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Isolation and *in vitro* chondrogenic potential of human foetal spine cells

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Abstract

Cell therapy for nucleus pulposus (NP) regeneration is an attractive treatment for early disc degeneration as shown by studies using autologous NP cells or stem cells. Another potential source of cells is foetal cells. We investigated the feasibility of isolating foetal cells from human foetal spine tissues and assessed their chondrogenic potential in alginate bead cultures. Histology and immunohistochemistry of foetal tissues showed that the structure and the matrix composition (aggrecan, type I and II collagen) of foetal intervertebral disc (IVD) were similar to adult IVD. Isolated foetal cells were cultured in monolayer in basic media supplemented with 10% Fetal Bovine Serum (FBS) and from each foetal tissue donation, a cell bank of foetal spine cells at passage 2 was established and was composed of around 2000 vials of 5 million cells. Gene expression and immunohistochemistry of foetal spine cells cultured in alginate beads during 28 days showed that cells were able to produce aggrecan and type II collagen and very low level of type I and type X collagen, indicating chondrogenic differentiation. However variability in matrix synthesis was observed between donors. In conclusion, foetal cells could be isolated from human foetal spine tissues and since these cells showed chondrogenic potential, they could be a potential cell source for IVD regeneration.

Keywords: foetal cells • intervertebral disc • cell therapy • alginate beads • extracellular matrix

Introduction

Up to 65% to 80% of the population will suffer from back pain in their lifetime. Degeneration of the intervertebral disc (IVD) is thought to be one main factor in the development of back pain and is responsible for approximately 90% of spine surgery procedures [1]. Degenerative changes, already observed in the second decade of life [2, 3], first occur in the nucleus pulposus (NP). Metabolic activity of the NP cells is modified and leads to a decrease in proteoglycan (PG) content and diminished water retention. Several factors contribute to the alteration of the cell metabolism, notably diminished nutrition, cell senescence, mechanical stress and inflammatory environment [4–7]. Current treatments for back pain aim at relieving pain but do not treat the causes. Although the initiating events of disc degeneration are unknown, it has been established that degeneration begins with the inability of the NP cells to maintain matrix homeostasis. Thus, it can be assumed that a cell-based therapy could be applied to early degenerated disc to increase matrix synthesis and delay degeneration. This hypothesis is supported by recent clinical and animal studies, where a cellular therapy was applied to degenerated discs with variable results [8–11]. One main key criteria in the success of cellular therapy is the choice of the source of cells. Cells used for cell-based therapy need to be readily expanded *in vitro* and to retain biological activity for restoring and maintaining organ functions. Due to immunocompatibility, autologous cells are usually preferred. However, disc cells have been shown to have poor proliferation capacity *in vitro* and dedifferentiate into fibroblasts in conventional monolayer culture [12–14]. In addition, the IVD offers a limited access

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Table 1 Name and origin of the cells used in the study

Cell name	Origin of the cells	Age of donor
FS 12w	Foetal spine	12 weeks
FS 13w	Foetal spine	13 weeks
FS 14w	Foetal spine	14 weeks
FS 15w	Foetal spine	15 weeks
FS 16w	Foetal spine	16 weeks
NP 41y	Adult NP	41 years
NP 30y	Adult NP	30 years
NP 39y	Adult NP	39 years

to retrieve cells for *in vitro* expansion which is complicated by the low cellularity of this tissue. In humans, autologous NP cell treatment was investigated at the clinical level in patients suffering from disc herniation and showed encouraging results although published data are limited [15]. On the other hand, stem cells are an alternative cell source for organ regeneration as they have high self-renewal capacity and can generate multiple cell lineages [16]. Stem cells represent only a small fraction of a tissue cell population and thus require an extensive *in vitro* expansion step. They were notably able to regenerate the NP in a rabbit model of IVD degeneration [8, 9, 17]. However, it was impossible to determine if stem cells could differentiate into NP cells due to the lack of a defined phenotype. Another potential source of cells for IVD regeneration is foetal cells. Unlike stem cells, foetal cells are differentiated cells with high expansion, regeneration and low immunogenicity properties [18, 19]. They can be isolated from foetal tissues, which follow embryonic stage at 9 weeks of development. Foetal cell therapies have been clinically applied to several degenerative conditions such as Parkinson's [20] or Huntington's diseases [21] and to damaged skin [22, 23]. Foetal cell therapy has also been investigated in spinal cord injury [24, 25], liver disorders, infarctions [26] as well as in bone regeneration [27, 28]. The aim of this study was to assess the feasibility of isolating foetal cells from foetal spine tissue and to assess their *in vitro* matrix production capacity.

Material and methods

Cell isolation

Biopsies for cell isolation were obtained in accordance with the Ethics Committee of University Hospital in Lausanne (Ethical Protocol 51/01). Human foetal spinal column tissues (1 cm length, $n = 5$) were obtained from foetuses after voluntary interruption of pregnancy at 12–16 weeks of gestation. Biopsies were first rinsed with 1% penicillin-streptomycin-phosphate buffer saline (PBS), cleaned of adjacent tissue and minced in small pieces. Foetal spine units, composed of one IVD and the two adjacent vertebrae, were put into culture in Dulbecco's Modified Eagle's medium (DMEM) (Gibco, Switzerland) supplemented with 10% FBS and 100 mM L-Glutamine (Gibco) (hereafter referred to as growth medium) at 37°C under 5% CO_2. A cell bank was established from each biopsy as described in De Buys Roessingh *et al.* [23] and Quintin *et al.* [29].

For comparison purpose, NP cells were isolated from adult tissues ($n = 3$) obtained from patients undergoing discectomy. Cells were retrieved after type II collagenase digestion and expanded. Cells were stored frozen in liquid nitrogen at passage 1 or 2. Then cells were thawed, expanded in monolayer at passage 3 and used for proliferation and alginate bead culture at passage 4. Table 1 presents the origin of the cells used in the study.

