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Comparison of cartilage specific markers in articular and differentiated chondrocytes in pellet system

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ABSTRACT

Autologous cartilage replacement has the inherent advantage that the transplanted tissue is immunogenically neutral. However, chondrocyte isolation, proliferation, and dedifferentiation limitations resulted in the search for a cell type that would overcome the aforementioned limitations. Here we investigated if adipose-derived stem cells (ADSCs), which are easy to isolate in large quantities, in combination with a three dimensional culture system and growth factor would be a suitable alternative for autologous cartilage replacement. In this study ADSCs and human articular chondrocytes were allowed to differentiate in pellet culture in the presence or absence of transforming growth factor-β3 (TGF-β3) for 14 days and their chondrogenic potential was evaluated. Pellets were microscopically and histologically evaluated for structure and morphology. Matrix production was assayed using immunohistochemistry for collagen types I and II, and aggrecan. Images were evaluated via custom-made routines programmed in MATLAB. Oval to round cell pellets were formed with an overall homogenous dense central structure that consisted of isogenic groups with walled-off chondrocytes in their lacunae and encircled by basophilic extracellular matrix (ECM). Our results show that in the presence of TGF-β3, collagen type I expression was 2-fold and aggrecan expression was 3-fold higher in differentiated ADSCs compared with articular chondrocytes, whereas collagen type II was reduced by a factor of 1.58. Furthermore, the absence of TGF-β3 nearly completely suppressed collagen type I in both cell types, whereas aggrecan expression remained unaffected in ADSCs and was reduced 1.87-fold in articular chondrocytes. Pellet culture of ADSCs-derived chondrocytes represents a viable alternative for autologous cartilage replacement. The high cell density and more natural three-dimensional environment of pellet culture attenuated dedifferentiation of cells. Although we obtained a mixed chondrogenic phenotype with increased collagen type I expression, the extent of this increase was lower compared to reports in the literature. Furthermore, ADSCs-derived chondrocytes expressed large amounts of aggrecan, which is an essential component of the ECM in articular cartilage. Finally, TGF-β3 might be used to direct chondrogenic differentiation and reduce collagen type I expression by controlling concentration and treatment duration with TGF-β3.

Keywords: *Differentiated Chondrocyte, Articular Chondrocyte, Adipose-derived Stem Cell, TGF-β3, Collagen type I, Collagen type II, Aggrecan, Pellet culture*.

1. INTRODUCTION

Cartilage is a specialized flexible connective tissue with a consistency that allows the tissue to withstand mechanical forces without being fragmented, and can be found in joints, thoracic cage, bronchial tubes, intervertebral discs, ear and nose [1]. More than 95% of the tissue volume consists of extracellular matrix (ECM) in which chondrocytes actively produce ECM components and maintain the ECM [2]. Water, collagen type II, different types of proteoglycans, especially aggrecan, as well as glycoproteins, such as chondronectin, are major components of the ECM in articular cartilage. Due to the lack of blood vessels, progenitor cell deficiency, low proliferation, but also the ECM's relative impotence to contribute to remodeling, the capacity of cartilage for self-repair is limited [3-5].

Recent epidemiological studies have shown an alarming increase in diseases associated with degeneration of cartilage tissue in different human populations and age groups, with congenital problems and sports injuries as the majority of causes of cartilage tissue defects. Autologous Chondrocyte Transplantation (ACT) is the foremost strategy for repairing cartilage-associated damage [6, 7]. With this method and during the biopsy, 150-300 mg of cartilage tissue sample is removed from an area of the body that does not carry weight, such as the femur's upper edge of the inner condyle, from which cells are cultured and multiplied in order to obtain transplantable replacement tissue. Subsequently, this tissue is transplanted into the location of cartilage damage to fill the lesion [8, 9]. This method offers the advantage that the patient's own tissue is used for treatment, which prevents immunological rejection of the transplant. However, loss of differentiated chondrocyte phenotype during proliferation and fibrocartilage formation after transplantation of chondrocytes in the damaged area are the main and significant limitations of this method [10-14].

