

Influence of collagen type II and nucleus pulposus cells on aggregation and differentiation of adipose tissue-derived stem cells

**Z. F. Lu^{a, d}, B. Zandieh Doulabi^{a, d}, P. I. Wuisman^{a, d}, R. A. Bank^{a, b, c, d},
M. N. Helder^{a, d}***

^a*Department of Orthopaedic Surgery, VU University Medical Center, Amsterdam, The Netherlands*

^b*Department of Oral Cell Biology, Academic Center for Dentistry, Amsterdam, The Netherlands*

^c*Division of BioSciences, TNO Quality of Life, Leiden, The Netherlands*

^d*Skeletal Tissue Engineering Group Amsterdam, Amsterdam, The Netherlands*

Received: October 24, 2007; Accepted: February 5, 2008

Abstract

Tissue microenvironment plays a critical role in guiding local stem cell differentiation. Within the intervertebral disc, collagen type II and nucleus pulposus (NP) cells are two major components. This study aimed to investigate how collagen type II and NP cells affect adipose tissue-derived stem cells (ASCs) in a 3D environment. ASCs were cultured in collagen type I or type II hydrogels alone, or co-cultured in transwells with micromass NP cells for 4 and 14 days. ASCs seeded in collagen type II gels acquired dentritic cell shapes, and orchestrated cell density-dependent gel contraction rates. Up-regulation of collagen type X, but not of other chondrogenic markers was observed at day 4, irrespective of the hydrogel type. Strikingly, in co-cultures with NP cells, more pronounced differentiation of ASCs along the cartilaginous lineage was observed (up-regulation of collagen IIA, IIB and aggrecan gene expression, as well as stronger alcian blue staining), when ASCs were embedded in collagen type II in comparison with type I hydrogels. Interestingly, strong cellular condensations/aggregations were observed in ASC-seeded type II, but not type I gels, and this aggregation was markedly delayed when the same gels were co-cultured with NP cells. The NP cell-mediated inhibition of ASC aggregation in collagen type II gels coincided with down-regulation of integrin subunit α_2 gene expression. We conclude that soluble factors released by NP cells can direct chondrogenic differentiation of ASCs in collagen hydrogels, and that combination with a nucleus-mimicking collagen type II microenvironment enhances differentiation towards a more pronounced cartilage/NP lineage relative to collagen type I hydrogels.

Keywords: adipose tissue-derived stem cells • microenvironment • nucleus pulposus cells
• collagen type II • chondrogenic differentiation • cell aggregation • disc regeneration

Introduction

Degenerated intervertebral disc disease (DDD) is thought to be one of the most prevalent causes for chronic low back pain, that currently has no identified cause [1, 2]. The traditional view during much of the last century was that DDD was primarily due to physical (over)loading as well as changes associated with the

normal aging process. In recent years, however, a dramatic advance has been made in our understanding of risk factors such as age, gender, genetics, environmental, chemical (smoking) and biomechanical influences for disc degeneration, thus changing our traditional views [3–6]. These advances allow exploration of new promising treatment modalities for the treatment of DDD, including cell-based therapies aiming to regenerate disc tissue and to restore the biological function of a disc rather than only alleviate the symptoms.

There are two distinct but interdependent cell populations in the intervertebral disc: nucleus pulposus (NP) cells in the inner region [7] having chondrocyte-like properties, and annulus

*Correspondence to: M. N. HELDER,
MOVE/Skeletal Tissue Engineering Group Amsterdam, VU
University Medical Center, Department of Orthopaedic Surgery,
P.O. Box 7057, 1007 MB Amsterdam, The Netherlands.
Tel.: +31-20-4445024; Fax: +31-20-4448683
E-mail: m.helder@vumc.nl

fibrous (AF) cells in the outer layer having a more fibrocyte-like phenotype. During the process of DDD, AF and NP cells react differently to the tissue degeneration. The NP is more vulnerable to degeneration, and the first pathological changes therefore start in the NP, with cellular loss and proteoglycan breakdown leading to diminished water-binding capacity and loss of disc height [8, 9].

So far, different concepts are under pre-clinical and clinical evaluation for the regeneration of NP tissue. For instance, re-implantation of NP cells was evaluated *in vitro* and *in vivo* for halting or retarding the process of DDD [10–13]. However, there are some potential drawbacks of using NP cells, for example degeneration of the adjacent discs due to NP cells harvesting and difficulties in obtaining enough NP cells for transplantation. By contrast, adult mesenchymal stem cells (MSCs) can overcome these drawbacks: they can be harvested from many sites, such as bone marrow, fat, muscle, skin without significant donor site morbidity [14]; MSCs can also be expanded *in vitro* to sufficient numbers for transplantation purposes [15].

Adipose tissue-derived mesenchymal stem cells (ASCs) have been identified as a new stem cell source having multilineage differentiation potential [16]. ASCs are particularly promising for clinical application due to their unique combination of minimally invasive procurement procedures and high stem cell yields [17, 18]. Together, these aspects allow one-step surgical procedures, in which ASCs are harvested, either or not triggered towards the desired differentiation lineage, and re-implanted at the site of interest within a time span of 2–3 hrs [19]. Although this procedure was described for spinal interbody fusion, we hypothesize that it might also be applicable for regeneration of degenerated discs by intradiscal injection of ASCs, particularly in early stages of disc degeneration.

It has been shown that tissue microenvironments play a critical role in guiding the differentiation of stem cells in normal regenerative processes [20, 21]. The NP is such an environment, characterized by a low density of NP cells and a highly abundant extracellular matrix (ECM), which contains a large amount of proteoglycans and predominantly collagen type II fibrils. We hypothesize that due to this high matrix-to-cell ratio, most of the ASCs will encounter matrix contacts and only indirect contact with NP cells (*via* soluble factors).

In a previous study, we demonstrated that NP cells, when cultured in a micromass configuration, can indeed induce a NP phenotype *in vitro* in micromass ASCs, and that they do so by soluble mediators [22]. The current study aims to more closely mimic the microenvironment the ASCs will encounter after intradiscal injection, by providing a true 3D nucleus-like environment through embedding in a collagen type II hydrogel. To assess cartilaginous matrix-mediated effects, differentiation-related gene expression profiles as well as histochemical and morphological parameters were compared with those of ASCs embedded in collagen type I gels. The results obtained in this study provide insight in the relative influence of the components separately (matrix or NP cells), and their possible interaction on the NP phenotype induction in ASCs.

Materials and methods

ASCs and NP cells isolation and culture

The human ethical committee of the Free University medical centre approved the retrieval of human specimens, and informed consent was obtained.

