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RESEARCH ARTICLE

Adipose stem cells differentiated chondrocytes regenerate damaged cartilage in rat model of osteoarthritis

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Abstract

Transplantation of mesenchymal stem cells (MSCs) or autologous chondrocytes has been shown to repair damages to articular cartilage due to osteoarthritis (OA). However, survival of transplanted cells is considerably reduced in the osteoarthritic environment and it affects successful outcome of the transplantation of the cells. Differentiated chrondroytes derived from adipose stem cells have been proposed as an alternative source and our study investigated this possibility in rats. We investigated the regenerative potential of ADSCs and DCs in osteoarthritic environment in the repair of cartilage in rats. We found that ADSCs maintained fibroblast morphology in vitro and also expressed CD90 and CD29. Furthermore, ADSCs differentiated into chondrocytes, accompanied by increased level of proteoglycans and expression of chondrocytes specific genes, such as, Acan, and Col2a1. Histological examination of transplanted knee joints showed regeneration of cartilage tissue compared to control OA knee joints. Increase in gene expression for Acan, Col2a1 with concomitant decrease in the expression of Col1a1 suggested formation of hyaline like cartilage. A significant increase in differentiation index was observed in DCs and ADSCs transplanted knee joints ($P = 0.0110$ vs. $P = 0.0429$) when compared to that in OA control knee joints. Furthermore, transplanted DCs showed increased proliferation along with reduction in apoptosis as compared to untreated control. In conclusion, DCs showed better survival and regeneration potential as compared with ADSCs in rat model of OA and thus may serve a better option for regeneration of osteoarthritic cartilage.

Keywords: ADSCs; DCs; osteoarthritis; rat model of osteoarthritis

Introduction

Osteoarthritis (OA) is caused due to damages in articular cartilage, synovitis and bone alterations due to edema, tissue granulation, cyst formation, and sclerosis (Centeno et al., 2008; Harris et al., 2010; Van Pham et al., 2013). Cell transplantation therapies with autologous chondrocyte (AC) (Harris et al., 2010; Niemeyer et al., 2014) and bone marrow derived mesenchymal stem cells (BMSCs) are used clinically to treat osteoarthritis (Centeno et al., 2008; Wong et al., 2013); however, limited availability of healthy chondrocytes and dedifferentiation during culture expansion has restricted its use (Dehne et al., 2010). Availability of bone marrow is restricted due to invasive procedures used in its aspiration. Direct transplantation of undifferentiated BMSCs leads to complications such as fibrogenesis, over-calcification, and heterotrophic tissue formation (Chen et al., 2009). These issues merit the development of other strategies with ability to address these limitations and treat cartilage injuries more effectively. Adipose tissue is rich alternate supply for isolation of stem cells and has the advantage that these can be obtained under local anesthesia with relatively little discomfort and less donor site morbidity (Van Pham et al., 2013; Perdisa et al., 2015). Adipose stem cells (ADSCs)

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Abbreviations: ADSCs, adipose tissue derived stem cells; Acan, aggrecan; AC, autologous chondrocytes; β-actin, beta actin; BM-MSCs, bone marrow derived mesenchymal stem cells; Col2a1, collagen type 2 alpha 1; Col1a1, collagen type 1 alpha 1; DCs, differentiated chondrocytes; DMEM, Dulbecco's Modified Eagle's medium; EDTA, ethylenediaminetetraacetic acid; Fig, figure; MSCs, mesenchymal stem cells; OA, osteoarthritis; P3, passage 3; PRP, platelet rich plasma; PCNA, proliferating cell nuclear antigen; SD, Sprague–Dawley

can be differentiated into mesodermal lineages, such as osteocytes, chondrocytes, and adipocytes (Zuk, 2010). Attention in clinical use of these cells arose from a case study reported by Pak (2011), where intra-articular injection of autologous ADSCs with Platelet Rich Plasma (PRP), hyaluronic acid, and dexamethasone was used to treat osteoarthritis. The author reported increased volume of meniscus cartilage in osteoarthritis, which suggests that regeneration is attributable to the ADSCs treatment. Use of ADSCs not only provides alternative cells but also allows manipulation to provide the best cells for implantation.

