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Short Report

Isolation of human nasoseptal chondrogenic cells: A promise for cartilage engineering

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Abstract In cartilaginous tissues, perichondrium cambium layer may be the source of new cartilage. Human nasal septal perichondrium is considered to be a homogeneous structure in which some authors do not recognize the perichondrium internal zone or the cambium layer as a layer distinct from adjacent cartilage surface. In the present study, we isolated a chondrogenic cell population from human nasal septal cartilage surface zone. Nasoseptal chondrogenic cells were positive for surface markers described for mesenchymal stem cells, with exception of CD146, a perivascular cell marker, which is consistent with their avascular niche in cartilage. Although only Sox-9 was constitutively expressed, they also revealed osteogenic and chondrogenic, but not adipogenic, potentials *in vitro*, suggesting a more restricted lineage potential compared to mesenchymal stem cells. Interestingly, even in absence of chondrogenic growth factors in the pellet culture system, nasoseptal chondrogenic cells had a capacity to synthesize sulfated glycosaminoglycans, large amounts of collagen type II and to a lesser extent collagen type I. The spontaneous chondrogenic potential of this population of cells indicates that they may be a possible source for cartilage tissue engineering. Besides, the pellet culture system using nasoseptal chondrogenic cells may also be a model for studies of chondrogenesis.

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Introduction

Cartilaginous tissue lacks an intrinsic regeneration capacity. The tissue engineering and cell-based approaches can be thus excellent alternatives to treat cartilage lesions (Ahmed and Hincke, 2010). Choosing the appropriate cell type for these therapies is a critical step. Chondrocytes are highly specialized cells responsible for the production of cartilage extracellular matrix (Huang, 1977), but articular chondrocytes are considered difficult to culture, have low proliferation capacity and may dedifferentiate in monolayer cultures, losing their chondrogenic phenotype (Kuo et al., 2006). In addition to articular cartilage, there are other sources of chondrocytes, such as ear (Malicev et al., 2009) and nasal septum, with some advantages as compared to articular chondrocytes, since they are easy to harvest with low iatrogenic morbidity (Chia et al., 2004).

Perichondrium surrounds all mammalian cartilage tissues, with exception of fibrocartilage and articular cartilage, where the synovial fluid is present. Despite not having a perichondrium, articular cartilage contains a progenitor/stem cell population on its surface zone (Archer et al., 1990; Dowthwaite et al., 2004). In other cartilaginous tissues, perichondrium cambium layer seems to be the source of new cartilage (Upton et al., 1981) where chondroprogenitor cells dwell (Engkvist et al., 1979). However, some authors do not recognize the perichondrium internal zone or cambium layer as a separate layer from the adjacent cartilage (Bairati et al., 1996). In particular, human nasal septal perichondrium is considered to be a homogeneous structure without clearly distinguishable zones (Bleys et al., 2007), and the border between cartilage and perichondrium is not very clear (Bairati et al., 1996).

The aim of the present study was to isolate and characterize a chondrogenic cell population from human nasal septal cartilage surface zone, a niche similar to the articular cartilage progenitor cell niche, pointing them as a possible source for cartilage tissue engineering approaches.

Results and discussion

Nasoseptal chondrogenic cells dwell in surface region of human nasoseptal cartilage

Before enzymatic digestion with collagenase IA, nasoseptal cartilage samples exhibited two distinguishable zones: the superficial and inner zones. The superficial zone showed low affinity with Safranin O and elongated fibroblast-like cells positioned with their long axes parallel to the cartilage surface. The inner zone was stained with Safranin O and cells showed a more rounded morphology organized in randomly distributed isogenic groups (Fig. 1A). Collagen type I was mainly detected on the surface zone (Fig. 1B). After the enzymatic digestion procedure, the superficial layer could no longer be observed (Fig. 1C).

Due to the abundance of collagen type I in nasal septal perichondrium and the difficulty to define its transition to cartilage (Bleys et al., 2007), we suggest that the superficial zone of the cartilage samples may correspond to perichondrium cambium layer. Van Osch et al. showed that ear perichondrium cells had chondrogenic potential superior to nasal perichondrium cells, but also suggested that the perichondrium cambium layer may have remained attached to the septum cartilage during the nasal perichondrium harvesting, leading to low chondrogenic capacity of the isolated tissue (Van Osch et al., 2000). Even though we have harvested cartilage samples without perichondrium, it seems that a similar event occurred in our work, *i.e.*, the perichondrium cambium layer remained attached to the septum cartilage during the sample harvesting.