ASCs were isolated from subcutaneous adipose tissue that was harvested from patients undergoing elective surgical procedures, as described previously [22]. Briefly, adipose tissue was cut into small pieces of about 25–50 mm³, washed with phosphate-buffered saline (PBS) to remove red blood cells and digested for 60 min. at 37°C with 0.5 U/ml Liberase Blendzyme 3 (Roche Diagnostics, Almere, The Netherlands) in PBS to dissociate the cells from their ECM. The digested material was passed over a 100- μ m-mesh filter to remove tissue debris, washed several times with PBS and resuspended in ASC culture medium (Dulbecco's modified Eagle's medium [D-MEM, Gibco, Paisley, UK]) supplemented with 500 μ g/ml streptomycin sulphate (Sigma, St. Louis, MO, USA), 600 μ g/ml penicillin (Sigma), 50 μ g/ml gentamycin (Gibco), 2.5 μ g/ml fungizone (Gibco) and 10% foetal bovine serum (FBS, Hyclone, Logan, UT, USA). Cells were plated in 25-cm² culture flasks at 37°C with 5% CO₂-in air in a humidified incubator, cultured for 24–48 hrs, washed to remove unattached cells and subsequently re-fed with fresh medium every 3–4 days. Upon reaching 80–90% confluency, ASCs were harvested with 0.25% trypsin and 0.1% ethylenediaminetetraacetic acid in PBS, replated in 75-cm² culture flasks (Greiner Bio-One, Kremsmuenster, Austria), and cultured until reaching confluency again. All cells used were from passage 3 or earlier.

NP cells were derived from a female scoliosis patient who was 16 years old, and from NP material obtained during posterior lumbar interbody fusion surgeries from two patients (one 42-year-old male, one 49-year-old female). Isolation was performed as described for the ASCs, only the Liberase digestion time was reduced to 30 min., and DMEM-F12 (Gibco) was used instead of DMEM when preparing the culture medium NP cells at passage 1 or 2 were used for our experiments. No apparent differences in NP cell inductive properties were observed.

Preparation of collagen type I/II hydrogels

Collagen type I/II hydrogels were prepared by mixing six parts of either rat tail collagen type I (R&D Systems, Minneapolis, MN, USA) or chicken sternal collagen type II (Sigma), one part of 10 \times DMEM (Gibco), one part of reconstitution buffer (2.2g NaHCO₃ in 100 ml of 0.05 N NaOH and 200 mM HEPES), and two parts of cell suspension. All components were mixed in ice and applied in 100- μ l volumes in wells of a 96-well plate for gel contraction analysis, or as 100- μ l droplets in wells of a 6-well culture plate for co-culture studies. The collagen gel lattices were allowed to form by placing the plates in a humidified incubator at 37°C in an atmosphere of 5% CO₂ for 1 hr. Subsequently, gels were carefully submerged in ASC culture medium. The medium was refreshed every 3 days.

Confocal analysis and measurement of gel contraction

Shape and distribution profile of ASCs in collagen type II gels were determined by confocal microscopy imaging (TCS SP5 Confocal microscope, Leica microsystems, Wetzlar, Germany) after a culture period of 12 hrs. For this purpose, ASCs were labelled according to the manufacturer's

Table 1 The primers used for real-time PCR

Gene		Oligonucleotide sequence	Product size (bp)
18S	Forward	5'-GTA ACC CGT TGA ACC CCA TT-3'	151
	Reverse	5'-CCA TCC AAT CGG TAG TAG CG-3'	
Collagen type IIA	Forward	5'-GGA TGG GCA GAG GTA AAT G-3'	255
	Reverse	5'-GGT CCT TTG GGT CCT ACA A-3'	
Collagen type IIB	Forward	5' AGG GCC AGG ATG TCC GGC A 3'	195
	Reverse	5' GGG TCC CAG GTT CTC CAT CT 3'	
Collagen type X	Forward	5' CAC TAC CCA ACA CCA AGA CA 3'	225
	Reverse	5' CTG GTT TCC CTA CAG CTG AT 3'	
Aggrecan	Forward	5'CAA CTA CCC GGC CAT CC 3'	160
	Reverse	5'GAT GGC TCT GTA ATG GAA CAC 3'	
α_1 integrin	Forward	5' AGG ACA GTG CCT ATA ACA CC 3'	234
	Reverse	5' CGG TGT CAC TTG TTG CAC TT 3'	
α_2 integrin	Forward	5' ACA GAC AAG GCT GGT GAC A 3'	258
	Reverse	5' TGC CGT TAG CTG TAC TGT CT 3'	
α_{10} integrin	Forward	5' TCC GAA CGA AGG AAG AAG TG 3'	193
	Reverse	5' GGA AGC TCC TCT CCA TCA T 3'	

instructions with CellTracker[®] Green CMFDA (Invitrogen, Carlsbad, CA, USA) prior to collagen type II gel encapsulation. Three different densities (0.5×10^6 , 1.5×10^6 and 5.0×10^6 /ml gel) were tested.

Gel contraction was assessed for the same gel-cell combinations in 96-well plates. Contraction was monitored at regular intervals during 14 days by taking digital photographs of the gels with a Nikon Coolpix digital camera (Nikon, Melville, NY, USA), and quantitation of the gel diameters.

Indirect co-culture system

Indirect co-culture of ASCs and NP cells was carried out by growing ASCs in six-well plates and growing NP cells in culture inserts (Becton Dickinson Labware, NJ, USA) having a 0.4- μ m pore-size filter, allowing communication between these two cell types *via* soluble signals only. Micromass culturing was applied for NP cells by seeding one droplet of medium (400,000 cells/50 μ l) on the insert as described previously [22]. Five groups were studied: (i): ASCs cultured in monolayer as control; (ii): ASCs in collagen type I gels; (iii): ASCs in collagen type II gels; (iv): ASCs in collagen type I gels + NP cells and (v): ASCs in collagen type II gels + NP cells. The cell seeding density of ASCs in the gels was 1.5×10^6 /ml gel. Cell distribution was monitored at regular intervals during 14 days by taking digital photographs of the gels with a Nikon Coolpix digital camera (Nikon). After culture for 4, 14 or 21 days, the gel constructs were harvested and processed for either real time-PCR analysis or histochemical analysis.

Real-time PCR analysis

Total RNA was extracted from cultured cells using Trizol (Invitrogen) according to the manufacturer's instructions. Seven hundred and fifty nanograms of total RNA was reverse transcribed in 20 μ l of reverse transcriptase-polymerase chain reaction (RT-PCR) mix (5U transcriptase reverse transcriptase [Roche Diagnostics]), 0.08 A260 units random primers (Roche Diagnostics), 1mM of each of dNTP (Invitrogen), 10U protector Rase inhibitor (Roche Diagnostics) and 1 \times transcriptor RT buffer] at 55°C for 30 min., and then at 85°C for 5 min. to inactivate the transcriptase. Chondrogenic gene expression (aggrecan, collagen type X and collagen type IIA and IIB) were determined by real-time PCR by light cycler (Roche LC 480).

The striking effect of matrix composition and the presence or absence of NP cells on the aggregation of ASCs within the gels prompted us to also determine the expression profiles of particular matrix-interacting integrins. Because the integrins $\alpha_1\beta_1$, $\alpha_2\beta_1$ and $\alpha_{10}\beta_1$ were previously shown to interact with collagen type II matrices, and were hypothesized to participate in cartilage homeostasis [23–25], expression levels of the integrin subunits α_1 , α_2 and α_{10} were quantified to provide some initial clues on their possible involvement in the underlying mechanisms governing these aggregation phenomena. All target genes were normalized to housekeeping gene 18S to obtain the relative gene expression. Primers used in real-time PCR are listed in Table 1.