It has been reported that during chondrogenic differentiation in vitro stem cells synthesize aggrecan link protein, collagen type II, and other proteins, which are component of normal articular cartilage (Moulharat et al., 2004; Wajid et al., 2013). Previously, it was reported in sheep model that autologous chondrogenically induced ADSCs and BMSCs can regenerate osteoarthritic cartilage (Ude et al., 2014). We used rat model of OA because they are inexpensive and easy to handle and their cartilage is thick enough to induce both partial and full thickness cartilage defects (Gerwin et al., 2010). Rat models has been used for study of cartilage restoration techniques like stem cells transplantation (Kuroda et al., 2006), gene therapy (Gelse et al., 2003), and growth factor treatments (Nishida et al., 2004). Rats have very little spontaneous degeneration in their knee joints so lesions observed are generally a result of the surgical manipulation only (Smale et al., 1995). In the background of the above, this study was planned to assess the potential of differentiated chondrocytes (DCs) and ADSCs to regenerate damaged cartilage in osteoarthritic environment in a rat model.

Material and methods

Animals

Three to four months old female Sprague–Dawley (SD) rats were used. Animals were handled according to guidelines of the animal care committee, National Center of Excellence in Molecular Biology, University of the Punjab, Lahore, Pakistan.

Isolation and expansion of stem cells from adipose tissue

Lower abdominal adipose tissue was used to isolate stem cells by digesting with collagenase 1 (Sigma–Aldrich, USA) as reported previously (Meric et al., 2013). Cells were cultured in DMEM (Sigma Cat No. D 5523) low glucose supplemented with 15% fetal bovine serum (FBS; Sigma Cat No. F9665). Cells were sub-cultured at 70–80% confluent. All the subsequent experiments were performed at passage 3 (P3).

Immunophenotyping

Immunophenotyping of P3 ADSCs was performed to check the mesenchymal nature by flow cytometry (Dominici et al., 2006). Briefly, cells were stained with conjugated antibodies for CD90-PE, CD29-FITC, CD106-FITC, and CD45-FITC (BD Biosciences, USA). Samples were analyzed in triplicates along with isotype controls by fluorescent activated cell sorting (FACS) calibur flow cytometer (BD Biosciences).

Chondrogenic differentiation of ADSCs

ADSCs were differentiated in chondrocytes as reported earlier (Johnstone et al., 2006; Wajid et al., 2013). Briefly, ADSCs were subjected to DMEM HG-serum free media (high glucose; Sigma–Aldrich) containing 0.1% bovine serum albumin (BSA; MP Biomedicals, France), 1% insulin, transferrin, and selenium (Sigma–Aldrich), 100 mM sodium pyruvate (Sigma–Aldrich), 4 mM L-glutamine (Invitrogen, USA), 10 ng/mL transforming growth factor-β1 (Invitrogen, USA), 0.05 mM L-ascorbic acid-2-phosphate (Sigma–Aldrich), and 100 nM dexamethasone (Sigma–Aldrich). Chondrogenic medium was replaced at alternate days. At day 7, chondrogenesis was evaluated by gene expression, Safranin-O staining, and immunocytochemistry (Johnstone et al., 2006).

Immunocytochemistry

DCs were characterized by immunocytochemistry analysis for hyaline cartilage specific markers Acan and Col2a1 as reported (Bhatti et al., 2013). Col2a1 (ab116242, UK) and Aggrecan (ab36861, UK) in a dilution of 1:1000 and 1:100 were used, respectively. Twenty-five to thirty images per sample were randomly taken by Olympus IX51 microscope (Olympus, USA) and analyzed.

Animal model of surgically induced osteoarthritis

Female SD rats aged 3–4 weeks weighing 180 ± 20 g were kept under controlled temperature and light conditions, with food and water ad libitum. Rats were randomly separated into four groups ($n = 12$ per group) as illustrated in Table 1. OA Rat model was developed by transaction of

Table 1 Rat control and experimental groups used in in vivo studies

Group	Description
N	Normal control rat
OA	Model osteoarthritis control
ADSCs	Model osteoarthritis ADSCs transplanted
DCs	Model osteoarthritis differentiated chondrocytes transplanted

anterior cruciate ligament and medial meniscus resection (ACLT + MMx) as reported (Kaufman et al., 2011; Xu et al., 2013). Systemic analgesia (0.1 mg/kg buprenorphine-HCl) was provided by subcutaneous injection. Surgical procedure was performed under a surgical microscope. Operated animals were left for 21 days in separate cages before cell transplantation.

Transplantation of DCs and ADSCs

DCs and ADSCs were labeled with PKH26 (Sigma–Aldrich) according to the manufacturer's instructions. Animal was anesthetized, right knee was shaved, disinfected with povidone-iodine, and a parapatellar 1 cm skin incision was made on the medial side of joint. The knee joint was maintained in extension and 28G needle was inserted under the patellar tendon into the joint space. 1×10^5 PKH26 labeled cells in 100 μL 1× PBS were transplanted by direct intra-articular injection into the knee joint space in respective groups. One hundred microliter 1× PBS was injected in each control OA animal model. Animals were euthanized by chloroform (Merck, Germany) after 4 weeks of transplantation for further analysis.