Cell proliferation

Foetal spine cells and adult NP cells at passage 4 were seeded at 3000 cells per cm^2 in triplicate in 96-well plates with growth medium. At days 0, 3, 7, 10, 14 and 21, 10 μl of CellTiter (Promega, Switzerland) was added to each well and incubated for 2 hrs at 37°C before measuring optical density at 492 nm with a microplate reader. The assay is based on the formation of formazan by cell activity after addition of tetrazolium. The amount of formazan formed is measured by optical density at 492 nm and is dependent on the number of cells and the specific cell activity.

Alginate bead culture

Foetal spine cells and adult NP cells at passage 3 were routinely cultured in growth medium in monolayer. Cells were trypsinized and suspended in 1.2% alginate solution (Fluka, Switzerland) at the density of 5 million cells per millilitre. Cell suspension was gently expressed in a dropwise fashion through a 26 G needle into 102 mM chloride calcium solution. Beads were left to polymerize for 15 min., washed three times in growth medium, then cultured in growth medium which had 50 μg/ml of ascorbic acid at 37°C under 5% O_2. Medium was changed twice a week.

Histology of foetal spine tissue and alginate beads

When possible, parts of the foetal spine tissue biopsy (12–16 weeks) were flash frozen in liquid nitrogen. Then, 7-μm-thick sections were cut and used for histology and immunohistochemistry. Paraffin sections of older foetal spine tissues (19–22 weeks) were provided by the University Institute of Pathology of Lausanne as numbered and coded tissues. Alginate beads were fixed in 0.1 M cacodylate buffer, 10 mM calcium chloride containing 4% paraformaldehyde overnight at 4°C and then washed in 0.1 M cacodylate, barium chloride at 4°C overnight. Then beads were dehydrated by successive incubation in 70%, 94%, 100% ethanol and xylol. Dehydrated beads were embedded in paraffin and 7-μm sections were cut. Frozen and paraffin sections were stained with alcian blue pH = 2.5 for 20 min. and nuclear fast red for 5 min. or with Von Kossa. Sections were then dehydrated and mounted for light transmission microscopy.

Immunohistochemistry of foetal spine tissue and alginate beads

For aggrecan staining, sections were digested with hyaluronidase (Sigma, St. Louis, MO, USA) and with pepsin (Sigma) for type II and type I collagen

Table 2 Sequences of the primers used in this study. An additional sequence of 18 bp was added to the 5′ end of forward primer to be used with the Amplifluor Uniprimer system

Gene	Forward primer	Reverse primer
GAPDH	CCACCCATGGCAAATTCC	TGGGATTTCCATTGATGACAAG
Aggrecan	AGTATCATCAGTCCCAGAATCTAGCA	AATGCAGAGGTGGTTTCACTCA
Type II collagen	GGGCAATAGCAGGTTCACGTA	CGATAACAGTCTTGC CCCACTT
Type I collagen	CTCCTCAAGGGCTCCAACG	CATCGACAGTGACGCTGTAGGT
Type X collagen	CAGATTTGAGCTATCAGACCAACAA	AAATTCAAGAGAGGCTTCACATACG
SOX-9	AGACCTTTGGGCTGCCTTAT	TAGCCTCCCTCACTCCAAGA
	(source: http://medgen.ugent.be/rtprimerdb)	(source: http://medgen.ugent.be/rtprimerdb)
RUNX-2	AACCCACGAATGCACTATCCA	CGGACATACCGAGGGACATG

staining. After washing, sections were treated with 0.1% phenylhydrazine (Merck, Switzerland), washed and blocked in PBS containing triton X-100, foetal calf serum and normal goat serum. Sections were then incubated with primary antibodies, aggrecan (1/2000) (Biosource, Nivelles, Belgium), type I collagen (5 μg/ml) (MP Biomedicals, Solon, OH, USA), type II collagen (1 μg/ml) (Chemicon, Temecula, CA, Switzerland) and type X collagen (1/250) (Sigma) washed and incubated with goat anti-mouse biotinylated secondary antibody (Vector Laboratories, Burlingame, CA, USA). After washing, sections were treated with Vectastain ABC kit and DAB revelation system (Victor Laboratories). Tissue sections and alginate bead sections were, respectively, counterstained with Papanicolaou and Fast Red, dehydrated and mounted for microscopic observation.

Glycosaminoglycan (GAG) and DNA analysis

After 28 days of culture in alginate beads in growth medium with 50 μg/ml of ascorbic acid, foetal spine cells and adult NP cells were analysed for GAG and DNA content.

Ten beads were digested in 2.3 U/ml of papain in 100 mM Na_2HPO_4, 10 mM ethylenediaminetetraacetic acid, 10 mM cysteine HCl at pH to 6.5 at 60°C for 14 hrs. The digest solution was assayed for GAG and DNA quantification. GAG content (μg/ml) was measured following the method of Enobakhare *et al.* [30]. Two hundred microlitres of DMMB solution at pH = 1.5 was mixed to 20 μl of digested beads. Absorbance was read at 595 nm and GAG concentrations were calculated by using a standard curve of chondroitin sulphate C (Sigma). DNA concentration (μg/ml) of digested beads was measured using Hoechst 33258 and calf thymus DNA as a standard. Then GAG/DNA ratio was calculated by normalizing the GAG concentration to DNA concentration for each sample.