Multipotent stem cells, such as bone marrow derived mesenchymal cells have the inherent ability to differentiate into various cell types, including osteoblasts, chondrocytes, myocytes and adipocytes. Currently, mesenchymal stem cells are isolated from a variety of sources, such as adipose tissue, amniotic fluid, and the periosteum [15]. There are no considerable differences between mesenchymal stem cells isolated from different bodily origins, as far as shape, safety cross-section and the ability to differentiate are concerned [15]. Adipose-derived stem cells (ADSCs) are considered to be suitable and easily obtainable for cell therapy and tissue engineering [16-19]; the stromal vascular fraction of adipose tissue contains an abundant population of ADSCs. In terms of surface markers and the ability to differentiate into various tissues, fat-derived and bone marrow-derived stem cells (BMSCs) share many similarities; albeit that ADSCs have an inferior chondrogenesis potential compared to BMSCs [20]. However, ADSCs have been shown to commit to chondrogenesis when cultured in transforming growth factor-β3-containing (TGF-β3) chondrogenic medium [21, 22]. Furthermore, the correct choice of one of the various three-dimensional culture systems seems to be essential for chondrogenic differentiation induction [23]. Monolayer culture is not appropriate to induce stem cell to chondrocyte differentiation and overcome phenotypic instability, which strictly requires three-dimensional culture systems for successful chondrogenic differentiation. For this purpose, two types of three-dimensional culture systems are available, i.e., scaffold-dependent and scaffold-free systems, such as micromass and pellet systems [24, 25]. Several studies have previously used pellet cultures for hyaline cartilage chondrocyte culture and studied the effects of exogenous agents, such as bone morphogenetic protein (BMP) on the chondrocyte phenotype [26], properties of the ECM [27], and bioenergetics of chondrocytes [28, 29].

In this study, we compared the production of key ECM components in, i.e., type I and II collagens and aggrecan in a pellet culture system of adipose-derived stem cells and chondrocytes isolated from human articular cartilage. Furthermore, the influence of the presence of TGF-β3 in the chondrogenic medium was evaluated. These initial investigations will serve as a basis for development of effective cartilage replacement strategies based in this three dimensional culture system and adipose-derived stem cells.

2. EXPERIMENTAL SECTION

2.1. Tissue collection and isolation of human adipose-derived stem cells and articular chondrocytes. Human adipose-derived stem cells (ADSCs) were obtained from the subcutaneous abdominal adipose tissue of four patients (30-50 years) during abdominoplasty surgery. Informed written consent was obtained from the patients prior to tissue removal. After mincing and washing the adipose tissue three times with phosphate buffered saline (PBS) to remove debris and red blood cells, 20 g tissue was digested with a 0.075% type I collagenase (Sigma, St. Louis, MO, USA) solution (1 mg for each mg fat) at 37°C for 30 min. Inactivation of the collagenase was performed by adding DMEM-LG (Sigma) supplemented with 10% fetal bovine serum (FBS; Invitrogen-Life technologies, Carlsbad, CA, USA). Subsequently, the cell solution was centrifuged at 1500 rpm (270×g) for 10 min. The supernatant was removed and the resultant pellet was resuspended in DMEM-LG supplemented with 10% FBS, 1% penicillin and streptomycin (Gibco-Life technologies. Darmstadt, Germany) and then cultured at 37°C in a humidified atmosphere, 5% CO2. The medium was replaced every 4 days. After 80% cell confluency was reached, cells were detached with 0.05% trypsin/0.53 mM EDTA (Sigma) solution and passaged. For the preparation of the pellet culture, cells at passage 3 were detached by trypsin/EDTA, centrifuged, and counted.