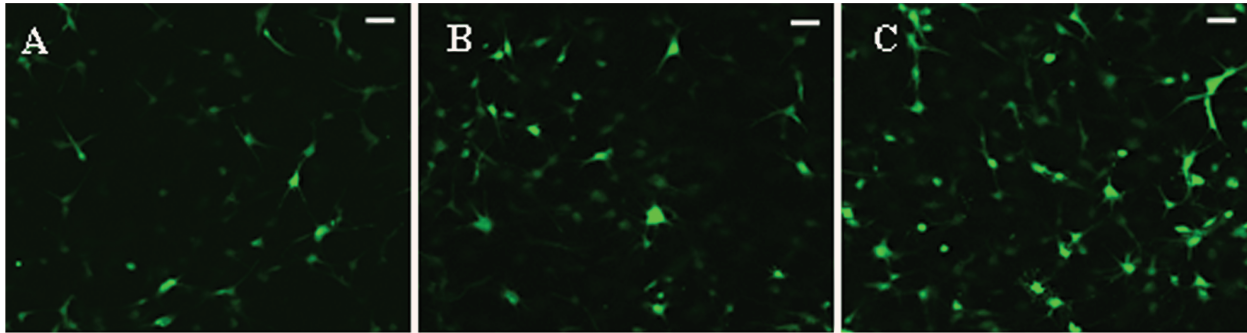


Fig. 1 Distribution and cell shapes of ASCs seeded with different cell density in collagen type II gel. ASCs distribution was analysed after being encapsulated with different cell densities in collagen type II gels for 12 hrs by confocal microscopy. All the cells, independent of seeding density, had attached to collagen type II and evenly distributed in the matrix from top to bottom with dendritic or stellate shapes. **(A)**: 0.5×10^6 cells/ml gel; **(B)**: 1.5×10^6 cells/ml gel; **(C)**: 5×10^6 cells/ml gel. Bar: 50 μ m.

Histology

The collagen gels containing cells were fixed in 10% neutral buffered formalin for 10 min. at room temperature, mounted in Tissue Tek O.C.T. compound, and frozen in liquid nitrogen. Sections of 7 μ m were prepared, which were stained for proteoglycans with Alcian blue-periodic acid Schiff (AB-PAS) staining. The pH of the Alcian blue used for the staining was 2.0.

Statistical analysis

Data were obtained from five independent donors, and one gel per group was analysed in each donor. For statistical analysis, first Levene's test was performed to determine the homogeneity of variance for all the data, and then Turkey or Tamhane's T2 *post-hoc* multiple comparison was performed for the comparisons of collagen type X and aggrecan gene expression among the five groups; a paired t-test or two-related samples nonparametric test was performed to compare integrin subunits α_1, α_2 and α_{10} gene expression among ASCs with and without co-culturing with NP cells. SPSS 11.0 program was employed for all statistical analysis and differences were considered significant if $P < 0.05$.

Results

Distribution and cell shape of ASCs in collagen type II gels

Preliminary experiments were aimed at determining the cell distribution in different concentrations of collagen type II gels. A homogenous distribution of ASCs was found in a range of concentrations of collagen type II gels (1.5–4 mg/ml gel; data not shown). The middle concentration (2.5 mg/ml) of collagen was used in all following experiments.

Next, Celltracker[®] Green labelled ASCs were encapsulated in different densities (0.5×10^6 , 1.5×10^6 and 5×10^6 cells/ml, respectively) in collagen type II gels. It was observed that all cells had already attached to the collagen type II matrix after 12 hrs, and distributed homogeneously in the matrix from top to bottom independent of the cell seeding density. Cells had dendritic or stellate shapes, and connected to each other *via* spindle-shaped extensions as observed under confocal imaging (Fig. 1).

Cell density-dependent contraction of collagen type II gels

Collagen type II gels showed an ASC seeding density dependent contraction rate from day 1 to day 14 ($0.5 \times 10^6 < 1.5 \times 10^6 < 5 \times 10^6$ cells/ml, respectively) (Fig. 2). Collagen type II gels with the highest cell density (5×10^6 cells/ml gel) started to contract as early as day 1, reached their peak at day 3 and stabilized thereafter. In contrast, when cell seeding density were 1.5×10^6 cells/ml gel or 0.5×10^6 cells/ml gel, the starting time of gel contraction delayed to day 3 or later, and reached the lowest level only at about day 14. When no cells were seeded, gel contraction was negligible (Fig. 2).

NP cells direct ASCs in collagen type II gels into a more pronounced cartilage/NP lineage than those in collagen type I gels

The effects of collagen type II on chondrogenic differentiation of ASCs were investigated relative to collagen type I gels. Monolayer-cultured ASCs served as controls.

When ASCs were cultured in monolayer, gene expression of collagen type X and aggrecan did not significantly change

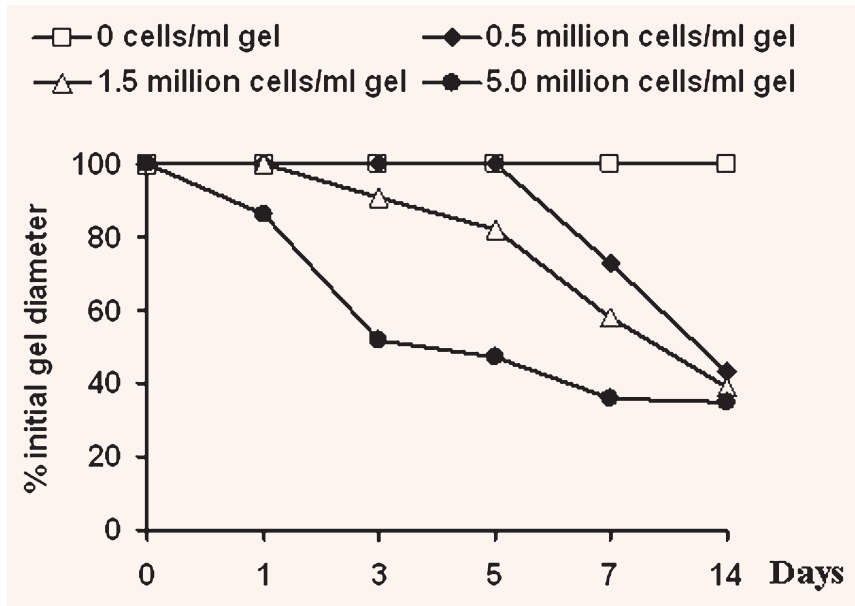


Fig. 2 Contraction of collagen type II gels seeded with different cell densities of ASCs. ASCs were encapsulated with different cell densities in collagen type II gels, and the diameters of the gels were measured from day 1 to day 14. There was a cell density dependent gel contraction in collagen type II gels, whereas gel contraction was not observed in the collagen type II gel without seeding cells.