RNA extraction and reverse transcription

Beads were dissolved in 55 mM sodium citrate and 30 mM ethylenediaminetetraacetic acid for 10 min. at 37°C. Cells were centrifuged and the cell pellet recuperated. RNA was extracted from the cell pellet using the Trizol method. RNA quantity and quality was assessed with Agilent Bioanalyser 2100 (Agilent Technologies, Waldbronn, Germany). One microgram of RNA was reverse transcribed using the Taqman Universal PCR kit with random hexamers (Applied Biosystems, Branchburg, NJ, USA) following manufacturer instructions. Primers for Glyceraldehyde 3-Phosphate dehydrogenase (GAPDH), SOX-9, RUNX-2, aggrecan, type I, type II and type X collagens genes were designed using the PrimerExpress software. Primer sequences are presented in Table 2. PCR reactions were performed with the UniprimerR technology (Intergen Discovery Products, Purchase, NY, USA) performed in a total volume of 25 μl (5 μl of first strand DNA, 12.5 μl of TaqMan Universal PCR Master Mix (Applied Biosystems), and 7.5 μl of the primers working solution with the ABI Prism 7700 (Applied Biosystems). Primers working solution included forward primer with an additional sequence of 18 bp added to the 5′ end, reverse primer and Amplifluor Uniprimer (Intergen Discovery Products). Thermal cycle conditions were 50°C 2 min., 95°C 10 min., then 50 cycles at 95°C 15 sec., 60°C 1 min. Measurements were performed in duplicates for each sample and mean of the Cts was used for calculation. For each sample, target and housekeeping gene expressions were calculated using the 2^{-Ct} formula with GAPDH as housekeeping gene. Then the target gene expression normalized to the housekeeping gene expression was calculated for each sample by using the $2^{-\Delta Ct}$ method.

Results

Histology and immunohistochemistry of foetal spine tissue

Alcian blue sections of foetal spine showed that IVD were already differentiated tissue where the gelly NP and fibrous annulus fibrosus (AF) were already present in the 15-week tissue donor (Fig. 1A). The NP contained notochordal cells in clusters with a polysaccharide-rich ECM (Fig. 1B). Fibres with fibroblastic cells were present in the outer AF (Fig. 1D) whereas the inner AF was more a hyaline structure with chondrocytic cells (Fig. 1C). Primary ossification

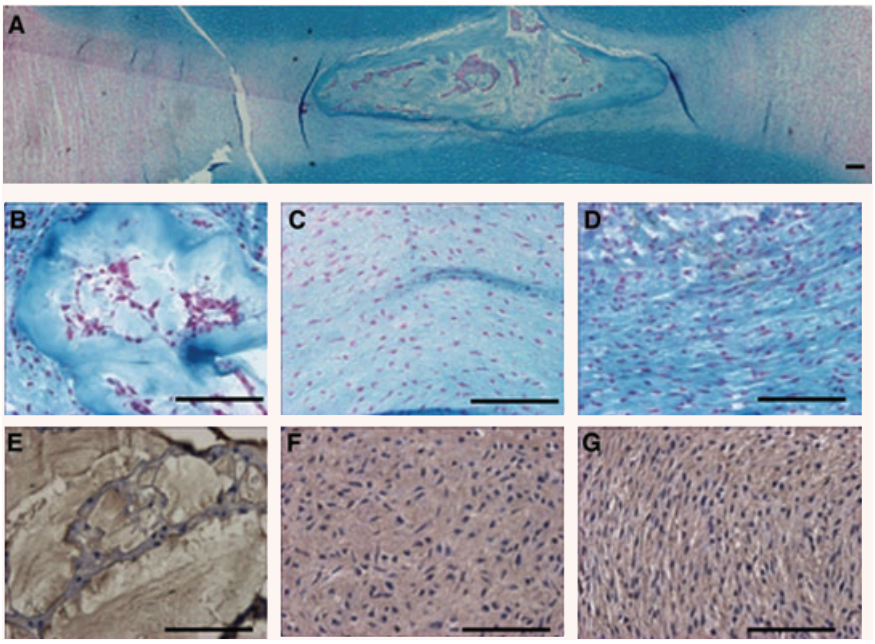

Fig. 1 Alcian blue staining (**A**, **B**, **C**, **D**) and aggrecan (**E**, **F**, **G**), immunostainig of a 15-week intervertebral disc (**A**), nucleus pulposus (**B**, **E**), inner annulus fibrosus (AF) (**C**, **F**) and outer annulus fibrosus (**D**, **G**). Scale = 100 μm.