Articular cartilage was obtained via arthroscopy from knee joints of 3 patients (30-50 years). The cartilage specimens were isolated from non-weight bearing areas and transported to the laboratory in PBS. Approximately 1-2 mm pieces of cartilage slices were digested with type II collagenase solution (350 U/ml) for 4 h at 37°C. Then, the solution was centrifuged and cells were cultured in DMEM/F12 (Gibco) supplemented with 10% FBS and 1% penicillin-streptomycin. Medium was replaced every 4 days. When the cells reached 90-95% confluency, cells were detached with trypsin/ EDTA solution and passaged. For the preparation of the pellet culture, cells at passage 2 to 4 were detached with trypsin/EDTA, centrifuged, and counted.

2.2. Pellet Culture System. For pellet culture, 25×104 ADSCs or articular chondrocytes were centrifuged at 1500 rpm (270×g) for 10 min. Chondrogenic medium was added to the centrifuged cells in polypropylene conical tubes. Falcon tubes were transferred to a 37°C incubator (5% CO2). Medium was refreshed every 3 days and on day 14, specimens were used for histological and immunohistochemical assays. Chondrogenic culture media contained: DMEM-HG (High Glucose; Gibco), 1% penicillin and streptomycin , 10-7 M dexamethasone (Sigma), 50 µg/ml ascorbate-2-phosphate (Sigma), 1% bovine serum albumin(Sigma), 5 µg/ml linoleic acid (Sigma), 1% insulin-transferrin-selenium (ITS; Sigma), 10 ng/ml transforming growth factor-β3 (TGF-β3; Sigma). The medium of the control group lacked TGF-β3.

2.3. Histological Assay. After 14 days, pellets of differentiated ADSCs and articular chondrocytes were fixed in 10% formalin for 2 h at room temperature. Subsequently, these were dehydrated, cleared and embedded in paraffin. Then sections of 5 µm thicknesses were prepared on a microtome for Hematoxylin and Eosin (H&E) staining. Glycosaminoglycan (GAG) was assessed by toluidine blue staining.

2.4. Immunohistochemistry Assay. For the evaluation of type I and II collagens and aggrecan, immunohistochemistry (IHC) was performed. Briefly, pellet sections were deparafffinized, and treated with 8 mg/ml hyaluronidase for 120 min at 37 ° C for antigen retrieval. To suppress endogenous hydrogen peroxidase, activity samples were placed in 3% hydrogen peroxide in ethanol

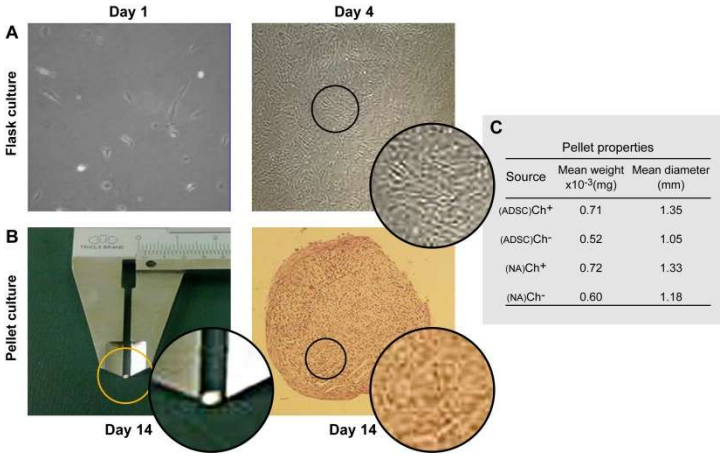
for 10 min. After washing three to four times with Tris-buffered saline (TBS), the primary antibodies against type I collagen and aggrecan (1:50) and type II collagen (1:70) were added to the sections. Following incubation for 24 h at 4 °C and washing again with TBS, horseradish peroxidase-conjugated goat anti-rabbit IgG (1:200; Boiss, Inc., Beijing, China) was added to the samples for 60 minutes and, finally, after applying diaminobenzidine, hematoxylin and eosin (H&E) staining was used to stain the field. Images were acquired with a Nikon TS100 microscope (Nikon, Tokyo, Japan).

2.5. Comparison and Semiquantitative Analysis of Images. Images were evaluated with a custom made routine in MATLAB Release 2013a (Mathworks, Natick, MA, USA). The routine consisted of background assessment and an initial target definition via edge detection, followed by main image edge reinforcement. After appropriate background corrections and morphological operations, intensity values for the various color channels were extracted to quantify the extent of staining.

