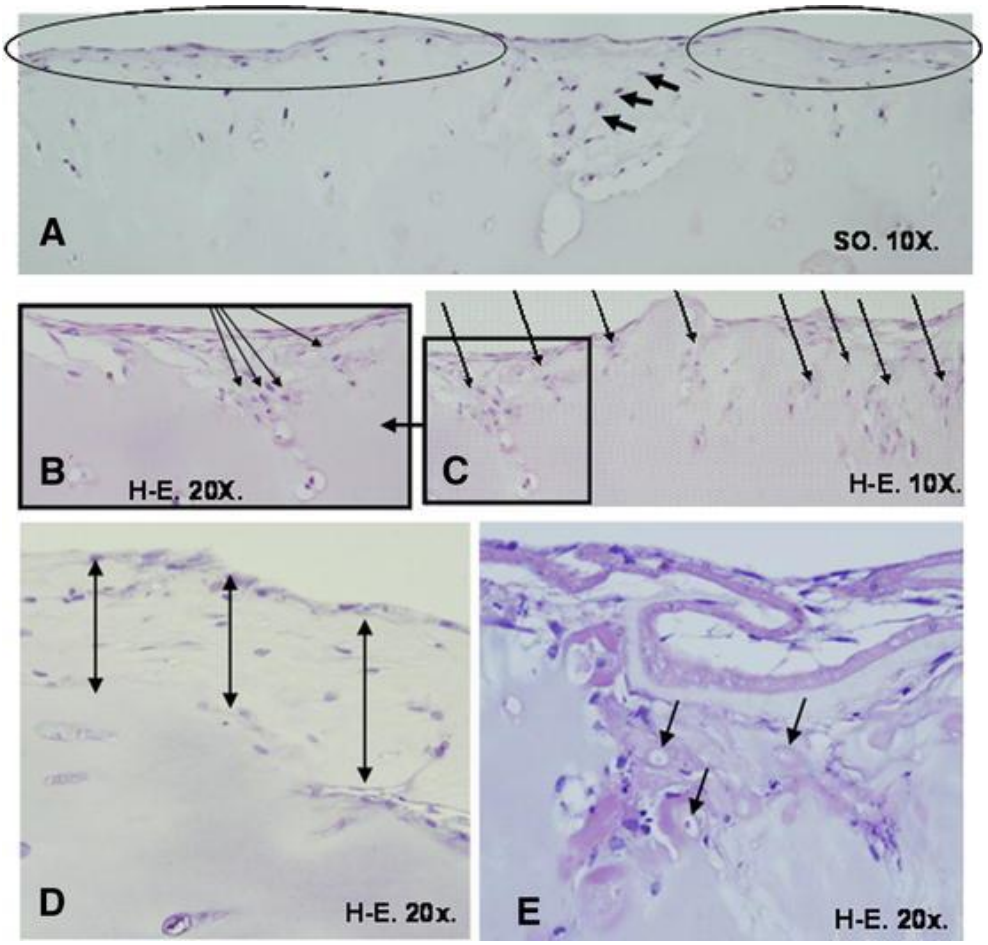

Fig. 6 In vitro repair model of human osteoarthritis (OA) articular cartilage. Human articular chondrocytes cultured on human amniotic membranes (HAMs) provided a smooth surface that filled the fissures and cavities providing a superficial cell cover that decreased the irregularities of the damaged cartilage surface of human articular OA cartilage. The newly-formed HAM tissue integrated well with native cartilage and formed bi-layers and monolayers of cells with a round morphology similar to chondrocytes (**a**). Chondrocytes, from the HAM, migrated and penetrated into the depth of the cavities or fissures of the OA cartilage (**b** and **c**). The newly-formed tissue showed high cell density and a thickening of the basement membrane of the HAM (**d**). Repair tissue also showed formation of lacunae that contained cells with a round morphology similar to chondrocytes (**e**). Safranin O staining (**a**), H–E (hematoxylin–eosin) staining (**b**, **c**, **d**, **e**)

In many of the transplants examined, chondrocytes from the HAM migrated to penetrate into the depths of the cavities or fissures in the OA cartilage (Fig. 6b, c).

The morphology of the repair tissue in the majority of experiments exhibited a fibrous appearance and high cellularity. We could not define the boundary between native cartilage and new tissue because that boundary appeared to be much diffused and not easily differentiated.