Histological analysis

Whole knee joints were fixed in 10% buffered formalin and decalcified in ethylenediaminetetraacetic acid (EDTA, Merck Germany) for 8–10 weeks as reported (Schmitz et al., 2010). Samples were embedded by immersing in fresh molten paraffin. Sections were mounted on glass slides. Proteoglycan content was examined by Safranin-O/Fast green staining and scored semi-quantitatively by modified Mankin's histological score (Little et al., 2010). Twenty-five to thirty images per sample were taken by an Olympus BX61 microscope (Olympus).

Semi-quantitative real time PCR

Rat's knee joint cartilage (n = 6 rats each group) was crushed in Trizol reagent (Invitrogen; Cat No. 15596–018) by a homogenizer to extract RNA. cDNA synthesis kit was used to synthesize cDNA from 1 μg of total RNA. Semi quantitative real time gene analysis (Supplementary Table S1) was performed by using SYBR Green PCR Super Mix (BioRad Lab, USA) on Piko Real™ Real-Time Polymerase Chain Reaction detection system (Thermo Scientific, USA). As control, beta actin (β-actin) gene was used.

Statistical analysis

Graph Pad Prism 5.00 for Windows (Graph Pad Software, USA) was used for statistical analysis. Data were expressed as mean ± SD. Shapiro–Wilk test and Levene's test were used for normal distribution and equality of variance of data respectively. One-way ANOVA followed by Dunnett's multiple-comparison post-test was performed for the comparison of group mean differences against a control group. Student's *t*-test was applied for unpaired comparison between two groups. Statistical significance was considered at $P \leq 0.05$.

Results

Characterization of ADSCs

ADSCs showed expression of mesenchymal stem cell makers, CD90 (84.77 ± 4.19%) and CD29 (94.22 ± 2.0%), low level expression for hematopoietic stem cells marker CD45 (2.31 ± 1.40%) and endothelial marker CD106 (0.32 ± 0.12%) by FACS analysis (Figure 1). Cultured ADSCs had good proliferation rate, formed monolayer when confluent and maintained fibroblast-like morphology in repeated sub-cultures as observed under phase contrast microscope (Figure 2A).

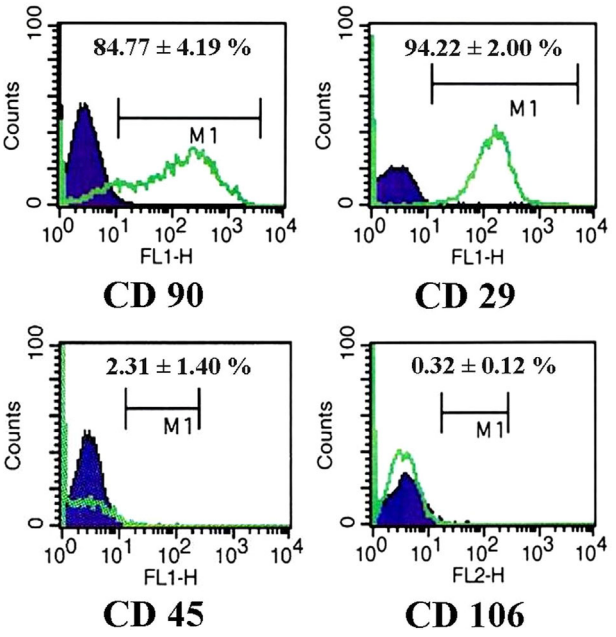

Figure 1 Characterization of ADSCs. ADSCs were characterized by Mesenchymal and hematopoietic markers, CD 90, CD 29, CD 106, and CD 45 by flow cytometry. Blue represents isotype control; green represents marker expression and M1 showing Marker designating positive events. The percentage of expression is recorded from three different sets of experiments and data are expressed as means ± SD (n = 3).