2.6. Statistical Analysis. All data are expressed as means ± SD (standard deviation). In calculations, resulting SDs were determined via the error propagation formula. One way analysis of variance (ANOVA) and the Tukey`s post-hoc test were used for data analysis. Differences between groups were considered statistically significant when $P<0.05$.

3. RESULTS SECTION

3.1. Primary Chondrocyte Flask Culture versus Pellet Culture. After isolation of primary cells and cell culturing in flasks (25 cm2), ADSCsand articular chondrocytes eventually adhered to the bottom of the flask and showed elongated cell morphologies (Fig. 1A). After several days in culture, a uniform sub-confluent monolayer of increasingly compact cells was obtained. However, monolayer flask cultures are inherently accompanied with dedifferentiation of chondrocytes [12-14] that result in fibroblast-like morphologies [14], which we indeed observed when passaging cells at low densities. Maintaining sufficiently high confluencies ensures that the chondrocytes phenotype is maintained [30, 31]. Therefore, we opted to maintain the isolated articular chondrocytes at a higher density than ADSCs before pellet culture. As expected, isogenic groups were distinctly absent in the monolayer flask cultures.



Source	Mean weight x10^{-3}(mg)	Mean diameter (mm)
(ADSC)Ch^{+}	0.71	1.35
(ADSC)Ch^{-}	0.52	1.05
(NA)Ch^{+}	0.72	1.33
(NA)Ch^{-}	0.60	1.18

Figure 1. Comparison of flask and pellet cultures. (A) Monolayer culture of adipose-derived stem cells (ADSCs) in flasks (magnification: 40×). (B) Pellets after 14 days in culture. (C) Weigh and diameter of the pellets. (ADSC)Ch+: ADSCs-derived chondrocytes in TGF-β3 medium; (ADSC)Ch–: ADSCs-derived chondrocytes in normal medium; (NA)Ch+: Natural articular tissue-derived chondrocytes in TGF-β3 medium; (NA)Ch–: Natural articular tissue-derived chondrocytes in normal medium; TGF-β3: transforming growth factor-β3.

In pellet culture, isolated stem cells and articular chondrocytes were initially concentrated at the bottom of the tube via centrifugation, and during the second and third days, cell pellets became free floating in the growth medium and matrices were formed by cell integration.White and round to oval pellet masses were formed over the 2 weeks in culture (Fig. 1B). During the first days in culture, cells typically were elongated. After several days, columnar chondrocytes, which are the most proliferative, were observed, whereas at later stages, oval/cubic cells (hyperthropic) predominated. Pellet samples were weighed and their dimensions determined as shown in Fig. 1C.

3.2. Histological and Immunohistological assessment. The presence of cartilage tissue was assessed via H&E staining of cross sections of both ADSCs-derived chondrocytes ((ADSC)Ch) and natural articular tissue-derived chondrocytes ((NA)Ch) pellets. As shown in Fig. 2, the central part of the samples contained isogenic groups with single or double rounded walled-off chondrocytes in their lacunae and encircled by basophilic extracellular matrix. Furthermore, an irregular collagenous layer is observable in peripheral parts of the samples, especially in the (ADSC)Ch sample.