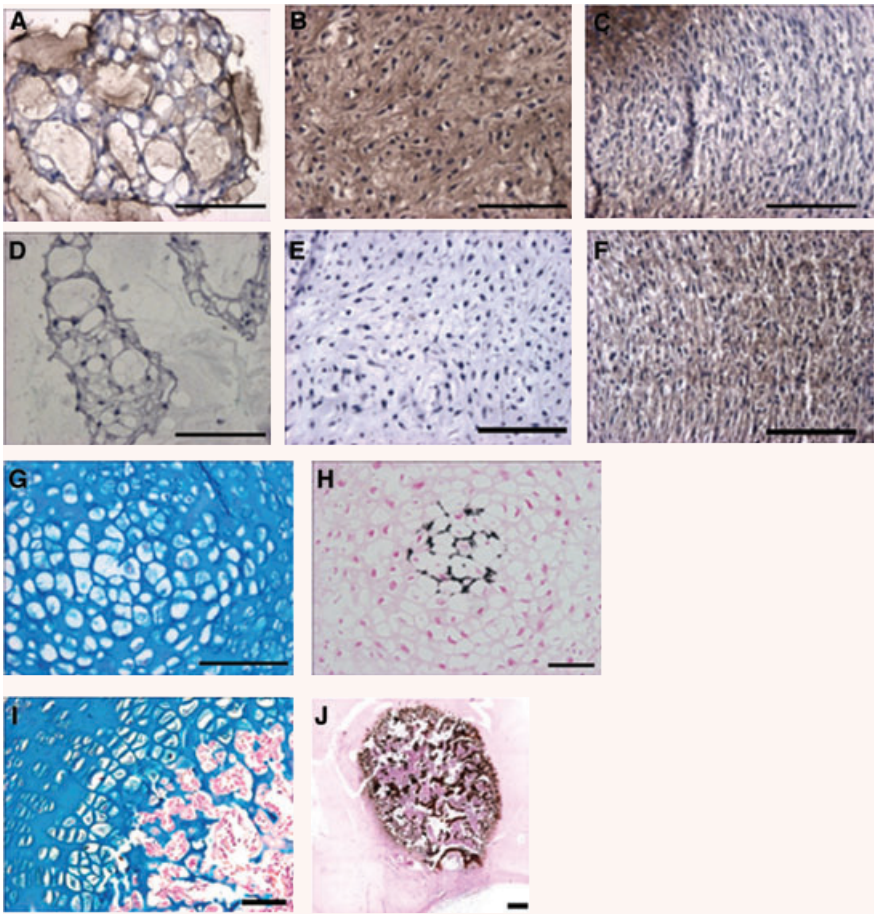

Fig. 2 Type II collagen (**A**, **B**, **C**) and type I collagen (**D**, **E**, **F**) immunostainig of a 15-week intervertebral disc (**A**), nucleus pulposus (**A**, **D**), inner annulus fibrosus (AF) (**B**, **E**) and outer annulus fibrosus (**C**, **F**). Alcian blue (**G**, **I**) and Von Kossa (**H**, **J**) staining of the ossification centre of a 12-week old (**G**, **H**) and 20-week old (**I**, **J**) foetal vertebrae. Scale = 100 μm.

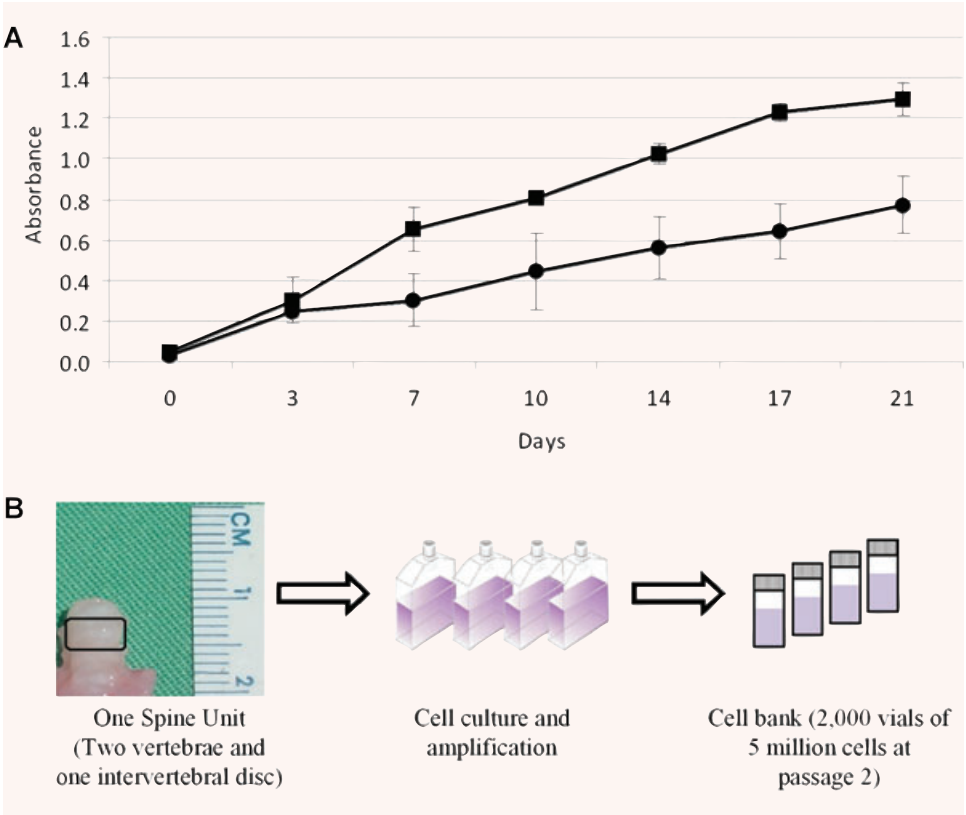

Fig. 3 (**A**) Proliferation curve of foetal spine cells and adult nucleus pulposus (NP) in monolayer at passage 4 during 21 days. Mean ± 1S.D. of five foetal spine cells (■) and three adult NP cells (●). (**B**) Establishment of a foetal spine cell bank from one foetal spine tissue donation. Cells were isolated from spinal unit (one intervertebral disc and two vertebrae) and amplified in monolayer.

centre of body vertebrae could be observed in the five samples but with variable degree of maturation (Fig. 2G–J). In the youngest sample (12 weeks), hypertrophic chondrocytes could be observed but without any blood invasion. However a small area of calcification can be identified by Von Kossa staining (Fig. 2G and H). In older tissues, calcification zone with blood marrow invasion could be observed (Fig. 2I and J). Aggrecan was present in the NP as well as in the inner and outer AF (Fig. 1E–G). Type II collagen was also present in the NP and inner AF (Fig. 2A and B). In contrast, type I collagen was present in the outer annulus but gradually disappeared towards the NP (Fig. 2D–F).

Cell proliferation

Foetal and adult cells were cultured in monolayer in growth medium at an initial density of 3000 cell/cm^2. From the third day of culture, foetal spine cells started to proliferate more rapidly than adult NP cells (Fig. 3A). From each foetal tissue donation, a cell bank of foetal spine cells at passage 2 was established and was composed of around 2000 vials of 5 million cells (Fig. 3B). For comparison, average yield of adult NP cells ranged from 5 to 10 million cells at passage 0. By monolayer expansion of cell bank of 200 to 400 million cells at passage 2 can be established for each adult donor.

Histology of foetal and adult cells in alginate beads

Foetal cells encapsulated in alginate were present as single cells or in clusters. Clusters of cells were only observed in the FS 14w, FS 15w and FS 16w (Fig. 4A). Production of sulphated glycosaminoglycan (sGAG), stained in intense blue by alcian blue, was observed around clusters of cells as well as isolated cells. However, some cells were not surrounded by alcian blue stained matrix.