Characterization of nasoseptal chondrogenic cells after *in vitro* expansion

The population of nasoseptal chondrogenic cells was positive for CD105, CD73 and CD44 (Fig. 2A–C), surface markers described for mesenchymal stem cells populations of different origins (Dominici et al., 2006). A perivascular niche has been suggested for mesenchymal stem cells in various tissues (Da Silva Meirelles et al., 2008; Crisan et al., 2008), but as expected, nasoseptal chondrogenic cells, that dwell in avascular niche, were negative for CD146 (Fig. 2D), a perivascular cell marker first described in bone marrow samples (Sacchetti et al., 2007). In addition, CD44, a hyaluronan receptor that organizes the pericellular matrix, was highly expressed (Fig. 2C) and its expression is concomitant with the reduction of intercellular spaces at early sites of future cartilage formation (Rousche and Knudson, 2002).

On monolayer culture and in the absence of inducing media, nasoseptal chondrogenic cells were positive only for

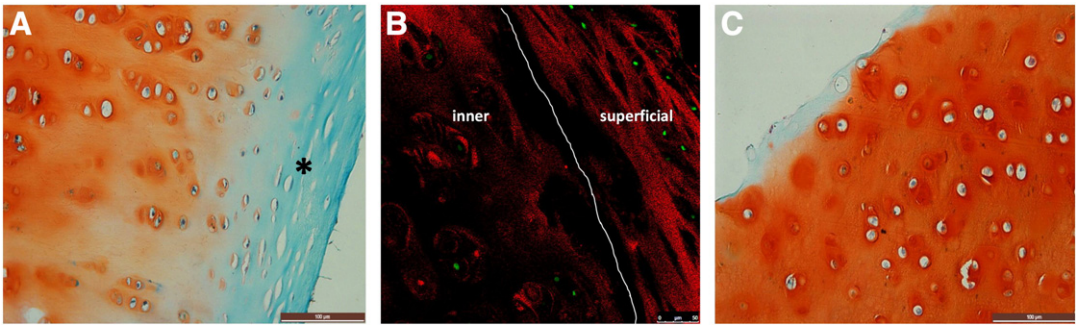

Figure 1 Effect of enzymatic digestion on human nasoseptal cartilage samples. Images of histological sections before (A, B) and after (C) enzymatic digestion with collagenase type IA. Safranin O (A, C). Asterisk indicates superficial zone. Bar size, 100 μm. Collagen type I (B) (in red) was detected using immunofluorescence protocols. Nuclei stained by Sytox green (in green). Superficial and inner zones are indicated. Bar size, 50 μm.