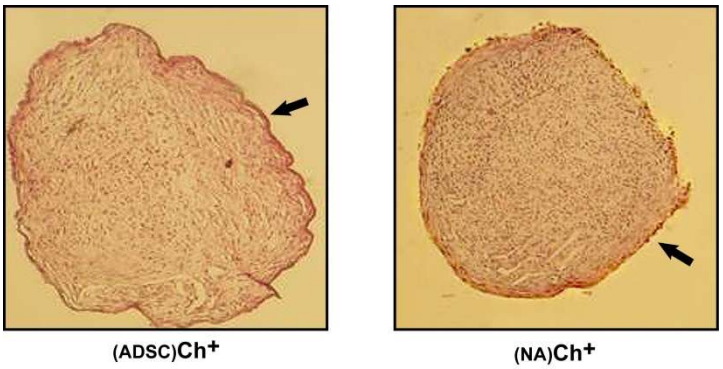


Figure 2. H&E stained chondrocyte pellets of differentiated adipose-derived stem cells (ADSCs) and articular chondrocytes. (magnification: 60×). Pellet core: single/double rounded walled-off chondrocytes in their lacunae. Arrows: irregular edges that display more staining. (ADSC)Ch+: ADSCs-derived chondrocytes in TGF-β3 medium; (NA)Ch+: Natural articular tissue-derived chondrocytes in TGF-β3 medium.

Both ADSCS-derived and articular chondrocyte-derived pellet samples cultured in chondrogenic medium with TGF-β3 had more texture and a smoother and more rounded appearance. In contrast, groups that were deprived of TGF-β3 were significantly more wrinkled with rough edges and had less texture (see Fig.

3B); although the effect was more dramatic in articular chondrocyte-derived pellets.

The suitability of ADSCs for cartilage replacement was evaluated by semi-quantitative determination of key ECM components of cartilagenous tissue, i.e., collagen types I (Col-I) and II (Col-II), and aggrecan (Agg), via immunohistochemical staining of pellet cross-sections (Fig. 3). Aggrecan is a cartilage-specific proteoglycan core protein, which through its cylindrical structure and self-assembly into large supramolecular complexes is responsible for cartilage's capacity to withstand compression [32], Together with type-II collagen,aggrecan forms a major structural component of cartilage and in particular articular cartilage. The results in Fig. 3A show that Col-I (2.02 ± 0.05 times) and Agg (3.05 ± 0.22 times) expression was significantly higher in differentiated ADSCs ((ADSC)Ch)compared with articular chondrocytes ((NA)Ch), whereas Col-II expression was reduced by a factor of 1.58 ± 0.09.