Gene expression and immunohistochemistry of foetal cells in alginate beads

Foetal cells were culture for 28 days in alginate beads at 5 million cell/ml in growth media supplemented with ascorbic acid. Cells were collected at 7, 14 and 28 days of culture to assess gene expression of the transcription factors SOX-9, RUNX-2 (Fig. 5) and matrix component aggrecan, type II, type I and type X collagens (Fig. 6). Individual values are presented as large donor to donor variations were observed.

Cells from two donors (FS 14w and FS 15w) showed a large increase in SOX-9 expression and a decrease in RUNX-2 expression. One donor's cells (FS 16w) showed an inverse pattern with

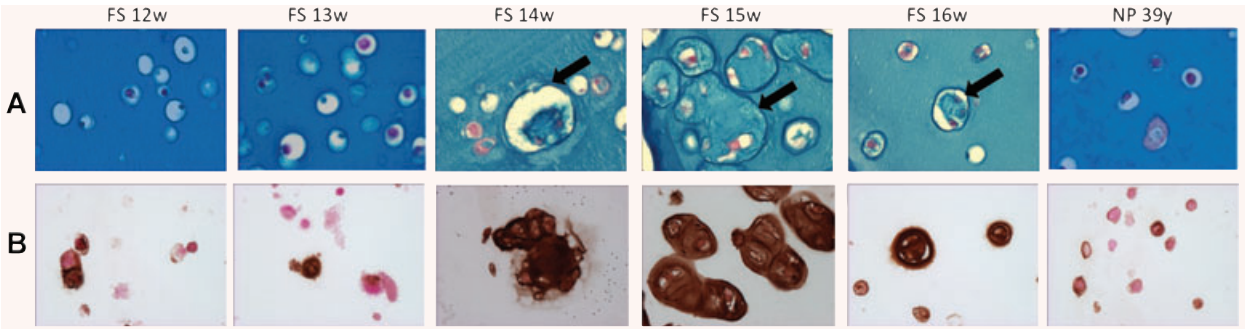

Fig. 4 (**A**) Alcian blue staining and (**B**) aggrecan immunostaining of foetal spine cells and adult nucleus pulposus cells after 28 days of culture in alginate beads. Arrows point to alcian blue stained matrix.

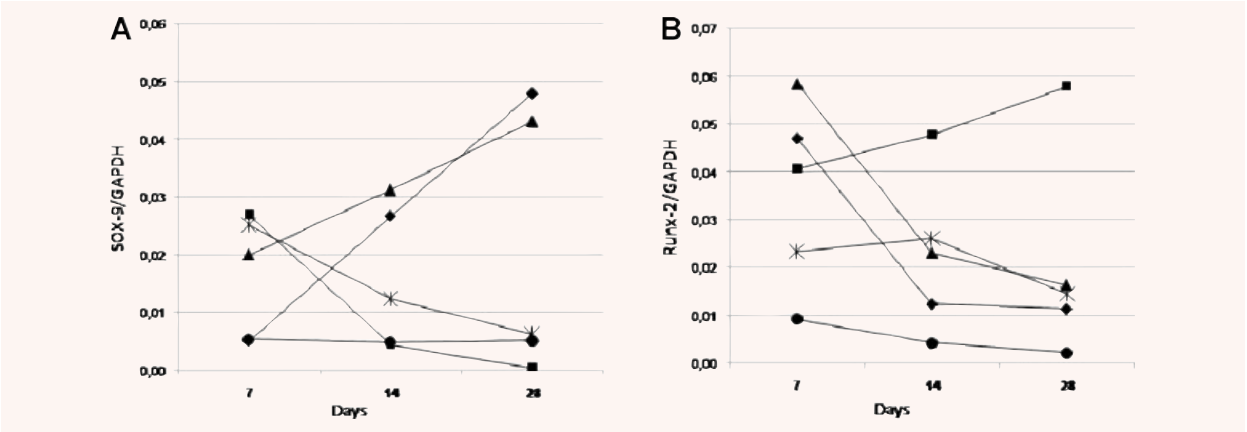

Fig. 5 (**A**) SOX-9 and (**B**) RUNX-2 gene expression was measured by real-time PCR at 7, 14 and 28 days of culture in alginate beads. Target gene expressions were normalized to housekeeping gene GAPDH by using the $2^{-\Delta Ct}$ method.

a decrease in SOX-9 and an increase in RUNX-2 expression. Whereas another donor's cells (FS 12w) showed a decrease in both transcription factors and the last donor's cells (FS 13w) showed a stable SOX-9 expression accompanied by a decrease in RUNX-2. Aggrecan gene expression was stable in four cell donors and was increased in only one sample (FS 14w) (Fig. 6A). However, aggrecan protein was detected in all the five cell donors but with high variation between donors (Fig. 4B). Type II collagen expression was largely increased between 14 and 28 days of culture in all the five cell donors (Fig. 6B) but staining was heterogeneous between donors (Fig. 7A). High staining was observed in the two cell donors with the highest increase in gene expression (FS 14w, FS 15w). These cells also showed a heterogeneous pattern for type II collagen expression. Some cells did not express type II collagen at all whereas neighbouring clusters or isolated cells were highly positive. Time course of type I collagen gene expression was variable between donors (Fig. 6C). It moderately increased in two cell donors (sixfold for FS 15w, twofold for FS 16w) and low level of the protein was detected in the matrix (Fig. 7B). Type I collagen remained stable in the 12w, 13w and 14w donors where very low protein stain could be observed. When measuring the ratio of type II to type I gene expression, it increased during the culture period for the five cell donors (Fig. 6E). Type X collagen gene expression decreased during the culture period in four cell donors and remained stable in the older donor cells (FS 16w) (Fig. 6D). In these cells, type X collagen was strongly detected (Fig. 7C). In the case of adult NP cells, staining of aggrecan, type II, I and X collagen was very low to absent (Figs. 4 and 7).