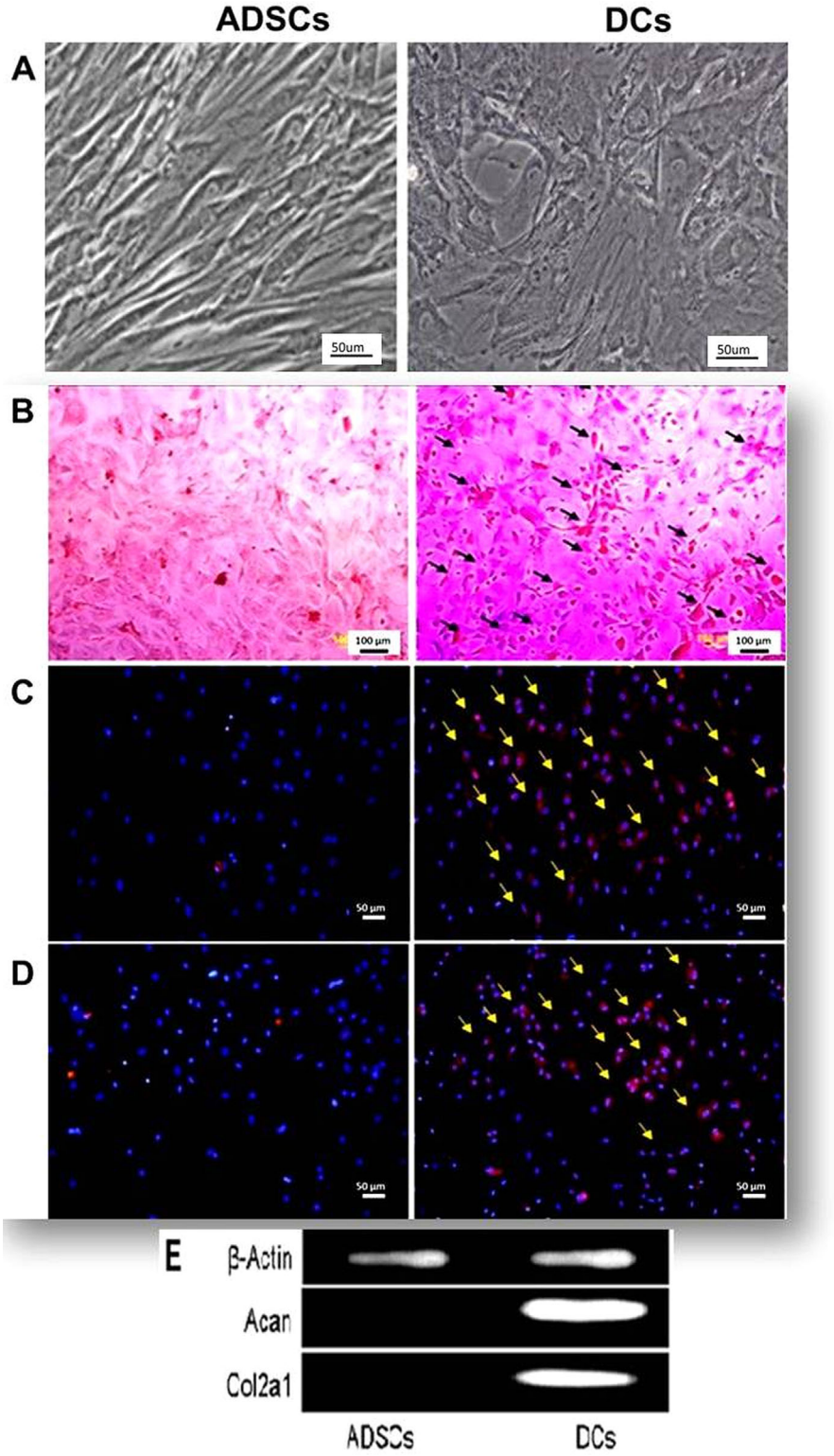

Figure 2 Differentiation of ADSCs into chondrocytes. (A) Gross morphology (B) Safranin O staining of adipose stem cells (ADSCs) and differentiated chondrocytes (DCs) for Proteoglycan content. (C) Immunocytochemical analysis of Col2a1 and (D) Acan. Arrows showing Acan and Col2a1 positive cells. (E) RT-PCR analysis of Col2a1 and Acan gene.

In vitro chondrogenesis of ADSCs

In vitro morphology revealed increased aggregation of cells and matrix of proteoglycans in chondrogenic induction medium that demonstrates differentiation of ADSCs into chondrocytes. Safranin-O staining confirmed the synthesis of proteoglycans (Figure 2B). Positive expression of chondrogenic markers Acan and Col2a1 by immunocytochemistry (Figures 2C and 2D) along with gene expression analysis (Figure 2E) further supported chondrogenesis of ADSCs.