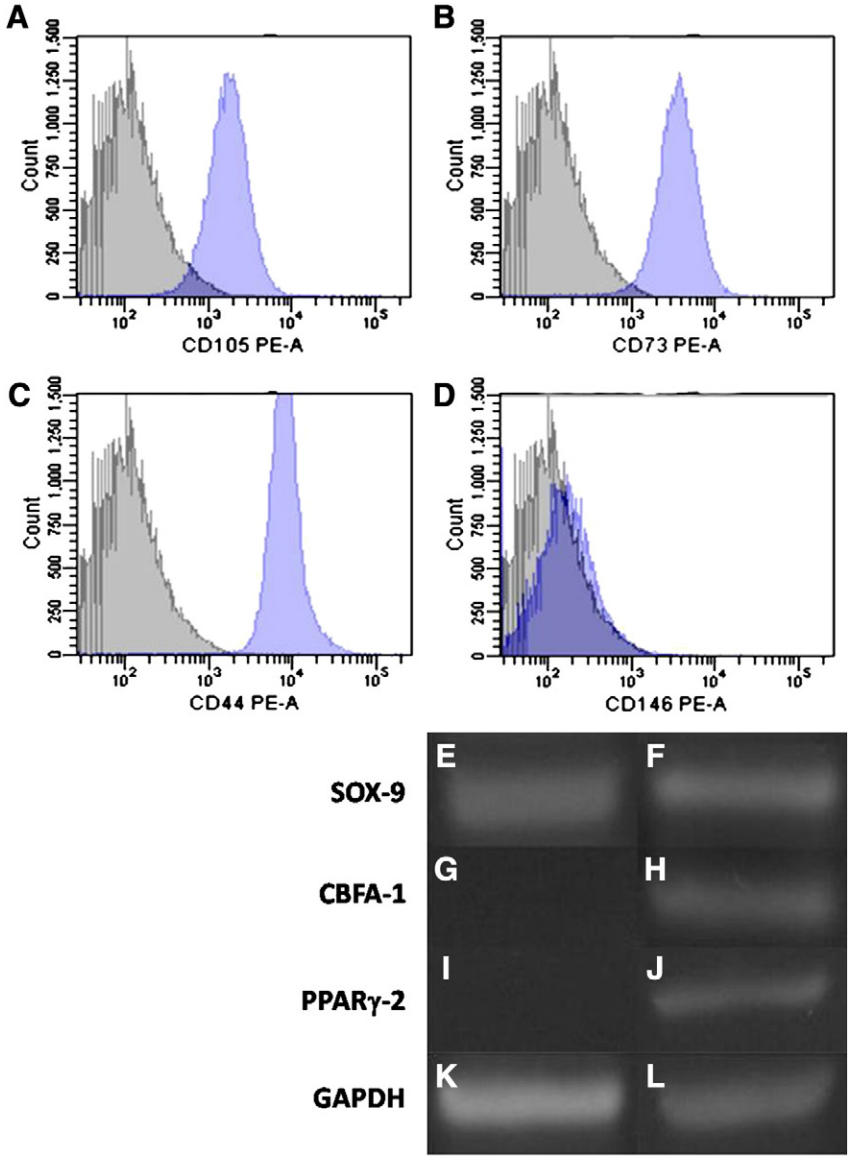

Figure 2 Molecular characterization of nasoseptal chondrogenic cells. Flow cytometry analysis of cells harvested after third passage (A–D). The population of cells was positive for mesenchymal markers CD105 (A), CD73 (B), CD44 (C), except for CD146 (D). Gray solid histograms represent negative controls (isotype controls). Reverse-transcriptase PCR (E–L). Amplified products of chondrogenic cells (E, G, I, K) and of mesenchymal cells from adipose tissue (F, H, J, L), used as positive control. Chondrogenic cells are positive for SOX-9 (E) and negative for CBFA-1 (G) and PPARγ2 (I). Constitutive expression of GAPDH (K, L) as control for RT-PCR reaction.

the chondrogenic master gene (Sox-9) (Fig. 2E). Although negative for the osteogenic master gene (CBFA-1) (Fig. 2G), nasoseptal chondrogenic cells could deposit considerable amounts of extracellular calcium under osteogenic inducing medium (Fig. 3B). It is possible that CBFA-1 expression had been modulated during differentiation. The mechanisms involved in this process should be better understood. On the other hand, absence of the adipogenic master gene (PPAR-γ2) (Fig. 2I) was correlated to the inability of nasoseptal chondrogenic cells to differentiate into adipocytes. Although these cells changed their morphology to a more rounded shape under adipogenic induction, no cytoplasmic lipid droplets were accumulated (Fig. 3D).

The surface markers and gene expression profile, but also the inability of multilineage differentiation *in vitro* may indicate nasoseptal chondrogenic cells as a population committed to the condrogenic lineage, while mesenchymal stem cells from different sources have more differentiation plasticity.

Pellet culture generated a three-dimension structure that resembled a cartilaginous tissue

In monolayer culture, cells isolated from superficial zone showed a fibroblastoid morphology *in vitro*, reaching confluence in approximately 10 days after isolation (Fig. 4A). When these cells were maintained in high cellular density pellet culture, a 3D system, even in the absence of chondrogenic growth factors, they switched from a fibroblastoid to an