GAG and DNA content

To measure the quantity of sGAG produced by the cells, GAG was quantified by DMMB and normalized to DNA content of the beads. Adult cells entrapped in alginate beads tended to show a slightly higher DNA content than foetal cells (Fig. 8). Two foetal cell donors (FS 12w and FS 16w) tended to have a smaller DNA content than the three other foetal cell donors. Two foetal cell donors

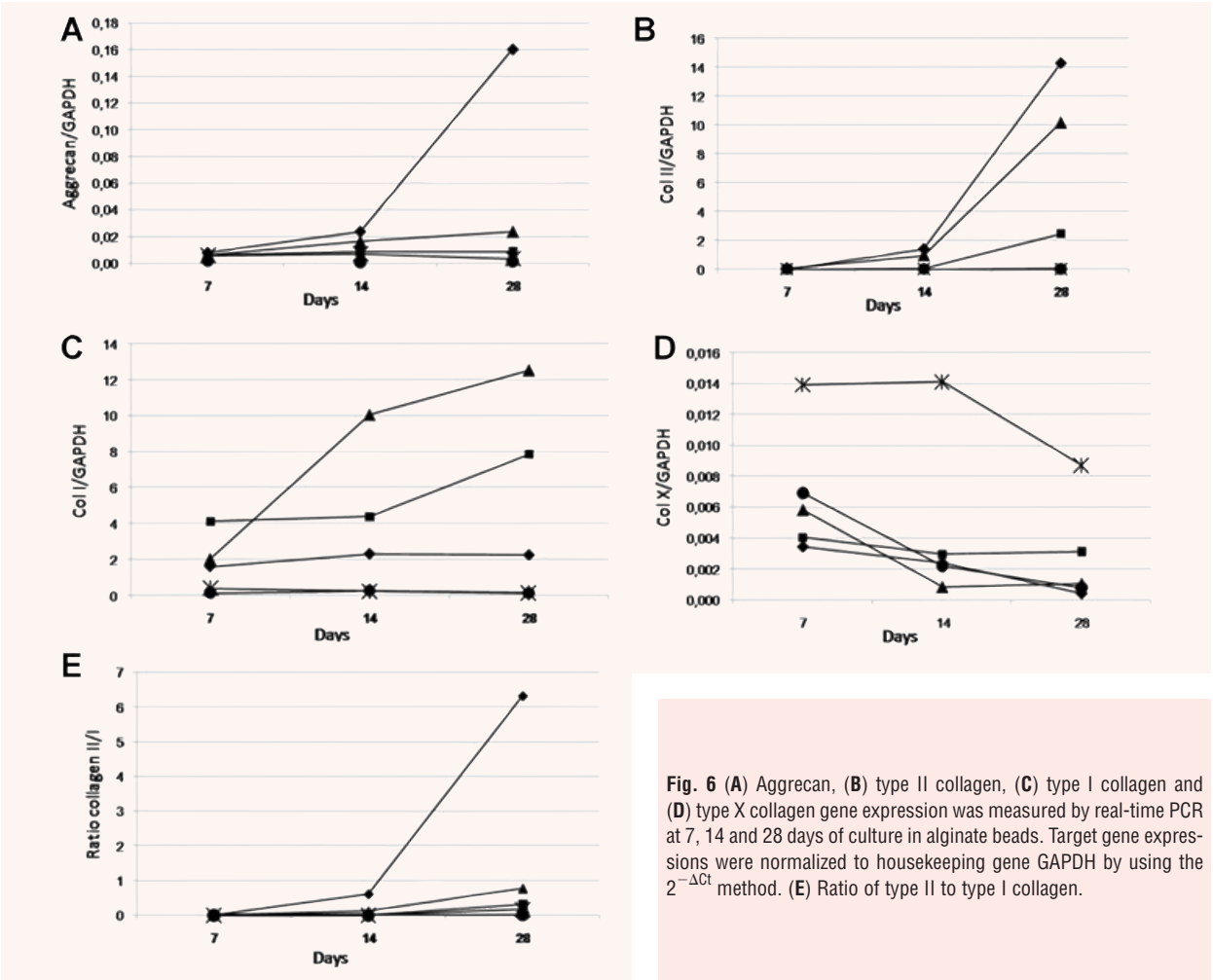

Fig. 6 (**A**) Aggrecan, (**B**) type II collagen, (**C**) type I collagen and (**D**) type X collagen gene expression was measured by real-time PCR at 7, 14 and 28 days of culture in alginate beads. Target gene expressions were normalized to housekeeping gene GAPDH by using the $2^{-\Delta Ct}$ method. (**E**) Ratio of type II to type I collagen.

(FS 14w and FS 15w) showed 3.6 times higher GAG content than adult NP cells. The three remaining foetal cell donors had a GAG content similar to the one of the adult cells. Results were similar when the GAG content was normalized to the DNA content. In addition, GAG/DNA ratio correlated well with the intensity of the alcian blue stain. The two samples showing an intense stain, FS 14w and FS 15w gave the highest ratio of GAG to DNA.

Discussion

This study presents the spontaneous matrix production of human foetal spine cells in alginate beads. The hypothesis of cell regeneration of the NP has strengthened with publications of NP regeneration by autologous, allogenous or stem cells transplantation in animal models [8, 11, 31–33]. One of the main criteria for its translation into the clinic is the choice of the cells. According to previous work performed with foetal skin and foetal bone cells, we speculate that foetal IVD could also be a good source of cells for NP regeneration. As foetal tissue donation after voluntary interruption of pregnancy is considered as an organ donation, it follows ethical rules applied to organ donation. Benefits of foetal cell therapy reside in the fact that it is possible to develop extensive cell banks using only one foetal organ donation. We first investigated the histology of foetal spine tissue from which foetal cells were isolated. IVD structures, *i.e.* NP, inner AF, outer AF could be histologically identified. Aggrecan was present in the whole IVD. Type II collagen was present in NP and inner AF whereas type I collagen was only present in the outer AF. These results are in agreement with previous studies on the matrix composition of foetal spine [34–37] and are similar to that of adult IVD. One main difference is the presence of notochordal cells as the only cells present in the foetal NP. In a first attempt, we tried to establish separate cultures of pure NP and AF cells. Due to the small size (1 mm thickness) and the very soft consistency of the foetal spine,