Gross evaluation and histological score of knee joints

Gross morphological features of the knee joints from various treatment groups were compared with untreated normal group. Osteoarthritic knee joint showed formation of hard, thick, yellowish fibrotic tissue and osteocyte formation over the whole joint space. Transplantation of DCs and ADSCs resulted in marked reduction in fibrosis along with the formation of soft neo-cartilage (Figure 3). Proteoglycan contents of knee joints of each group were determined by Safranin-O/Fast Green staining (Figure 4A). Normal control group showed smooth cartilage with even margins and chondrocytes were evenly distributed. On the contrary, osteoarthritic knee joint showed severe proteoglycan loss, formation of osteophytes, and fibrillation. Transplantation of ADSCs and DCs resulted in marked reduction in fibrosis along with the formation of hyaline-like neo-cartilage. The repair of cartilage was semi-quantified by modified Mankin score that consists of a full score of 15 (Figure 4B). Higher histological score indicates repair of the damaged cartilage. DCs group showed formation of cartilage tissue at femorotibial groove with good integration, thickness and surface regularity (*Mankin score*: 9.00 ± 1.00 in group DCs vs. 4.00 ± 1.00 in group OA; $P = 0.0036$). In ADSCs group mild proteoglycan loss was observed along with slight fibrillation (*Mankin score*: 6.00 ± 1.00 in group ADSCs vs. 4.00 ± 1.00 in group OA; $P = 0.0705$). Histological score of DCs transplanted group was more significant as compared to ADSCs transplanted group (*Mankin score*: 9.00 ± 1.00 in group DCs vs. 6.00 ± 1.00 in group ADSCs; $P = 0.0213$; Figure 4B). DCs transplanted group showed significant regeneration of cartilage as compared to ADSCs transplanted group.

Chondrogenic profile of cartilage tissue

Semi quantitative real time PCR analysis of cartilage tissue also correlated with histological observations (Figure 5). OA control group demonstrated down-regulation in the expression level of both hyaline cartilage specific genes, Acan (0.08 ± 0.02-fold) and Col2a1 (0.30 ± 0.04-fold) and up-regulation of fibro cartilage gene Col1a1 (2.72 ± 0.74-fold). Transplantation of DCs and ADSCs into knee joints significantly enhanced the expression of Acan. Significant difference between expression of Acan in DCs (7.20 ± 1.40-fold; $P = 0.0009$) and in ADSCs transplanted group (3.40 ± 0.52-fold; $P = 0.0004$) was observed as compared to OA control (0.08 ± 0.02-fold; Figure 5A). The expression level of Col2a1 between DCs and ADSCs transplanted knee joints (1.90 ± 0.91-fold vs. 1.20 ± 0.48-fold; $P = 0.2704$; Figure 5D) was not significant. On the other hand there was significant down regulation of Col1a1 gene expression in DCs (0.40 ± 0.08-fold; $P = 0.0058$) and ADSCs (0.71 ± 0.14-fold; $P = 0.01$) transplanted groups as compared to OA control (Figure 5E). Decrease differentiation index of OA knee joints (0.11 ± 0.021-fold) as compared to normal

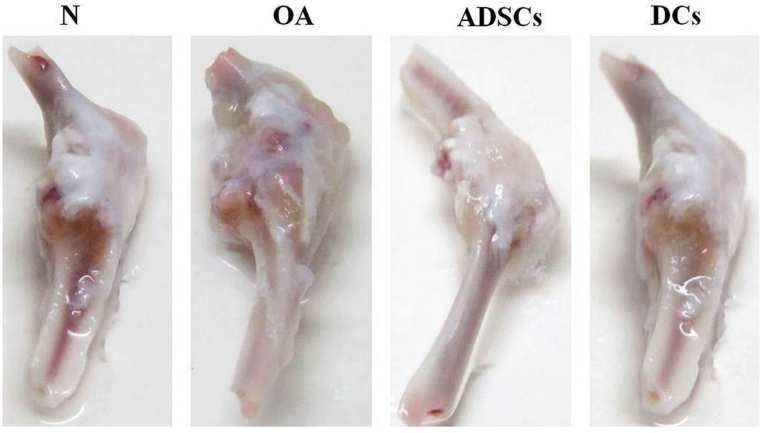

Figure 3 Morphological analysis of rat knee joint. The differentiated chondrocytes injected in the osteoarthritis rat model showed better efficiency in regenerating the injury and is closer to the normal knee morphology as compared to adipose stem cells. Normal (N), osteoarthritic (OA), adipose stem cells transplanted (ADSCs), differentiated chondrocytes transplanted (DCs).