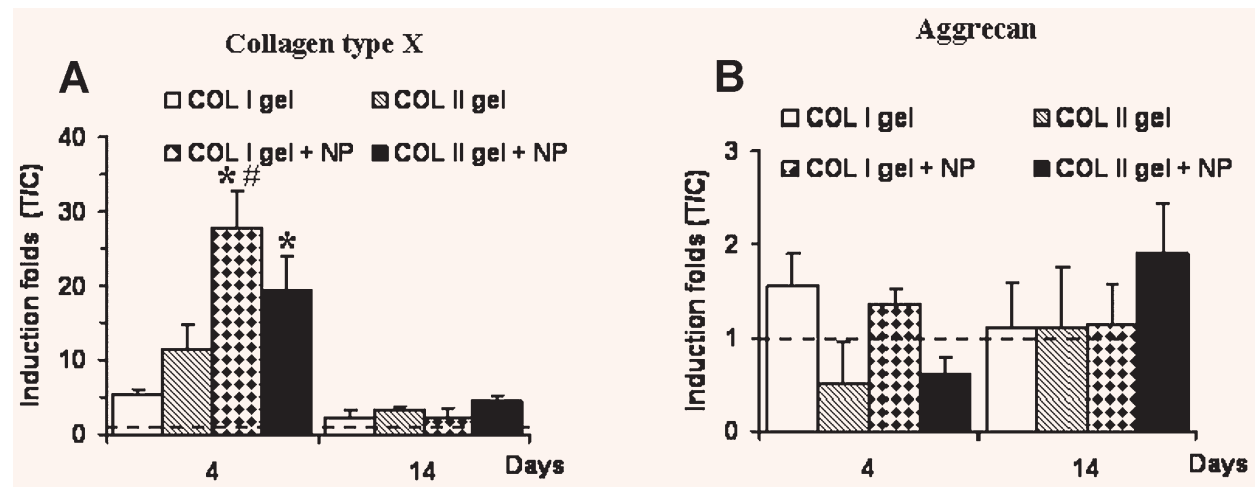


Fig. 3 Effects of collagen type I/II 3D environment and NP cells on collagen type X and aggrecan gene expression of ASCs. ASCs in collagen type I/II gels were cultured alone or co-cultured with NP cells for 4 and 14 days. **(A)**: In comparison to monolayer control, collagen type I and type II gel embedment up-regulated the gene expression of collagen type X up to 5- and 11-fold at day 4, respectively; co-culturing with NP cells resulted in further up-regulation of gene expression of collagen type X in ASCs in both gel types (about 28- and 20-fold higher in collagen type I and type II gel compared to monolayer ASCs, respectively), and significant up-regulation of collagen type X gene expression by NP cells was also found by comparison with ASCs cultured alone in collagen type I gels. **(B)**: There was a highest gene expression of aggrecan in ASCs that were grown in collagen type II gels and co-cultured with NP cells at day 14 (although not significant); T/C: treatment/control, dashed line represents monolayer control. Data are expressed with mean \pm SEM, $n = 5$. Abbreviations: COL I: collagen type I; COL II: collagen type II. *Comparing with monolayer control, $P < 0.05$. #Comparing with ASCs cultured alone in collagen type I gels, $P < 0.05$.

compared to starting time point, and no induction of collagen IIA and IIB gene expression was observed (data not shown). Compared to monolayer controls, embedment of ASCs in collagen type I and type II gels up-regulated gene expression of collagen

type X up to 5- and 11-fold at day 4 (although not significant), respectively, but these values returned to monolayer levels at day 14 (Fig. 3A). No significant changes were observed in aggrecan gene expression, irrespective of which collagen type and configuration

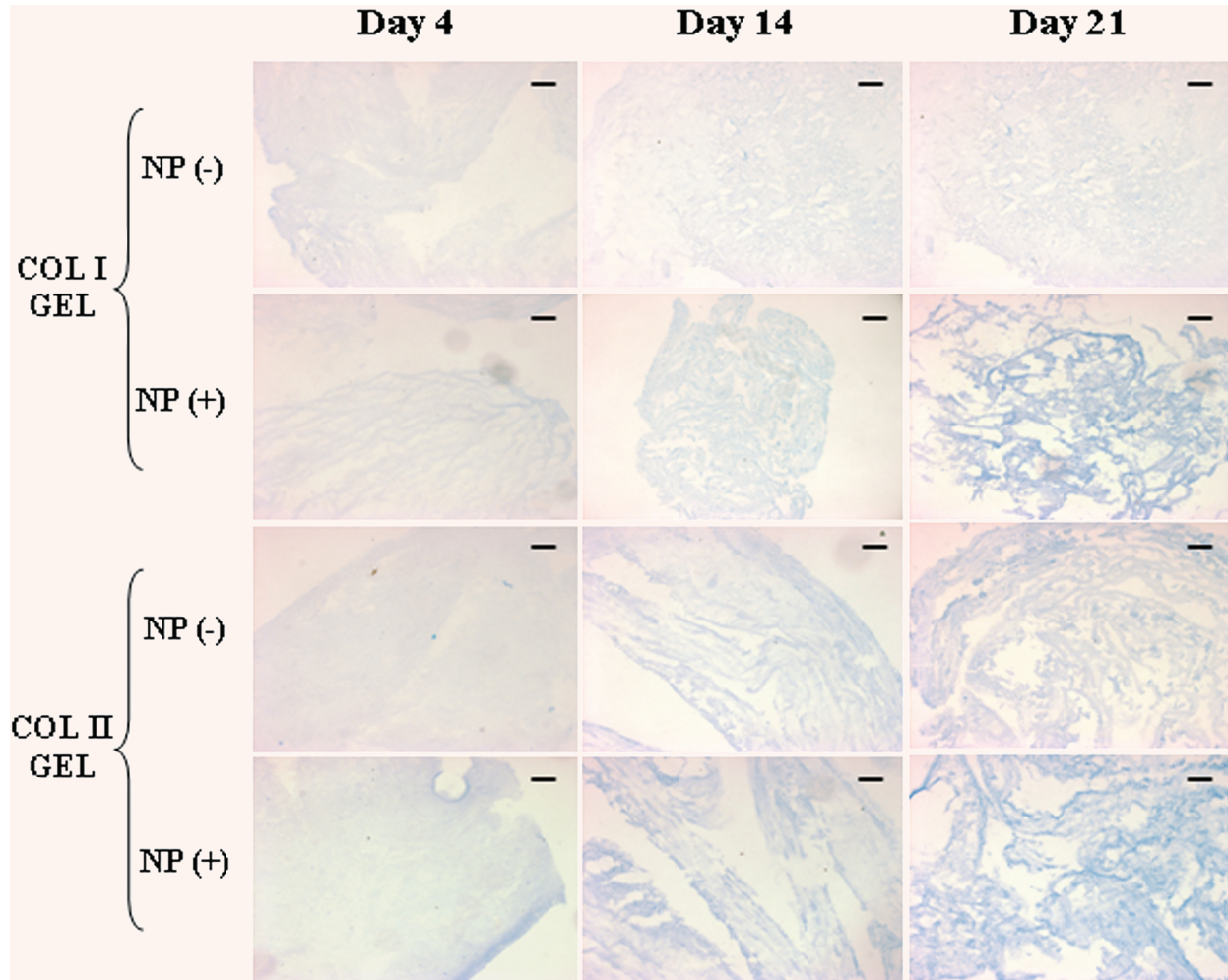


Fig. 4 Effect of collagen type I/II 3D environment and NP cells on alcian blue staining of ASCs. ASCs were cultured in collagen type I or type II gels alone, or indirectly co-cultured with NP cells for 4, 14 and 21 days. NP cells could greatly enhance the alcian blue staining of ASCs in either collagen type I and type II gels; collagen type II gels displayed stronger staining than collagen type I gels independent of whether ASCs were cultured alone or co-cultured with NP cells. Abbreviations: COL I: collagen type I; COL II: collagen type II. Bar: 250 µm.

was used (Fig. 3B). The same was true for collagen type IIA/IIB gene expression in ASCs (data not shown).