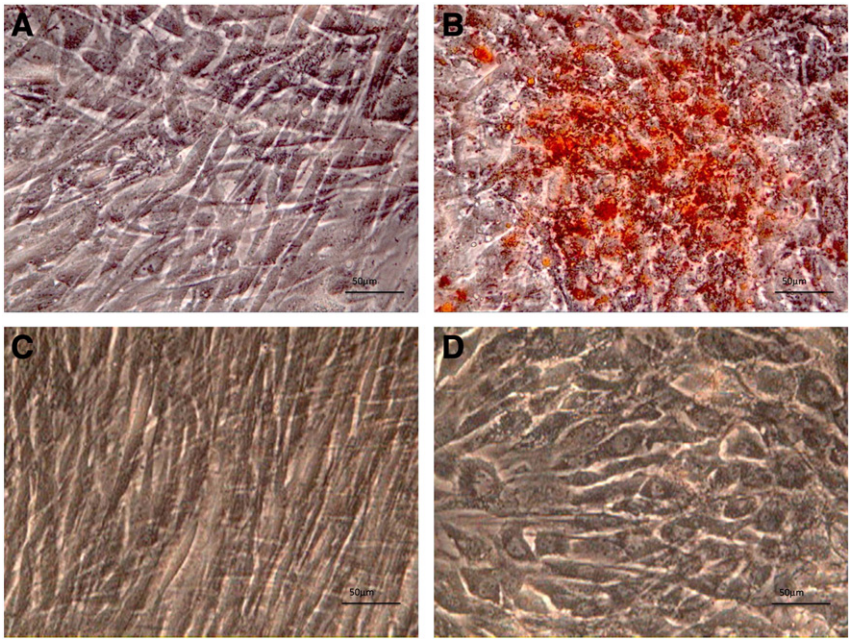

Figure 3 Nasoseptal chondrogenic cells differentiation potential to osteogenic and adipogenic lineages. Monolayers of cells maintained under osteogenic (B) or adipogenic (D) inducting media for 21 days. After this period, they were fixed and stained with Alizarin Red to reveal calcium deposits, and with Oil Red O to detect lipid droplets. Control media (A, C); inducing media (B, D). Bar size, 50 μm.

oval morphology and synthesized sulfated glycosaminoglycans, as shown by a strong Safranin O staining (Fig. 4B). Elongated cells, present on the pellet surface, can also be detected on agarose clusters surface prepared with surface articular chondrocytes, but not on agarose clusters prepared with deep zone articular chondrocytes (Archer et al., 1990).

Collagen type I was less detected throughout the pellet (Fig. 4C) while collagen type II was clearly present (Fig. 4D). For a successful chondrogenic differentiation, *i.e.* with high levels of collagen type II and sulfated glycosaminoglycans, bone marrow-derived mesenchymal stem cells (Johnstone et al., 1998), adipose-derived mesenchymal stem cells (Estes et al., 2010) and dedifferentiated articular chondrocytes (Barbero et al., 2003) must be culture in a 3D system with chondrogenic growth factors as TGF-βs and BMPs. For nasoseptal chondrogenic cells grown up to the third passage, the addition of such growth factors was not necessary for a successful chondrogenic differentiation, which highlights its chondrogenic commitment.

This chondrogenic commitment, together with inability of multilineage differentiation, could be explained, in part, by nasoseptal chondrogenic cells niche, which may correspond to perichondrium cambium layer, and surface markers and gene expression profile, as high CD44 expression but not CD146, and expression of only the chondrogenic master gene mRNA, as already discussed. More specifically, the absence of PPAR-γ2 mRNA expression on nasoseptal chondrogenic cells may have strong implications. Some reports on literature suggest a mutually antagonistic relationship between reduced PPAR-γ expression and increased TGF-β signaling in a lung model of development (Nicola et al., 2011) and fibrosis (Kulkarni et al., 2011). Chondrocytes seem to respond in a similar way to high levels of PPAR-α, β/δ or γ, whereas PPAR agonists reduce the stimulating effect of TGF-β1 on extracellular matrix components synthesis (Poleni et al., 2007; Poleni et al., 2010). Exogenous TGF-β is not required for cartilage matrix synthesis on nasoseptal chondrogenic cells perhaps because this signaling pathway is not hampered by PPARs.

In pellet culture system nasoseptal chondrogenic cells revealed a typical pattern of organelles described on superficial chondrocytes, as a prominent Golgi complex and enlarged rough endoplasmic reticulum cistern, which distinguish them from other perichondrium cells (Bairati et al., 1996). Small lipid droplets and large amounts of glycogen in their cytoplasm can also be observed (Fig. 4E, F). The pattern of organelles observed suggests that this population of cells belongs to the chondrogenic lineage.