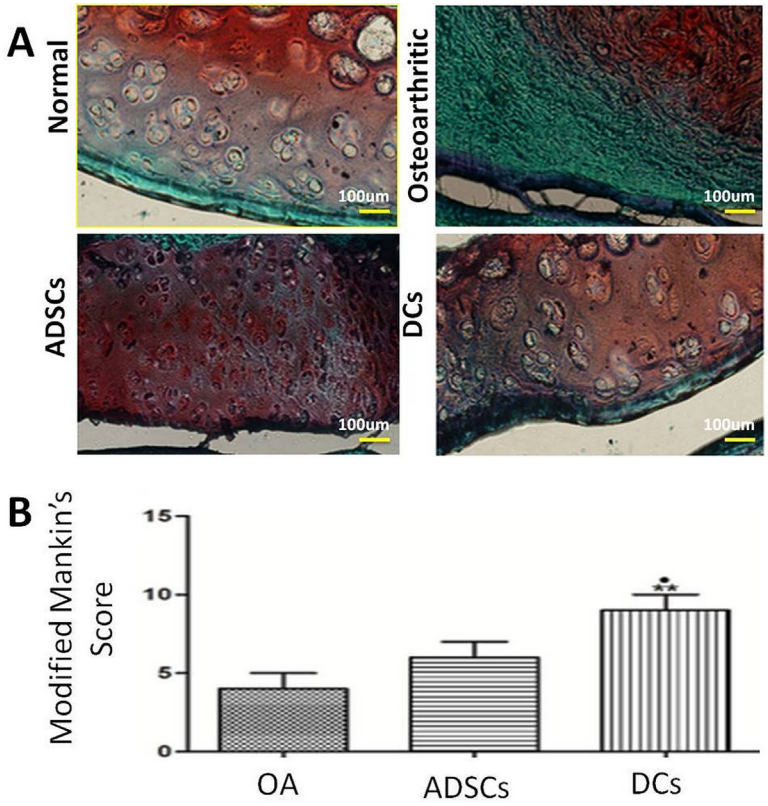

Figure 4 Histology of DCs and ADSCs transplanted rat knee model. (A) Proteoglycan content of knee joints stained by Safranin-O/Fast Green (N), normal, (OA) osteoarthritic, (ADSCs) ADSCs transplanted and (DCs) differentiated chondrocytes transplanted knee joint. (B) Histological score of knee joints (OA) osteoarthritic, (ADSCs) ADSCs transplanted and (DCs) differentiated chondrocytes transplanted. Data are presented as mean ± SD (n = 3) •$P < 0.05$ versus osteoarthritic knee joint (OA). *$P < 0.01$ versus osteoarthritic knee joints.

knee joints was a sign of deterioration. Differentiation index improved significantly in DCs (4.70 ± 1.80-fold; $P = 0.0110$) and ADSCs transplanted group (1.80 ± 0.99-fold; $P = 0.0429$) as compared to OA knee joints.

Analysis of apoptosis in cartilage tissue

Expression level of Casp3 gene (Figure 5C) revealed up-regulation in OA control group as compared to normal control (5.30 ± 0.97-fold). Casp3 expression level significantly decreased in DCs (0.66 ± 0.14-fold; $P = 0.0012$) and ADSCs transplanted knee joints (2.20 ± 0.73-fold; $P = 0.0111$). Significant difference was also observed between DCs and ADSCs transplanted knee joints (0.66 ± 0.14-fold vs. 2.20 ± 0.73-fold; $P = 0.0226$).

Cellular proliferation in cartilage tissue

Cellular proliferation in the newly formed cartilage tissue was analyzed by PCNA gene expression (Figure 5B). There was more significant fold increase in PCNA gene expression in DCs transplanted knee joints (12.00 ± 3.50-fold; $P = 0.0040$). ADSCs transplanted joints (6.10 ± 0.69-fold; $P = 0.0001$) also revealed increase PCNA level. Although down-regulation in the expression of PCNA (0.40 ± 0.02-fold) gene was observed in OA knee joints as compared to normal knee joints.

Discussion

Articular cartilage has reduced capacity for repair, hence its regeneration potential is considered as an important challenge. There are two cell-based treatments for cartilage repair, the stem cell-based cell therapy, and autologous chondrocyte implantation (ACI; Viste et al., 2012; Wu et al., 2013; Perdisa et al., 2015). Transplantation of undifferentiated mesenchymal stem cells can lead to complications such as fibrogenesis, heterotrophic tissue formation, and over-calcification (Chen et al., 2009; Puetzer et al., 2010). Outcome of autologous chondrocytes transplantation has also been unsatisfactory due to decrease in their proliferative capacity with increasing age (Kuo et al., 2006) and difficulties in maintaining chondrocyte specific phenotype. Hence, the differentiation of stem cells before transplantation is a