To study the potential contribution of NP cells in the differentiation process of ASCs cultured in either collagen type I or type II gels, transwell co-cultures with micromass NP cells were performed, and at day 4 and day 14 ASCs were harvested and analysed for expression of NP-phenotype related markers. It was found that at day 4 the presence of NP cells resulted in further enhancement of collagen type X gene expression in ASCs in both gel types (about 28- and 20-fold higher in collagen type I and type II gel compared to monolayer ASCs, respectively). Up-regulation of collagen type X gene expression by NP cells was also found to be significant when compared to ASCs cultured in collagen type I gels (Fig. 3A). Aggrecan gene expres-

sion was highest when ASCs were grown in collagen type II gels and co-cultured with NP cells, although there were no significant differences found among these five groups (Fig. 4B). Most striking differences were observed in the gene expression profiles of collagen type II: Collagen type IIA (early chondrogenic marker) was only induced in collagen type I-embedded ASCs from two out of five donors at day 4, and not at other time points. In contrast, both collagen type IIA and IIB (late chondrogenic marker) were induced in ASCs in collagen type II gels at day 4 or/and day 14, with a shift from collagen type IIA to collagen type IIB in three of five donors between day 4 and day 14 (Table 2).

Alcian blue staining demonstrated enhancement of proteoglycan staining intensity by NP cells in both collagen type I and

Table 2 Induction of collagen type IIA and IIB gene expression of ASCs embedded in collagen type I or type II gels and co-cultured with NP cells

Collagen gel type	Donor	Day 4		Day 14	
		Col IIA	Col IIB	Col IIA	Col IIB
I	1	–	–	–	–
	2	+	–	–	–
	3	+	–	–	–
	4	–	–	–	–
	5	–	–	–	–
II	1	–	–	+	–
	2	+	+	–	+
	3	+	–	–	+
	4	+	–	–	+
	5	–	–	+	+

Note: –: undetectable in 45 PCR cycles; +: detectable in 45 PCR cycles.

type II ASC-loaded matrices. Collagen type II gels displayed stronger staining than collagen type I gels in both situations (absence and presence of NP cells) (Fig. 4).

NP cells modulate ASCs distribution pattern in collagen type II gels

During our cultures, striking differences could be observed in the aggregation behaviour between the various experimental groups. Starting from around day 7 of culturing, strong cellular condensation/aggregation and ‘island formation’ was observed when ASCs were embedded in collagen type II (Fig. 5, upper panel), but not when embedded in collagen type I (data not shown). However, to our surprise, collagen type II- embedded ASCs in the presence of NP cells did show a severe delay or even prevention of these aggregation phenomena (Fig. 5, lower panel). The absence of aggregation events in collagen type I gels was not influenced by NP cells (data not shown).

NP cells down-regulate integrin subunit α_2 gene expression of ASCs in collagen type II gels

To gain insight on why NP cells modulated the striking ASCs distribution pattern in collagen type II gels, gene expression profiles of collagen type II receptor-integrin subunits α_1 , α_2 and α_{10} were determined in both collagen type II-embedded ASC groups (1+/-

NP cells). It was found that co-culture with NP cells significantly down-regulated gene expression of integrin subunit α_2 , but not the integrin subunits α_1 , α_{10} at day 4 (Fig. 6B). At day 14, although the trend was still pronounced, no significant changes for either integrin subunit was observed any more (Fig. 6A–C).

Discussion

Tissue microenvironments play a crucial role in normal regenerative processes. MSCs, as a part of normal regenerative processes, are believed to migrate and circulate away from their niches [26], and can differentiate into various anchorage-dependent cells types, such as osteoblasts [27, 28], neurons [29] and NP cell-like cells [30] *in vivo*. It is crucial to know how the individual components of the tissue microenvironment interact with each other to guide this anchorage-dependent cell differentiation.

In a previous study, we showed that micromass NP cells can successfully induce the NP phenotype in micromass ASCs (but not in monolayer ASCs) through soluble bio-factors in an indirect co-culture system [22]. This result is consistent with two other published reports in which chondrogenic differentiation was induced in embryonic stem cells or rabbit ASCs by soluble factors produced by primary chondrocytes or NP cells in indirect co-culture systems [31, 32]. The indirect co-culture setup was selected because our driving hypothesis was (and is) that, due to the high matrix-to-cell ratio in the NP, most of the ASCs will experience only indirect contact with NP cells *via* soluble factors. However, this at the same time implies that the microenvironment the ASCs will encounter after intradiscal injection will predominantly be a 3D matrix containing collagen type II. In the current study, we aimed to closely mimic this *in vivo* situation by seeding ASCs in 3D collagen type II gels and indirect co-culturing with NP cells. Our current data show: (i) A 3D environment *per se* (i.e. both collagen type I and type II gels) results in significant up-regulation of the gene expression of collagen type X but not of other markers associated with chondrogenesis (Fig. 3); (ii) Embedding of ASCs in collagen type II, but not in collagen type I gels, in the presence of NP cells provokes a more pronounced ASC differentiation towards the NP phenotype.

In general, a 3D environment, such as in collagen, fibrin and alginate gels, is required for chondrocytes to maintain their differentiated chondrogenic phenotype or for MSCs to undergo chondrogenic differentiation *in vitro* [33–36]. An important finding of the current study is that apparently an additional level of control of the chondrogenic response of ASCs may be achieved by optimizing the composition of the matrix environment they will encounter. Interestingly in this regard is that even though the effects of the matrix in itself appeared minor (only a modest difference in up-regulation (twofold) when comparing the collagen type X gene expression of collagen type I *versus* type II gels in favour of the latter, and no expression differences for the other chondrogenesis-associated markers), the matrix type appeared to ‘potentiate’ the ASCs