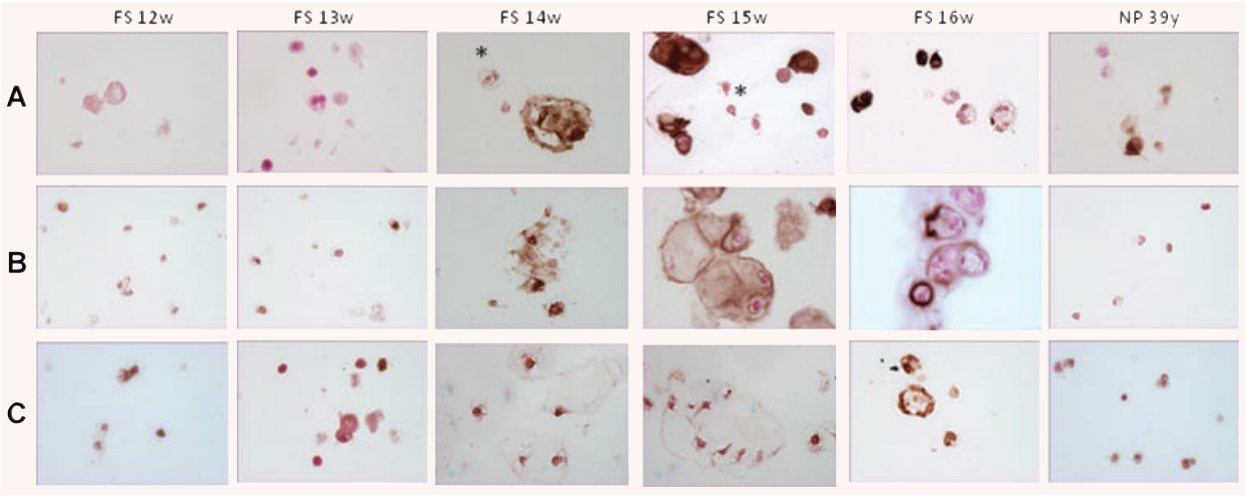

Fig. 7 (**A**) type II collagen, (**B**) type I collagen and (**C**) type X collagen immunostaining of foetal spine cells and adult nucleus pulposus cells after 28 days of culture in alginate beads. Asterisk indicates non-stained cells.

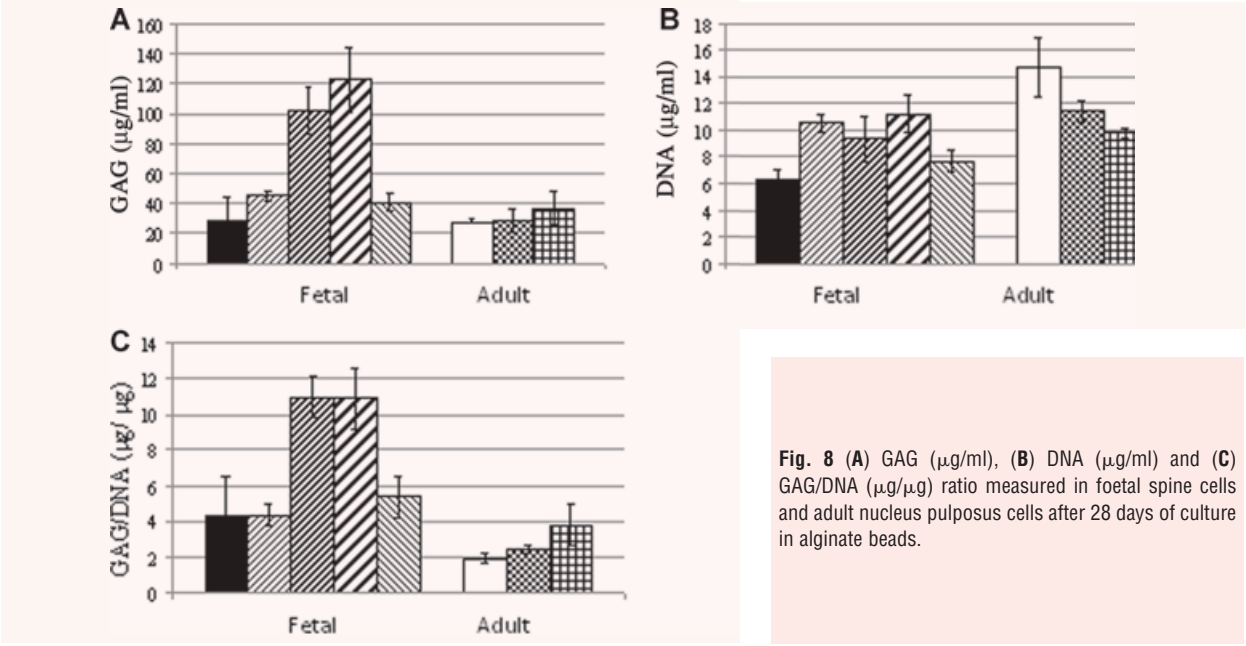

Fig. 8 (**A**) GAG (µg/ml), (**B**) DNA (µg/ml) and (**C**) GAG/DNA (µg/µg) ratio measured in foetal spine cells and adult nucleus pulposus cells after 28 days of culture in alginate beads.