In most cases the newly-formed tissue was so cellular that its cell density was even higher than that of the native cartilage (Fig. 6d). Some of the transplants showed a thickening of the basement membrane of the HAM, this may be because chondrocytes grown on the basement membrane penetrate, infiltrate and spread in a uniform manner throughout the thickness of the stromal matrix (Jin et al. *2007*) (Fig. 6d). In the repair tissue we saw the formation of lacunae containing cells with round morphology similar to chondrocytes that were well integrated with neighbouring cartilage. H–E staining corroborated that some repair areas appeared similar to hyaline cartilage (Fig. 6e).

Staining to identify the specific and main components of the cartilage ECM was performed. Specifically, we sought to determine the presence of molecules characteristic of hyaline cartilage, such as proteoglycans by staining with Safranin O and types I and II collagen using immunohistochemistry. Safranin O staining revealed no proteoglycans in nearly all instances. There were only a few showing positive staining, notably in areas where the newly-formed HAM tissue showed good integration with the native cartilage (Fig. 7a, b). The deepest areas of native cartilage showed positive staining with Safranin O, which disappeared in the more superficial areas. The low proteoglycan content would indicate that the quality of the repair tissue is low and that the newly-formed tissue was fibrocartilage. The newly-formed HAM tissue showed such good integration with native cartilage that in some of the in vitro transplants it was impossible to distinguish the border between the newly-formed and native tissues (Fig. 7c, d). In contrast to the lack of staining for proteoglycans that indicates fibrocartilage formation, the newly-formed tissues showed a positive immunoreaction for type II collagen (Fig. 7e) while immunohistochemical staining for collagen type I was weak or absent (Fig. 7f).