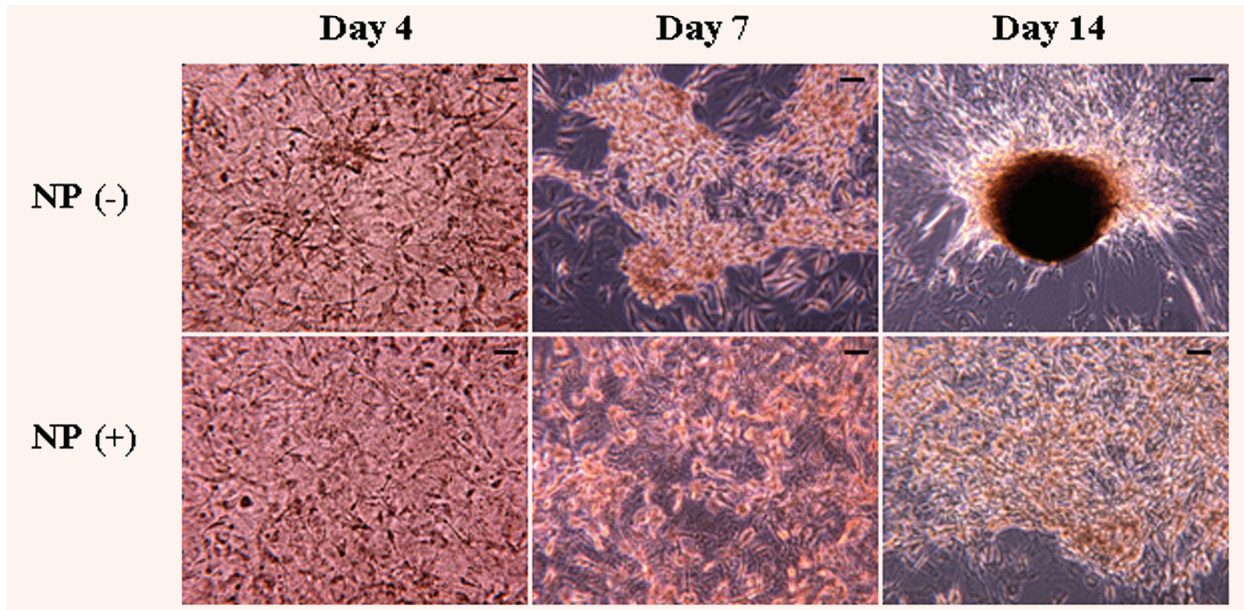


Fig. 5 Effect of NP cells on ASCs distribution pattern in collagen type II gel. ASCs in collagen type II gels were either cultured alone or co-cultured with NP cells over 14 days. When ASCs in collagen type II gels were cultured without NP cells (upper panel), ASCs began to aggregate around day 7 and formed striking 'islands' around day 14. This ASC aggregation was prevented/delayed by NP cells (lower panel). COL II: collagen type II. Bar: 150 μ m.

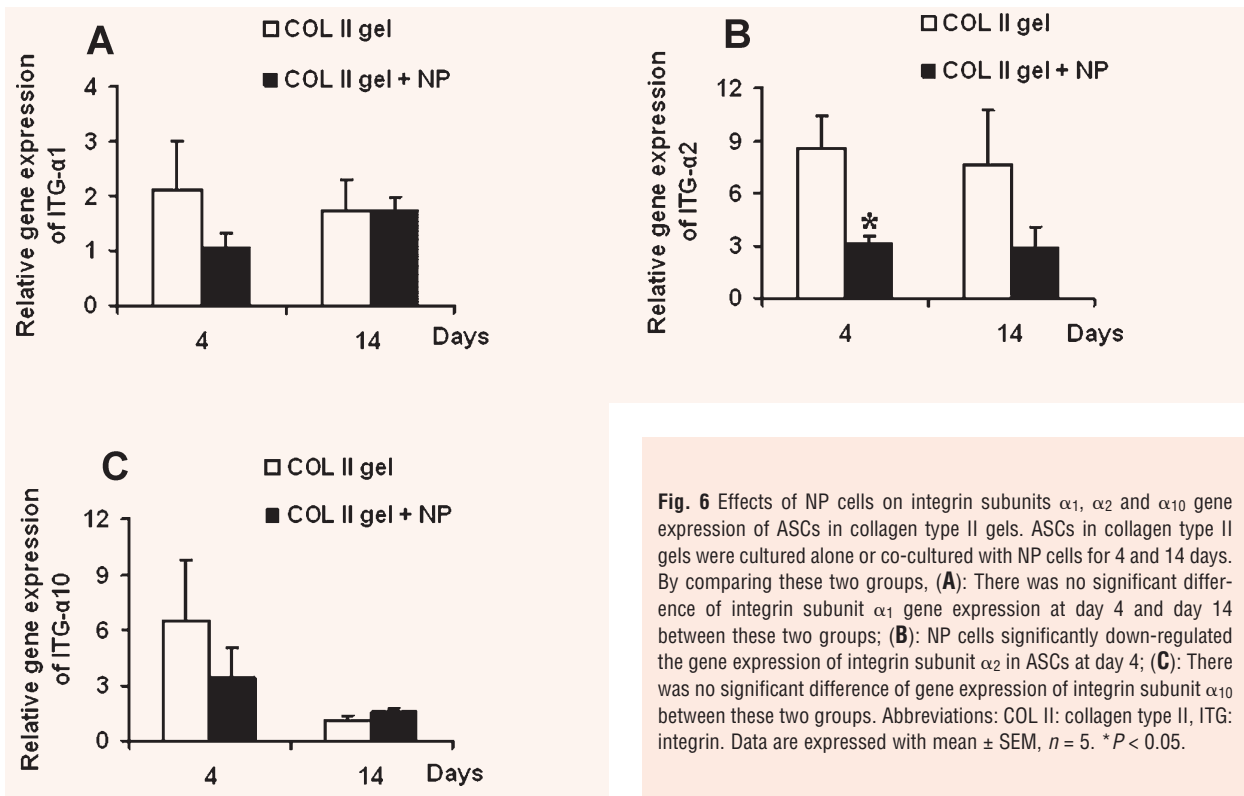


Fig. 6 Effects of NP cells on integrin subunits α_1 , α_2 and α_{10} gene expression of ASCs in collagen type II gels. ASCs in collagen type II gels were cultured alone or co-cultured with NP cells for 4 and 14 days. By comparing these two groups, (A): There was no significant difference of integrin subunit α_1 gene expression at day 4 and day 14 between these two groups; (B): NP cells significantly down-regulated the gene expression of integrin subunit α_2 in ASCs at day 4; (C): There was no significant difference of gene expression of integrin subunit α_{10} between these two groups. Abbreviations: COL II: collagen type II, ITG: integrin. Data are expressed with mean \pm SEM, $n = 5$. * $P < 0.05$.

for a stronger response to chondrogenic influences of the NP cells in our co-culture studies.

The early up-regulation of collagen type X expression as observed in this study does not match with the traditional view that this collagen should be regarded as a late marker, associated with hypertrophic, mineralizing chondrocytes [37]. In contrast, our data suggest that collagen type X induction is an early event, probably at least partly induced by placement of ASCs in a 3D environment, but less dependent of the collagen type the cells 'feel'. Also, the response to chondrogenic induction media appears to be relatively independent of the matrix type (Fig. 3A). Interestingly, and in accordance with our own findings, the early up-regulation of collagen type X has recently been recognized by other research groups as well [38, 39]. Although merely speculative, this might suggest a hitherto unrecognized role for collagen type X in early chondrogenic differentiation, or even a response to a changed environment (see above; from 2D-cultured to 3D-hydrogel embedded cells) *per se* that may be not or only indirectly be linked to chondrogenic events.