Much of our current understanding concerning the molecular mechanisms of chondrogenesis has been delineated *in vitro* utilizing high cellular density culture systems. Nasoseptal chondrogenic cells under pellet culture system had an extracellular matrix content very similar to cartilage, being a potential model for studying chondrogenesis. Besides, the chondrogenic commitment observed in nasoseptal chondrogenic cells may be very useful for cartilage engineering, since it has been reported that chondrogenic differentiation of mesesenchymal stem cells usually results in a significant proportion of fibrocartilage (Steck et al., 2005).

Material and methods

Human cartilage sampling

Cartilage fragments from nasoseptal were obtained from donors (n = 12) from 25 to 40 years old that underwent esthetic surgery procedures. The study has been approved by the

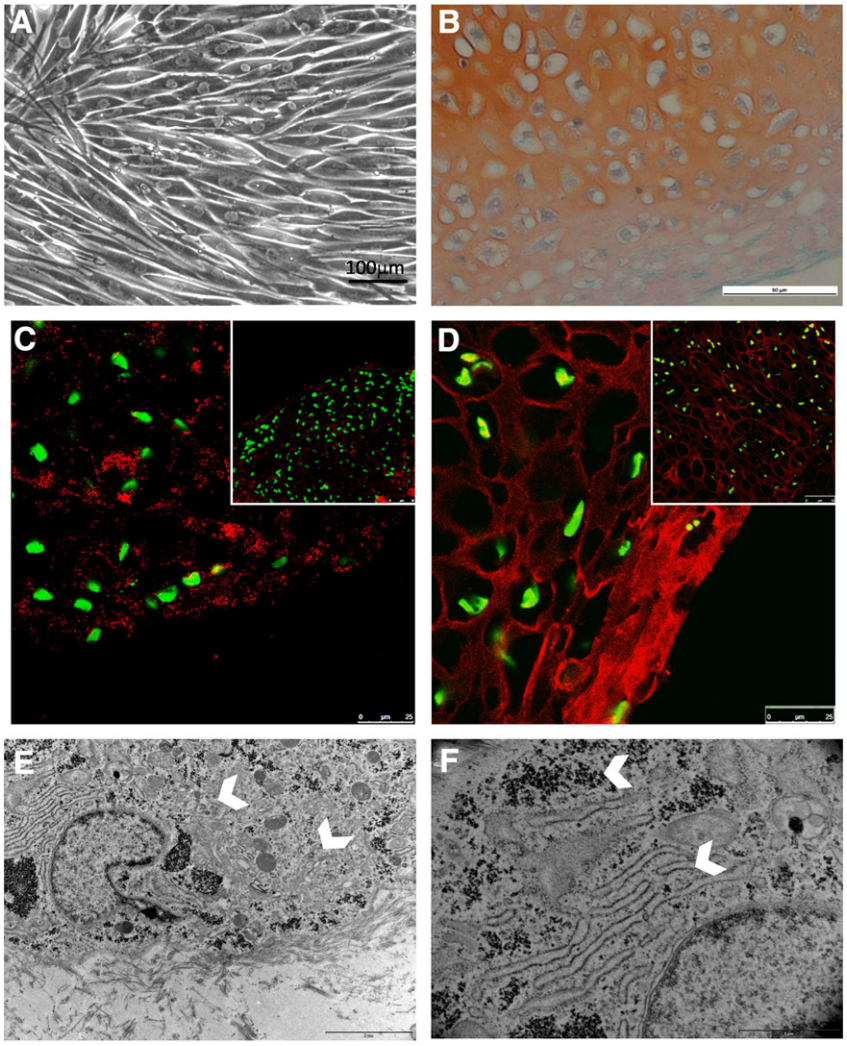

Figure 4 Spontaneous differentiation potential into the chondrogenic lineage. Nasoseptal chondrogenic cells in monolayer culture revealed fibroblastoid morphology (A). Phase contrast microscopy — bar size, 100 μm. Pellet culture maintained under control media (no chondrogenic induction) for 28 days. After this period, they were fixed and stained with Safranin O (B) to reveal sulfated glycosaminoglycans. Bar size, 60 μm. Immunofluorescence protocols for collagen type I (C) and type II (D) (in red) were also performed. Nuclei stained by Sytox green (in green). (C, D) Bar size, 25 μm. Insets (lower power image) — bar size, 50 μm. Pellet culture maintained under control media for 15 days, observed by transmission electron microscopy (E, F). Arrowheads in E represent small cytoplasmic lipid droplets and prominent Golgi complexes, in F an enlarged rough endoplasmic reticulum cistern and large amounts of glycogen. (E, F) Bar size, 2 μm and 1 μm, respectively.