it was not feasible to isolate IVD from the adjacent vertebrae. In addition, there is no specific marker available to assess the purity of the cell population obtained. Hence, we have chosen to establish cell culture from whole spine units (one IVD and the two adjacent vertebrae). Notochordal cells were observed in the culture media but they did not proliferate neither were adherent. As a result, our foetal spine cell cultures are mixed cultures of cells coming from disc annulus and vertebrae. As consistency of cell cultures to be used for tissue-engineering project is of primary importance, stability of the foetal spine cell phenotype was checked at passages 4, 6 and 8 by microscopic observation of cell morphology and by FACS analysis of HIF-1 and galectin-3 expression (data not shown) [29]. From the histological analysis, foetal vertebrae between 12 and 16 weeks were still cartilaginous tissue but ossification centres could be identified. In the 12-week spine, ossification centre could be observed but with very small area of calcified region, whereas in the 15-week foetal spine, bone formation and calcification could be observed. Further investigation

performed on older foetal spine (19–22 weeks of gestation) showed presence of calcified area in the vertebrae, which were not observed in younger samples. A recent study performed with MRI on foetal lumbar spine showed that the lumbar vertebral area of ossification appears around the age of 14–15 weeks [38], which confirms our histological observations. These data indicate that our cell populations may contain hypertrophic chondrocytes, raising the risk of endochondral bone formation.

In vitro assessment of matrix production by chondrocyte cells requires a three-dimension system such as alginate beads. Alginate has been shown to be an appropriate matrix to support chondrogenesis of articular cartilage [39–41], IVD [42, 43] and stem cells [44–46]. Alginate beads also support endochondral [47, 48] and osteogenic [49, 50] differentiation. Mechanisms of chondrogenic differentiation in alginate beads have yet not been elucidated. They do not likely rely on specific alginate-cell interaction as alginate does not promote protein adsorption [51] but more likely on cell cytoskeleton reorganization [52]. So we assumed that alginate bead culture of foetal spine cells would reflect the spontaneous activity of the cells in a three-dimensional system. Only ascorbic acid was added to the media as it is necessary for collagen production [53]. Aggrecan could be detected in all the five samples but to a various extent across donors. Intensity of aggrecan staining correlated well with the GAG/DNA ratio, indicating that aggrecan is one of the main sulphated PG produced by these cells. In addition, the GAG production of the higher producing foetal cells was much higher than that of adult NP cells cultured in the same conditions. As the DNA content was fairly similar in all samples, the GAG production is likely due to a high cellular activity rather than to a higher number of cells. Difference in donors was even more marked in type II collagen production. Age of the donors may explain this large variation. However, due to the low number of samples, it cannot be excluded that other factors, such as foetal tissue quality and cell isolation procedure play a role in sample variation.

In the same way, cells from a same donor were also heterogeneous for collagen type II staining, likely explained by the mix populations of cells in our foetal spine cells. It can be speculated that cells which do not produce type II collagen are derived from the outer AF, which is poor in type II collagen. It was not possible to assess the origin of the cells due to the lack of specific marker. We also investigated type I collagen as its expression is a usual result of chondrocyte dedifferentiation and the ratio of collagen II to I can be used as a marker of differentiation [54]. This ratio increased for all donors during culture in alginate beads indicating that cells were differentiating towards a chondrocyte phenotype. Confirmation of these results by increase in SOX-9 gene expression was only observed for two donors (FS 14w and FS 15w). As previously mentioned, the presence of vertebrae cells raises the risk of endochondral ossification. Thus RUNX-2 gene expression and type X collagen production were measured during the alginate culture [55]. Endochondral markers were very lowly expressed by four cell donors. Only the older donor (FS 16w) showed type X collagen protein production. This result could be explained by a more advanced stage of mineralization of the vertebrae leading to the majority of chondrocytes already directed in the hypertrophy pathway. Confirmation of the correlation between age of the foetal tissue donor and endochondral ossification require a larger number of donors. Nevertheless, these results indicate cells isolated from foetal spine can have spontaneous cartilage matrix synthesis properties. In this study, adult NP cells showed very low matrix synthesis capacity. Culture conditions of NP adult cells were not optimized as addition of growth factors such as BMP-2, BMP-12, OP-1, TGF-βs can partially restore matrix production after monolayer expansion [56–59]. However, the aim of the study was to observe spontaneous behaviour of cells as avoiding the use of growth factors for cell therapy could increase biosafety and could reduce cost of the final product. The use of animal products (bovine serum, trypsin) in cell culture to be used for clinical trials can hardly be avoided and represents a risk of contamination of the final product. Any animal products used should be virus tested and transmissible spongiform encephalopathies certified [60]. Countries with no reported cases of bovine spongiform encephalopathies, like New Zealand or Australia, are interesting source of serum for research and clinical purposes [61]. Otherwise, a traceability of each serum bottle to be used for cell culture for clinical usage is required. Up to now, the most attractive source of cells for NP regeneration is stem cells. Chondrogenic differentiation of stem cell requires the use of growth factors such as TGF-βs, which induce type X collagen production and drive chondrocyte hypertrophy [45, 48, 64–68]. In addition, stem cell cultures require the use of growth factors in order to maintain their stem cell property. For a clinical application, the use of growth factors is a potential risk for the final product biosafety and increases the cost for establishing a cell bank. If differentiated cells have to be used for cell treatment of the NP, this problem could be alleviated by the use of foetal spine cells, which matrix production does not require the use of growth factors and which escape from hypertrophy. If non-differentiated cells are to be used, the fate of these cells in the degenerated disc will depend on many uncontrolled environmental factors such as nutrition, mechanical loading, inflammation and interaction with endogenous cells. These factors will also have high impact on the long term fate of pre-differentiated cells. In both cases, the success of NP regeneration relies on the adaptation of the cells to such a complex environment, and this can only be assessed by *in vivo* trials.

In conclusion, we have shown that after a careful selection of the right donor, a foetal spine cell bank can be established, offering security and reproducibility for a clinical cell therapy. These cells showed high levels of *in vitro* matrix synthesis properties compared to adult NP cells. The next step of the cell characterization will be to test the *in vivo* efficacy of these cells in an appropriate animal model.

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