After ASCs were co-cultured with NP cells, we observed that ASCs in collagen type II gel displayed a more pronounced chondrogenic phenotype than ASCs in collagen type I gels: stronger alcian blue staining and induction of both collagen type IIA and IIB gene expression (Table 2, Fig. 4). It has been shown that collagen type IIA transcripts are synthesized by prechondrocytes whereas collagen type IIB transcripts are synthesized by mature chondrocytes [39, 40]. That collagen type II is more able to maintain or induce a chondrogenic phenotype has also been shown in several other studies [41–43]. To the best of our knowledge, mechanisms behind the property of collagen type II (maintenance or induction of chondrogenic phenotype) are still remaining unclear. Herein we only can speculate that collagen type I and collagen type II have different preferences to bind or induce different members of the integrin β_1 -family, and thereby follow a different signal pathway leading to chondrogenesis, because integrin β_1 family plays a crucial role in chondrogenesis [44] and has a cross-talk with growth factors such as transforming growth factor- β_1 (TGF- β_1) [45].

In this study, ASCs attached to collagen type II matrix as evidenced by different cell densities of ASCs homogeneously distributed in the different concentrations (from 1.5 to 4 mg/ml) of collagen type II matrix with the stellate shapes (Fig. 1, data not shown for the low and high concentrations of collagen type II gel) within

12 hrs. There was also a cell density-dependent gel contraction in collagen type II gels embedded with ASCs (Fig. 2). Integrins comprise a group of receptors on the cell surface that mediate cell–ECM interactions. All receptors consist of one α and one β subunit which together form a non-covalently bound heterodimer [46]. The integrins $\alpha_1\beta_1$, $\alpha_2\beta_1$ and $\alpha_{10}\beta_1$ have been identified to interact with collagen type II, and each of them separately or in combination might be involved in the attachment of ASCs. Because these integrins have distinct signalling functions due to their different short intracellular domains of the collagen-binding α units [23, 47, 48], we determined the expression patterns of all α subunits in an effort to assess which integrin might be primarily involved in the aggregation events observed in collagen type II gels, and its delay after co-incubation with NP cells (Fig. 5). Although far from conclusive, the significant down-regulation of integrin subunit α_2 gene expression in the collagen type II/NP cell combination (Fig. 6B) suggests that the change in ASC aggregation rate imposed by the NP cells may be mediated by NP cell-secreted factors affecting the expression level of the integrin $\alpha_2\beta_1$. Further studies are warranted to substantiate the potential role of this particular integrin in ASC condensation events, as well as the nature of the soluble factor(s) involved.

In conclusion, collagen type II can provide an appropriate scaffold for the attachment of ASCs. In addition, it was found that a microenvironment consisting of collagen type II hydrogels in combination with soluble factor-secreting NP cells, can direct ASC differentiation more efficiently towards a more advanced cartilage/NP lineage than ASCs in a collagen type I/NP cell combination. Furthermore, the different collagen type II receptors-integrin $\alpha_1\beta_1$, $\alpha_2\beta_1$ and $\alpha_{10}\beta_1$ in ASCs may exert different functions in response to collagen type II, where integrin $\alpha_2\beta_1$ is more likely involved in mediating ASC aggregation. The findings from this study allow us to proceed to *in vivo* experiments in which ASCs will be used for the injection treatment for mildly DDD.

Acknowledgements

The authors gratefully acknowledge F. J. van Milligen for providing the ASCs. This study is supported by Cytori Therapeutics, Inc. and by the Dutch Tissue Engineering Program (DPTE # BGT.6734).

Reference

1. **Buckwaller JA.** Spine update – aging and degeneration of the human intervertebral disc. *Spine.* 1995; 20: 1307–14.
2. **Manek NJ, MacGregor AJ.** Epidemiology of back disorders: prevalence, risk factors, and prognosis. *Curr Opin Rheumatol.* 2005; 17: 134–40.
3. **Ala-Kokko L.** Genetic risk factors for lumbar disc disease. *Ann Med.* 2002; 34: 42–7.
4. **Battie MC, Videman T, Gill K, Moneta GB, Nyman R, Kaprio J, Koskenvuo M.** 1991 Volvo award in clinical sciences – smoking and lumbar intervertebral-disk degeneration – an Mri study of identical-twins. *Spine.* 1991; 16: 1015–20.
5. **Battie MC, Videman T, Gibbons LE, Manninen H, Gill K, Pope M, Kaprio J.** Occupational driving and lumbar disc degeneration: a case-control study. *Lancet.* 2002; 360: 1369–74.