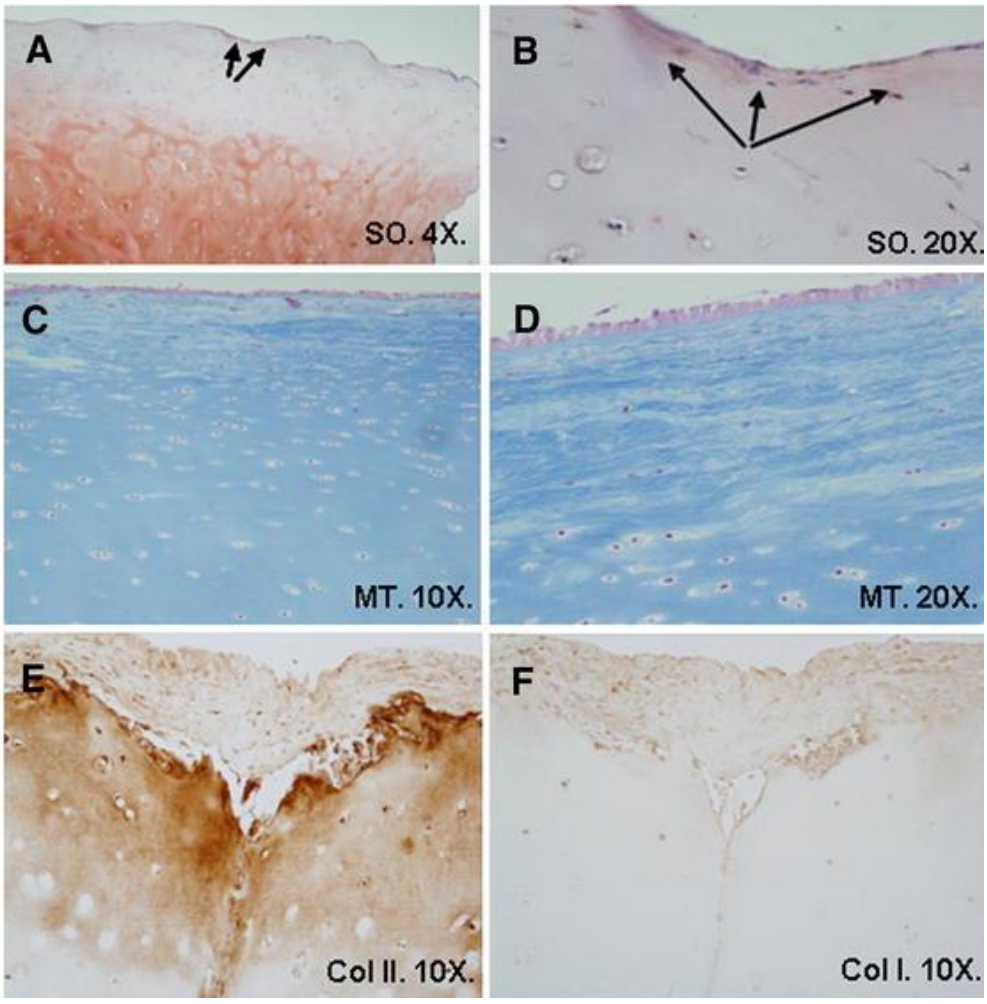

Fig. 7 Histology of the in vitro repair model of human osteoarthritis articular cartilage. Safranin O staining (**a**, **b**). Masson's Trichrome staining (**c**, **d**). Type II and I collagen staining respectively (**e**, **f**)

In the group of controls, amniotic membrane without chondrocytes was adhered to the surface of OA articular cartilage but it was not observed any newly-formed tissue neither in the fissures nor in the small cavities distributed along the edge of the lesion.

Discussion

The prevalence of OA in the human population underscores the importance of developing an effective and functional articular cartilage replacement. Recent research efforts have focused on tissue engineering as a promising approach for cartilage regeneration and repair (Kuo et al. *2006*). Cartilage tissue engineering is critically dependent on the selection of appropriate cells, suitable scaffolds for cell delivery and biological stimulation with chondrogenically bioactive molecules (Kuo et al. *2006*). A major prerequisite for choosing a scaffold is its biocompatibility, the property of not producing toxic, injurious, carcinogenic, or immunological responses in living tissue (Niknejad et al. *2008*). New tissue regeneration should occur as the scaffold degrades, so the new tissue assumes the shape and size of the original scaffold. Design criteria for scaffolds include controlled biodegradability, suitable mechanical strength and surface chemistry, ability to be processed in different shapes and sizes, and the ability to regulate cellular activities such as differentiation and proliferation (Kuo et al. *2006*). For cartilage tissue engineering, scaffolding has been fabricated from both natural and synthetic polymers, such as fibrous structures, porous sponges, woven or non-woven meshes and hydrogels (Kuo et al. *2006*). Researchers have recently proposed that the HAM is suitable as a scaffold for tissue engineering (Niknejad et al. *2008*; Wilshaw et al. *2006*).

The ECM components of the basement membrane from the HAM include collagen, fibronectin, laminin and other proteoglycans important for overlying cell growth. Other properties of the HAM include anti-inflammation, anti-fibrosis, anti-scarring, anti-microbial, low immunogenicity and adequate mechanical properties, all important requirements for tissue engineering (Niknejad et al. *2008*). The HAM can produce a wide array of growth factors and provide a healthy new substrate suitable for re-epithelization and epithelial healing (Wilshaw et al. *2006*). Amnion allografts are widely applied in ophthalmology, plastic surgery, dermatology, and gynecology (Tejwani et al. *2007*; Santos et al. *2005*; Rinastiti et al. *2006*; Meller et al. *2000*; Morton and Dewhurst *1986*). The low cost of amnion graft preparation and the very good clinical results in multipurpose applications have made it a valuable material for tissue banking and a viable alternative to other natural (i.e., preserved human skin) and synthetic wound dressings.