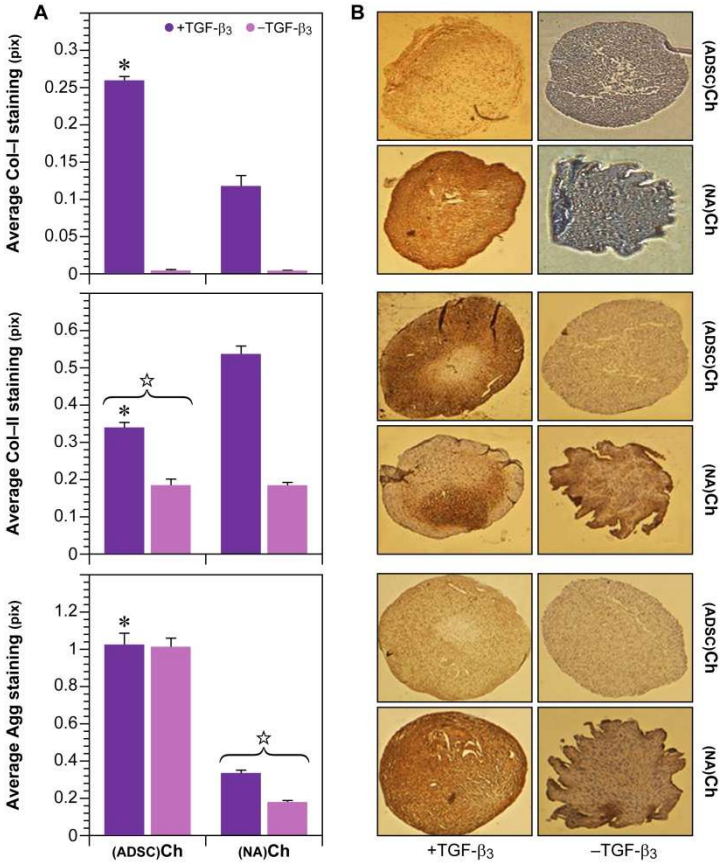


Figure 3. Immunohistochemical evaluation of extracellular matrix (ECM) proteins in pellet cultures of chondrocytes from adipose-derived stem cells (ADSCs) or articular origin. (A) Quantitative evaluation of the extent of the immunohistochemical staining for collagen-I (Col-I), collagen-II (Col-II), and aggrecan (Agg). (ADSC)Ch: ADSCs-derived chondrocytes; (NA)Ch: Natural articular tissue-derived chondrocytes; TGF-β3: transforming growth factor-β3. *: P<0.05 compared with (NA)Ch group; ☆ :: P<0.05 when comparing TGF-β3-treated and untreated groups. (B) Representative samples of immunohistochemical staining for the proteins of interest in pellet cultures of ADSC origin ((ADSC)Ch) or articular origin ((NA)Ch). Magnification 60×. Notice the distinctly wrinkled and rough edges in articular chondrocytes when cultured in the absence of TGF-β3.

Furthermore, the absence of TGF-β3 in the growth medium not only resulted in obvious morphological malformations in the pellet and inner structure (Fig. 3B), but also resulted in a measurable and significant reduction in ECM component expression. Most importantly, this effect was more pronounced in articular chondrocytes ((NA)Ch–). The absence of TGF-β3 nearly completely abolished Col-I expression in both ADSCs and articular chondrocytes (52.01 ± 1.44 and 25.80 ± 0.47 fold compared with TGF-β3-treated groups); the lack of Col-I expression in the (ADSC)Ch– and (NA)Ch– pellets is visually (grey-blue) obvious from Fig 3B because of the lack of brown staining. Col-II expression was reduced by a factor of 1.83 ± 0.15 ((ADSC)Ch) and 2.92 ± 0.16 ((NA)Ch) compared with TGF-β3-treated groups. Interestingly, Agg expression was independent of TGF-β3 in (ADSC)Ch, whereas Agg expression was reduced 1.87 ± 0.13 in (NA)Ch and no significant difference could be determined between Col-II expression in (ADSC)Ch or (NA)Ch pellets cultured in the absence of TGF-β3.

One of the major problems when growing and multiplying chondrocytes isolated for autologous cartilage replacement in culture systems is to maintain the chondrocyte phenotype whilst obtaining adequately high cell numbers for tissue engineering. In adhesion permissive culture systems, dedifferentiation of chondrocytes occurs after several days in culture in the absence of sufficiently high cell densities and their original tissue environment. Our microscopic observations showed that no chondrons and pericellular matrix is formed in monolayer culture. Furthermore, isolation from the femur's upper edge of the inner condyle results in isolation of relatively small numbers of cells that must be multiplied with the risk of dedifferentiation, may cause significant patient discomfort, and carries the risk of side-effects such as infections. Pellet culture provides the required high cell densities, formation of ECM, and allows cell-cell communication reminiscent of cell communication during cartilage formation in embryonic development [30, 33, 34]. Therefore, an alternative source of easily obtainable cells that would not be burdened by dedifferentiation in combination with a suitable three-dimensional culture system such as pellet culture would be highly desirable. Since adipose-tissue derived stem cells are easily obtainable in high quantities, not yet differentiated, and have been shown to commit to chondrogenesis when cultured in the presence of TGF-β3 [21, 22], we examined if ADSCs in a pellet culture would be a suitable alternative for autologous cartilage replacement.

Our results show that the cell populations in both (ADSC)Ch or (NA)Ch pellets are comparable with normal cartilage in terms of histological and morphological characteristics [1] and the pellet cross-sections show that this culture system is significantly more competent in retaining the chondrocyte phenotype. Similar observations were made by Bernstein et al. when these authors compared pellet culture with alginate scaffold-based culture of articular cartilage from pig knee [33] and Chang and co-workers when evaluating pellet culture of human mesenchymal stem cells [35]. Our data further show that in a growth factor competent (TGF-β3) chondrogenic medium, (ADSC)Ch express significantly more Col-I and Agg and less Col-II than (NA)Ch. Moreover, the increased Agg levels show that at the high cell density in the pellet, the unwanted gradual shift from synthesis of large aggregating proteoglycans such as Agg to lower molecular weight proteoglycans such as versican and thus dedifferentiation is prevented. Our results are overall consistent with the observations by a number of groups in stem cells from