6. **Videman T, Battie MC.** Spine update – the influence of occupation on lumbar degeneration. *Spine.* 1999; 24: 1164–8.
7. **Meacham G, Cornah MS.** Fine structure of juvenile human nucleus pulposus. *J Anat.* 1970; 107: 337–50.
8. **Cs-Szabo G, Juan DRS, Turumella V, Masuda K, Thonar EJMA, An HS.** Changes in mRNA and protein levels of proteoglycans of the annulus fibrosus and nucleus pulposus during intervertebral disc degeneration. *Spine.* 2002; 27: 2212–9.
9. **Pearce RH, Grimmer BJ, Adams ME.** Degeneration and the chemical-composition of the human lumbar intervertebral-disk. *J Orthop Res.* 1987; 5: 198–205.
10. **Ganey T, Libera J, Moos V, Alasevic O, Fritsch KG, Meisel HJ, Hutton WC.** Disc chondrocyte transplantation in a canine model: a treatment for degenerated or damaged intervertebral disc. *Spine.* 2003; 28: 2609–20.
11. **Meisel HJ, Ganey T, Hutton WC, Libera J, Minkus Y, Alasevic O.** Clinical experience in cell-based therapeutics: intervention and outcome. *Eur Spine J.* 2006; 15: S397–405.
12. **Nishimura K, Mochida J.** Percutaneous reinsertion of the nucleus pulposus – an experimental study. *Spine.* 1998; 23: 1531–8.
13. **Okuma M, Mochida J, Nishimura K, Sakabe K, Seiki K.** Reinsertion of stimulated nucleus pulposus cells retards intervertebral disc degeneration: an *in vitro* and *in vivo* experimental study. *J Orthop Res.* 2000; 18: 988–97.
14. **Caplan AI.** Mesenchymal stem-cells. *J Orthop Res.* 1991; 9: 641–50.
15. **Tuan RS, Boland G, Tuli R.** Adult mesenchymal stem cells and cell-based tissue engineering. *Arthritis Res Ther.* 2003; 5: 32–45.
16. **Zuk PA, Zhu M, Ashjian P, De Ugarte DA, Huang JI, Mizuno H, Alfonso ZC, Fraser JK, Benhaim P, Hedrick MH.** Human adipose tissue is a source of multipotent stem cells. *Mol Biol Cell.* 2002; 13: 4279–95.
17. **Aust L, Devlin B, Foster SJ, Halvorsen YDC, Hicok K, du Laney T, Sen A, Willingmyre GD, Gimble JM.** Yield of human adipose-derived adult stem cells from liposuction aspirates. *Cytotherapy.* 2004; 6: 7–14.
18. **Fraser JK, Wulur I, Alfonso Z, Hedrick MH.** Fat tissue: an underappreciated source of stem cells for biotechnology. *Trends Biotechnol.* 2006; 24: 150–4.
19. **Helder MN, Knippenberg M Klein-Nulend J, Wuisman PI.** Stem cells from adipose tissue allow challenging new concepts for regenerative medicine. *Tissue Eng.* 2007; 13: 1799–808.
20. **Djouad F, Delorme B, Maurice M, Bony C, Apparailly F, Louis-Plence P, Canovas F, Charbord P, Noël D, Jorgensen C.** Microenvironment changes during mesenchymal stem cell differentiation towards chondrocytes. *Arthritis Res Ther.* 2007; 29: R33.
21. **Engler AJ, Sen S, Sweeney HL, Discher DE.** Matrix elasticity directs stem cell lineage specification. *Cell.* 2006; 126: 677–89.
22. **Lu ZF, Zandieh Doulabi B, Wuisman PI, Bank RA, Helder MN.** Differentiation of adipose stem cells by nucleus pulposus cells: configuration effect. *Biochem Biophys Res Commun.* 2007; 359: 991–6.
23. **Camper L, Hellman U, Lundgren-Akerlund E.** Isolation, cloning, and sequence analysis of the integrin subunit alpha 10, a beta 1-associated collages binding integrin expressed on chondrocytes. *J Biol Chem.* 1998; 273: 20383–9.
24. **Knudson W, Loeser RF.** CD44 and integrin matrix receptors participate in cartilage homeostasis. *Cell Mol Life Sci.* 2002; 59: 36–44.
25. **Loeser RF, Sadiev S, Tan L, Goldring MB.** Integrin expression by primary and immortalized human chondrocytes: evidence of a differential role for alpha 1 beta 1 and alpha 2 beta 1 integrins in mediating chondrocyte adhesion to types II and VI collagen. *Osteoarthritis Cartil.* 2000; 8: 96–105.
26. **Katayama Y, Battista M, Kao WM, Hidalgo A, Peired AJ, Thomas SA, Frenette PS.** Signals from the sympathetic nervous system regulate hematopoietic stem cell egress from bone marrow. *Cell.* 2006; 124: 407–21.
27. **Otsuru S, Tamai K, Yamazaki T, Yoshikawa H, Kaneda Y.** Bone marrow-derived osteoblast progenitor cells in circulating blood contribute to ectopic bone formation in mice. *Biochem Biophys Res Commun.* 2007; 354: 453–8.
28. **Wang Z, Goh J, De SD, Ge ZG, Ouyang HW, Chong JSW, Low SL, Lee EH.** Efficacy of bone marrow – derived stem cells in strengthening osteoporotic bone in a rabbit model. *Tissue Eng.* 2006; 12: 1753–61.
29. **Deng J, Petersen BE, Steindler DA, Jorgensen ML, Laywell ED.** Mesenchymal stem cells spontaneously express neural proteins in culture and are neurogenic after transplantation. *Stem Cells.* 2006; 24: 1054–64.
30. **Sakai D, Mochida J, Iwashina T, Watanabe T, Nakai T, Ando K, Hotta T.** Differentiation of mesenchymal stem cells transplanted to a rabbit degenerative disc model – potential and limitations for stem cell therapy in disc regeneration. *Spine.* 2005; 30: 2379–87.
31. **Li XD, Lee JP, Balian G, Anderson DG.** Modulation of chondrocytic properties of fat-derived mesenchymal cells in co-cultures with nucleus pulposus. *Connect Tissue Res.* 2005; 46: 75–82.
32. **Vats A, Bielby RC, Tolley N, Dickinson SC, Boccaccini AR, Hollander AP, Bishop AE, Polak JM.** Chondrogenic differentiation of human embryonic stem cells: the effect of the micro-environment. *Tissue Eng.* 2006; 12: 1687–97.
33. **Batorsky A, Liao JH, Lund AW, Plopper GE, Stegemann JP.** Encapsulation of adult human mesenchymal stem cells within collagen-agarose microenvironments. *Biotechnol Bioeng.* 2005; 92: 492–500.
34. **Kavalkovich KW, Boynton RE, Murphy JM, Barry F.** Chondrogenic differentiation of human mesenchymal stem cells within an alginate layer culture system. *In Vitro Cell Dev Biol Anim.* 2002; 38: 457–66.
35. **Lemare F, Steimberg N, Le Griel C, Demignot S, Adolphe M.** Dedifferentiated chondrocytes cultured in alginate beads: restoration of the differentiated phenotype and of the metabolic responses to interleukin-1 beta. *J Cell Physiol.* 1998; 176: 303–13.
36. **Wayne JS, McDowell CL, Shields KJ, Tuan RS.** *In vivo* response of polylactic acid-alginate scaffolds and bone marrow-derived cells for cartilage tissue engineering. *Tissue Eng.* 2005; 11: 953–63.
37. **Mwale F, Billinghamurst C, Wu W, Alini M, Webber C, Reiner A, Ionescu M, Poole J, Poole AR.** Selective assembly and remodelling of collagens II and IX associated with expression of the chondrocyte hypertrophic phenotype. *Dev Dyn.* 2000; 218: 648–62.
38. **Mwale F, Stachura D, Roughley P, Antoniou J.** Limitations of using aggrecan and type X collagen as markers of chondrogenesis in mesenchymal stem cell differentiation. *J Orthop Res.* 2006; 24: 1791–8.
39. **Murdoch AD, Grady LM, Ablett MP, Katopodi T, Meadows RS, Hardingham TE.** Chondrogenic differentiation of human bone marrow stem cells in transwell cultures: generation of Scaffold-free cartilage. *Stem Cells.* 2007; 25: 2786–96.
40. **Sandell LJ, Morris N, Robbins JR, Goldring MB.** Alternatively spliced type-I procollagen messenger-Rnas define

- distinct populations of cells during vertebral development – differential expression of the amino-propeptide. *J Cell Biol.* 1991; 114: 1307–19.
41. **Bosnakovski D, Mizuno M, Kim G, Takagi S, Okumura M, Fujinaga T.** Chondrogenic differentiation of bovine bone marrow mesenchymal stem cells (MSCs) in different hydrogels: influence of collagen type II extracellular matrix on MSC chondrogenesis. *Biotechnol Bioeng.* 2006; 93: 1152–63.
42. **Scully SP, Lee JW, Ghert MA, Qi W.** The role of the extracellular matrix in articular chondrocyte regulation. *Clin Orthop Relat Res.* 2001; S72–89.
43. **van der Kraan PM, Buma P, van Kuppevelt T, van den Berg WB.** Interaction of chondrocytes, extracellular matrix and growth factors: relevance for articular cartilage tissue engineering. *Osteoarthr Cartil.* 2002; 10: 631–7.
44. **Shakibaei M.** Inhibition of chondrogenesis by integrin antibody *in vitro*. *Exp Cell Res.* 1998; 240: 95–106.
45. **Schneiderbauer MM, Dutton CM, Scully SP.** Signaling “cross-talk” between TGF-beta 1 and ECM signals in chondrocytic cells. *Cell Signal.* 2004; 16: 1133–40.
46. **Hynes RO.** Integrins: bidirectional, allosteric signaling machines. *Cell.* 2002; 110: 673–87.
47. **Camper L, Holmvall K, Wangnerud C, Aszodi A, Lundgren-Akerlund E.** Distribution of the collagen-binding integrin alpha 10 beta 1 during mouse development. *Cell and Tissue Res.* 2001; 306: 107–16.
48. **White DJ, Puranen S, Johnson MS, Heino J.** The collagen receptor subfamily of the integrins. *Int J Biochem Cell Biol.* 2004; 36: 1405–10.