Research Ethics Committee of the Clementino Fraga Filho University Hospital, Federal University of Rio de Janeiro, Brazil.

Isolation and culture of cells

Cartilage fragments were minced into small pieces and rapidly incubated with collagenase IA (Sigma). Cartilage samples were collected before and after digestion for histological evaluation.

Cells were harvested by centrifugation and plated in tissue culture flasks with alpha-minimum essential medium (alpha-MEM, Sigma Chemical Co) containing 10% fetal bovine serum (FBS, LGC), 100 U/mL penicillin, and 100 μg/mL streptomycin. Cultures were maintained at 37 °C in a humid atmosphere with 5% CO_2, and the medium was changed every 3–5 days until cell monolayer reached confluence. At confluence, cells were harvested with 0.78 mM EDTA (Gibco) and 0.125% trypsin (Gibco) and re-seeded at a density of 10^4 cells/cm^2. Dissociation with trypsin followed by re-seeding for cell expansion was denominated "passage".

Pellet cultures were performed as described previously (Johnstone et al., 1998; Baptista et al., 2009a), with cells from up to the third passage. For transmission electron microscopy analysis, pellet cultures (n = 3) were fixed at 15 days in 2.5% glutaraldehyde buffered with 0.1 M sodium cacodylate and post fixed with 1% OsO_4 (all from Electron Microscopy Sciences) in the same buffer. Samples were dehydrated in graded acetone (Merck) and embedded in the Epon resin (Electron Microscopy Sciences). Sections of 70 nm were obtained on ultramicrotome (EM UC6) and

examined under a transmission electron microscope (FEI-Tecnai Spirit 12).

Flow cytometry analysis

Cells were monitored for surface marker expression at the first passage (n=4) using flow cytometry. Harvested cells were washed with phosphate-buffered-saline containing 3% bovine serum albumin (PBS-BSA 3%) and incubated for 30 min at 4 °C with mouse monoclonal antibodies conjugated with fluorescent dies: CD44-phycoerythrin (PE), CD73-PE, CD146-PE (all from BD Biosciences) and CD105-PE (R&D Systems). Subsequently, cells were washed with PBS-BSA 3%. Fifty thousand events were acquired per antibody set, on FACSCanto flow cytometer equipped with FACS Diva Software 4.3.

RT-PCR analysis

Cells were monitored for mRNA gene expression at the second passage (n=3), as described previously (Baptista et al., 2009b). Mesenchymal stem cells from adipose tissue were used as positive controls. Reaction settings had an initial denaturation step of 5 min at 95 °C, followed by 36 cycles of 1 min at 95 °C, 1 min at annealing temperature for each primer used (Supplemental — Table 1), and 1 min at 72 °C. Finally, samples were maintained for 5 min at 72 °C. Reaction products were resolved by electrophoresis on a 1.2% agarose (Invitrogen) gel and incubated with GelRed (Biotium) for visualization under UV light.

Cell differentiation assays

Osteogenic and adipogenic potential was investigated *in vitro* using the appropriate inducting media with cells from up to the third passage (n=3, for each assay), as described previously (Baptista et al., 2009a). Following lineage differentiation induction, cells were fixed in 10% formaldehyde (Vetec) for 60 min. Adipogenic differentiation was assessed using Oil Red O staining (Sigma) as an indicator of intracellular lipid accumulation. Osteogenic differentiation was assessed using alizarin red staining (Sigma) as an indicator of extracellular calcium deposition (Baptista et al., 2009b). Chondrogenic potential was monitored in pellet cultures under absence of growth factors commonly used to induce chondrocyte phenotype. After 28 days, histological and immunofluorescence protocols were performed in these cultures.