various sources. Karlsson et al. reported that decreased expression of Col-I was accompanied by increased expression of Col-IIA and IIB in articular chondrocytes, whereas mesenchymal stem cells-derived chondrocytes upregulated Col-I, IIA, and IIB [36]. Equally, Mahmoudifara and Doran, when comparing human fetal chondrocytes and (ADSC)Ch grown in the presence of growth factors (in this case insulin and TGF-β1) on nonwoven-mesh polyglycolic acid (PGA) scaffolds, determined that (ADSC)Ch produced more total collagen and reduced Col-II [37]. Conversely, a study by SedaTigli et al. reported quite the opposite in the sense that Col-II expression levels in ADSCs were higher than in chondrocytes [38].

Increased Col-I production has been described to characteristically occur during differentiation of ADSCs [39-41] and BMSCs [40, 42] into chondrocytes, and co-expression of Col-I and II is characteristic of fibrocartilage formation rather than articular cartilage. On the other hand, these conflicting reports might be a direct consequence of a myriad of circumstances, including age of individuals, the anatomic location from which the samples were taken and therefore cell source differences are important to consider with regard to chondrogenic outcomes, and not in the least, the use of divergent culture conditions such as growth factors (and combinations thereof) and scaffold materials. For instance, Jurgens and co-workers showed that fat cells removed from the abdomen have a chondrogenic and osteogenic potential that is significantly higher than cells isolated from the hip or tight [43].In addition, research by Rada et al. revealed significant differences in chondrogenic potential in various subpopulations of ADSCs, which might be used to further augment chondrogenesis in pellet culture [44]. Finally, several of the aforementioned studies generally based their observations on gene expression of ECM components. Gene expression does not necessarily reflect protein expression or activity, since gene expression outcomes can still be modulated downstream through processes such as gene silencing or protein degradation [45]. Nonetheless, the presence of Col-I is undesirable in tissue-engineered articular cartilage constructs, since elevated levels of Col-I have been shown to promote ossification of transplanted stem cell-based constructs rather than establishment of a stable chondrogenic phenotype [42]. Overall, our results point in the direction of stem cell differentiation into a mixed cartilage phenotype; albeit that this is less pronounced than the results by the aforementioned groups. Furthermore, the data presented here underscore the importance of TGF-β3 (TGF-β1,2,3 have been shown to stimulate proteoglycan and Col-II synthesis [46]), since the absence of this growth factor nearly completely ameliorated protein expression of Col-I. It is likely that the concentration and/or duration of TGF-β3 exposure in our system might be used to steer the chondrogenic phenotype and ECM composition outcome, especially since TGF-β3 does not seem to affect Agg expression; in fact we obtained similar results as far as Agg is concerned when growing ADSCs in alginate beads [17]. Future investigations should clarify to which extent this fact can be used as a tool and if the dynamic presence of TGF-β3 affects other components of cartilage ECM and other proteoglycans. What is also obvious from our results is that when TGF-β3 is not present, (NA)Ch pellets show irregular edges, which might be a direct result of the nearly two-fold reduction in Agg. Finally, the fact that Col-I synthesis collapses when TGF-β3 is not present during pellet culture shows the importance of this growth factor in ECM synthesis.

4. CONCLUSIONS

Overall, it may be concluded that although less type II and more type I collagen was produced by ADSCs, these are still able to produce large amounts of Agg, which is an essential component of the ECM in articular cartilage. Low production of collagen type II by these cells may be compensated for by providing optimum conditions, such as optimal concentration and exposure duration of growth factors such as TGF-□3. Pellet culture clearly offers a stabile chondrogenic and three-dimensional environment for cartilage engineering, and ADSCs represent a viable, easily obtainable cell source for autologous cartilage replacement.

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6. ACKNOWLEDGEMENTS

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