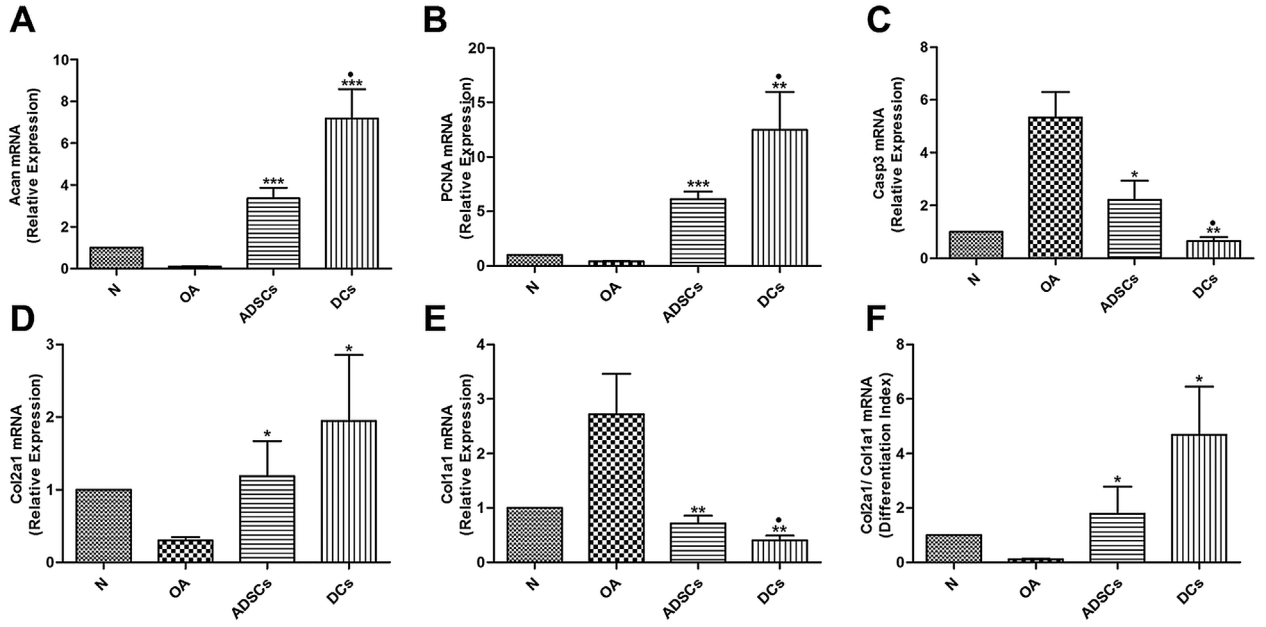

Figure 5 Gene expression analysis in transplanted rat knee model. Semi-quantitative expression of Acan, PCNA, Casp3, Col2a1, and Col1a1 in different groups, (N) normal control, (OA) osteoarthiric, (ADSCs) ADSCs transplanted, and (DCs) differentiated chondrocytes transplanted, respectively, differentiation index of Col1a1 and Col2a1. Data are presented as mean ± SD (n = 3). (*) represents DC versus OA and (•) represents DC versus ADSCs. $^{*}P$ or $^{\bullet}P < 0.05$, $^{**}P$ or $^{\bullet\bullet}P < 0.01$, and $^{***}P$ or $^{\bullet\bullet\bullet}P < 0.001$.

strategy to lock the mesenchymal stem cells into the desired differentiation state, that is, chondrocytes (Dickhut et al., 2009). Earlier in vitro studies have revealed that transforming growth factor beta (TGF-β), Ascorbate 2 phosphate (A-2P), and dexamethasone containing media support the synthesis of proteoglycans (Johnstone et al., 2006; Chubinskaya et al., 2011; Viste et al., 2012; Ude et al., 2014).

In this study, we selected ADSCs because they are easy to harvest with less donor site morbidity, rapid expansion and high proliferation rate. ADSCs are more abundant, about 5% of nucleated cells versus 0.0001–0.01% of BMSCs (Zuk et al., 2002; Spencer et al., 2012). We selected autologous ADSCs over autologous chondrocytes because osteoarthritis is a disease of late age and cartilage ageing drives cellular changes that result in damage-induced secretion of chemokines, cytokines, and proteases (Loeser, 2011, 2013). Further, autologous ADSCs has been shown to repair human bone osteonecrosis and cartilage damaged meniscus in human osteoarthritic knee (Pak, 2011; Pak et al., 2013; Jo et al., 2014; Koh et al., 2014).