The purpose of this study was to evaluate the potential use of cryopreserved HAMs as a support for human chondrocytes to repair human articular cartilage lesions. Recent studies have found that limbal, corneal and chondrocyte stem cells rapidly proliferate on HAMs (Koizumi et al. *2002*; Galindo et al. *2003*; Jin et al. *2007*). We have determined that human chondrocytes were able to grow on both the epithelial and chorionic sides of the HAM. The chondrocytes showed a characteristic monolayer cell growth. However, when grown on the epithelial side of the HAM the monolayer of chondrocytes separated from the extra-embryonic ectoderm, suggesting a possible competition between the chondrocytes and the epithelial cells. This indicates that the chorionic surface of the HAM is more suitable than the epithelial side for human chondrocyte growth. We propose that the HAM could be an excellent candidate for use as a scaffold for cell delivery and migration in order to achieve bonding to the adjacent host tissue, but when the cells are grown on the chorionic surface. The utility of this new biomaterial may be because the HAM promotes epithelialization and neovascularization and possesses immune privilege (Sippel et al. *2001*). It has been previously documented that HAMs may accelerate epithelialization of gingival wounds and ocular chemical and thermal injures when reconstructing damaged organs and corneal tissue (Rinastiti et al. *2006*; Madhira et al. *2008*; Sangwan et al. *2007*; Tejwani et al. *2007*). Also, Santos et al. (*2005*) have demonstrated the suitability of HAMs for treating limbal stem cell deficiency (LSCD). Although we found the chorionic surface of the HAM most suitable for chondrocyte growth in the in vitro repair model for human articular cartilage, other studies indicate that when HAM is used for support of human limbal epithelial cells (HLEC), the epithelial side of the HAM is more appropriate (Li et al. *2006*; Meller et al. *2002*).

Human chondrocytes are known to de-differentiate toward fibroblasts when cultured (Gimeno Longas and de la Mata Llord *2007*). Using immunohistochemistry, we have demonstrated that human chondrocytes cultured on HAMs expressed type II but not type I collagen. This confirms that the chondrocytes did not de-differentiate to fibroblasts or to a different cell type, but maintained the characteristic phenotype of human chondrocytes.

Human chondrocytes cultured on HAM and transplanted onto human osteoarthritis articular cartilage produced a more regular surface, filling the fissures and cavities of the OA cartilage. The chondrocytes grew in a linear arrangement and decreased the degree of damages of the OA articular cartilage surface.

Also, HAM with cultured chondrocytes showed good integration with the native cartilage and the newly synthesized tissue constituted bi-layers and monolayers of cells with round morphology and characteristics similar to chondrocytes. Chondrocytes migrated from the HAM to penetrate into the depths of the cavities and fissures in the OA cartilage. The morphology of the repair tissue exhibited a fibrous appearance and high cellularity. Also, we were not able to delineate the boundary between native cartilage and newly-formed tissue.

In most cases the newly-formed tissue was so cellular that it had a higher cell density than the native cartilage. Some of the transplants showed a thickening of the basement membrane of the HAM, probably because chondrocytes grown on the basement membrane penetrate, infiltrate and spread in a uniform manner throughout the thickness of the stromal matrix as previously described (Jin et al. *2007*). In the newly-formed tissue, we observed the formation of lacunae containing cells with the round morphology of chondrocytes to be well integrated with neighbouring cartilage. In fact, using H–E staining, it was possible to corroborate that some repair areas appeared similar to hyaline cartilage.

Staining was done to detect specific major components of the ECM to obtain more detailed information about the structure and composition of the repair tissue (Fuentes Boquete and López Armada *2007*). Safranin O staining showed no proteoglycans in almost all cultures, although a few did show positive staining, notably in areas where the HAM showed good integration with the native cartilage. The deepest area of the native cartilage showed positive staining with Safranin O, but this disappeared in the superficial area. The absence of proteoglycan in the outer areas of OA cartilage has been previously described (Fuentes Boquete and López Armada *2007*). The low content of proteoglycan indicates that the quality of the repair tissue is low, the newly-formed tissue being hyaline-like cartilage. Importantly, the newly-formed tissue showed a positive immunoreactivity for type II collagen, while the immunohistochemical staining for type I collagen was weak or absent.

Conclusion

Our results indicate that cryopreserved HAMs are useful for the support of human chondrocyte proliferation in cell therapy to repair human OA cartilage.

Acknowledgments

This study was supported by grants: Servizo Galego de Saúde, Xunta de Galicia (PS07/84), Catedra Bioiberica de la Universidade da Coruña and Instituto de Salud Carlos III CIBER BBN CB06-01-0040. Silvia Diaz-Prado is beneficiary of an Isidro Parga Pondal contract from Xunta de Galicia, A Coruna, Spain. Tamara Hermida-Gómez is beneficiary of a contract from Fondo de Investigación Sanitaria (2008), Spain. Emma Muíños-López is supported by Rheumatology Spanish Foundation, Spain. We would like to thank MJ Sánchez and P Filgueira for technical assistance.

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