Histological evaluation

For histological preparations, pellet and cartilage samples (n=4, each) were fixed in 10% formaldehyde. Samples were dehydrated in graded ethanol, cleared in xylol, and embedded in paraffin (all from Vetec). Cartilage samples were decalcified with a solution of formaldehyde/formic acid prior to dehydration process. Sections of 5 μm of both samples were obtained on American Optical Microtome. Histological sections were stained with Safranin O counterstained by Fast Green (all from Sigma) to assess glycosaminoglycan content, as described previously (Grogan et al., 2006). Sections were examined under an optical microscope (Leica DMI 6000 B) equipped with Leica DFC 500 digital camera.

Immunofluorescence analysis

For frozen sections, pellet and cartilage samples (n=4, each) were fixed in 4% paraformaldehyde (Vetec) for subsequent embedding in OCT compound (Sakura Finetek). Sections of 10 μm were obtained on Leica C1850 cryostat and mounted on microscope slides. Antigen unmasking was done by treatment with hyaluronidase (4800 U/ml — Sigma). Unspecific binding of immunoglobulins was blocked with 5% PBS/BSA, 5% goat serum and 0.5% Triton for 1.5 h. Sections were incubated overnight at 4 °C with primary antibodies (1:50) for type I collagen or type II collagen (both from Santa Cruz Biotech). Secondary antibody staining was performed using Alexa fluor 594 conjugate anti-mouse IgG for 1 h at room temperature. Nuclei were stained with Sytox green — 1:500, (Invitrogen — Molecular Probes). Slides were mounted in a commercial Vectashield® Mounting Medium (Vector Lab) and examined under a confocal fluorescence microscope (Leica TCS SP5).

Conclusions

There are various applications for cell-based therapies in cartilage repair. For knee cartilage lesion treatment, autologous chondrocyte transplantation has become the principal cell-based approach. The first human autologous chondrocyte transplantation was done by a Swedish group in 1994 (Brittberg et al., 1994). Although this has granted clinical benefits including pain relief with function improvement (Recht et al., 2003; Ferruzzi et al., 2008), it still remains unsatisfactory, mainly because of the morbidity in the donor site caused by the harvesting of the articular cartilage (Hunziker, 2002). It has also been reported that the repair tissue still contains a considerable proportion of fibrocartilage (McNickle et al., 2009).

In maxillofacial surgery, the repair and augmentation of craniofacial structures or repair of nose deformities is a challenge for surgeons. The current clinical practice is to treat these deformities with the combination of surgery and the use of autologous tissues. However, grafting autologous tissue is associated with difficulty to obtain a sufficient amount of tissue (Yanaga et al., 2006).

Some authors indicate perichondrium as a good cell sources for cartilage repair (Van Osch et al., 2000; Togo et al., 2006), while others do not, claiming inconsistent reproducibility and yield rate of neocartilage (Shieh et al., 2004). Also, the repair tissue formed by these perichondrium cells *in vivo* is different from the native one (Dounchis et al., 2000) and unstable after long periods (Ostrander et al., 2001). Apart from this discussion, it seems that no study worked specifically with cells of the cartilage superficial zone or perichondrium cambium layer, suggested to be the source of new cartilage (Upton et al., 1981).

Mesenchymal progenitor cells have been reported in normal and osteoarthritic articular cartilage (Alsalameh et al., 2004). The surface of articular cartilage contains pluripotent

progenitor cells (Dowthwaite et al., 2004). Although nasoseptal chondrogenic cells were not reported to present such plasticity, they also occupy a surface niche on cartilaginous tissue, belonging to chondrogenic lineage.

Further studies are necessary to prove that nasoseptal chondrogenic cells can efficiently generate cartilaginous tissue *in vivo* and to compare its chondrogenic potential against other progenitor cell types. However, engineering articular cartilage with biomimetic scaffolds is being pointed as a major strategy for success of cartilage engineering in the future (Klein et al., 2009). Therefore, combination of different cell types is an advantage, and nasoseptal chondrogenic cells may occupy the superficial niche in a construct. Finally, nasoseptal chondrogenic cells may not only be used for clinical implants, but also as a model for studying chondrogenesis.

Supplementary materials related to this article can be found online at doi:10.1016/j.scr.2011.09.006.

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