In background of the above, this study assessed the effect of ADSCs differentiated chondrocytes versus ADSCs transplantation into the rat model of OA. In vitro characterization of ADSCs exhibited mesenchymal stem cells markers, which is in agreement with the previous studies (Dominici et al., 2006; Khan et al., 2012). The main collagen and proteoglycan in articular cartilage are Col2a1 and Acan (Cha et al., 2013; Rosenzweig et al., 2013; Sakata et al., 2013). Hyaline cartilage is characterized by positive expression of these markers and they play a vital role to repair the damaged cartilage (Hu et al., 2012; Cha et al., 2013). Expression of Acan and Col2a1 in DCs evidently indicated that ADSCs were successfully differentiated into chondrocytes in vitro. Unique polygonal morphology in monolayer culture and staining of proteoglycans by Safranin-O is in agreement with earlier reports (Gosset et al., 2008). Morphological features, such as fibrosis and full-thickness defect, were observed in surgically induced rat model of OA involving transaction of anterior cruciate ligament (ACL) as reported previously (Mainil-Varlet et al., 2012; Naraoka et al., 2013). Yellow fibrotic thick tissue over whole joint was observed in OA control rat knee as compared to normal control knee. Transplantation of ADSCs and DCs diminished these effects with better gross morphology seen in DCs transplanted knee joints.

The stability of matrix of articular cartilage is maintained by proteoglycans and collagens (Halonen et al., 2013) and their loss is linked with cartilage damage associated with osteoarthritis (Wang et al., 2013). Histological analysis of OA control rat showed fibrotic tissue and damaged cartilage surface, whereas engineered cartilage displayed smooth cartilage surface as well distribution of lacunae and chondrocytes. DCs and ADSCs transplanted rat's knee displayed cartilage regeneration. The superficial area was smooth with nearly comparable cartilage thickness to the normal knee. These findings are similar to previous studies

that showed cartilage formation and proteoglycan synthesis after transplantation of differentiated chondrocytes. These chondrocytes were either differentiated from bone marrow or adipose-derive MSCs in sheep OA models (Al Faqeh et al., 2012; Ude et al., 2014). In addition to their findings, we observed significantly higher histological score in knee joints transplanted with DCs as compared with those transplanted with ADSCs.

Expression of Acan and Col2a1 is vital for the integrity and maintenance of cartilage tissue. They play a vital role in the repair of the damaged cartilage. Hyaline cartilage present at the articular surfaces is characterized by the expression of Col2a1 as the major collagen and Acan as the major proteoglycan. These components have key role in preventing hypertrophy of chondrocytes (Nugent et al., 2011). Semi quantitative gene expression analysis of control OA cartilage showed significantly increased expression of fibro-cartilage gene (Col1a1) with a corresponding decrease in hyaline cartilage genes, Col2a1 and Acan expression as reported earlier (Diaz-Romero et al., 2008; Aini et al., 2012). In DCs group, the increase in the expression of Col2a1 and Acan gene was more significant as compared to ADSCs transplanted group. Increase in the differentiation index of DCs group is indication of formation of hyaline-like cartilage in the transplanted knee joints (Marlovits et al., 2004; Diaz-Romero et al., 2008).

Apoptosis is a well recognized event of cellular death and increase in the expression of apoptotic marker Casp3 has been reported with the development of OA (Gao et al., 2011). Our data demonstrated that expression of Casp3 was increased in osteoarthritic knee joints with significant decrease in DCs as compared to ADSCs transplanted knee. Initiation of proliferation of chondrocytes to repair the damaged cartilage due to OA has been suggested (Pfander et al., 2001; Lugo et al., 2012). DCs and ADSCs transplanted knee joints showed increase in the expression level of PCNA as compared to OA thus indicates survival of transplanted cells, which helped to regenerate the damaged cartilage surface.

Conclusions

This study demonstrated that ADSCs differentiated chondrocytes has more proteoglycan, collagen, and aggrecan expression in vitro. Furthermore, DCs transplanted group showed significant reduction in the expression of fibrocartilage related gene Col1a1 and apoptotic associated gene Casp3 with a corresponding increase in hyaline cartilage specific genes Col2a1 and Acan. Safranin-O/Fast green staining of DCs and ADSCs-transplanted knee joints demonstrated improved cartilage surface as compared to OA control rats. We suggest that DCs may help to repair the damaged cartilage. Further studies are needed to compare DCs with ADSCs at the molecular level to elucidate the mechanisms involved in the repair of cartilage tissue.

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Author disclosure statement

No competing financial interests exist.

Conflict of interest

The authors declare that they have no competing interest.

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Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web-site.

Table S1. Primer sequences, product size and annealing temperature of genes used for expression analysis of genes. β-Actin: beta-actin, Col2a1: collagen type-II alpha, Acan: aggrecan, PCNA: proliferating cell nuclear antigen, Casp3: caspase 3, Col1a1: collagen type-I alpha. qPCR was performed according to the following conditions: initial denaturation at 95°C for 5 min, 35 cycles of amplification (denaturation at 95°C for 45 s, annealing at optimized temperature for specified marker for 45 s, and extension at 72°C for 45 s) and a final extension at 72°C for 10 min.