Acta Histochemica 116 (2014) 763–770

Contents lists available at ScienceDirect

Acta Histochemica

journal homepage: www.elsevier.de/acthis

Characterization of colony-forming cells in adult human articular cartilage

Ozlem Ozbey[a], Zeliha Sahin[b], Nuray Acar[a], Filiz Tepekoy Ozcelik[a], Alpay Merter Ozenci[c], Sadi Koksoy[d], Ismail Ustunel[a,*]

[a] Department of Histology and Embryology, College of Medicine, Akdeniz University, Antalya 07070, Turkey
[b] Department of Histology and Embryology, College of Medicine, Near East University, Nicosia 922022, Mersin-10, Turkey
[c] Department of Orthopedics and Traumatology, College of Medicine, Akdeniz University, Antalya 07070, Turkey
[d] Department of Medical Microbiology, College of Medicine, Akdeniz University, Antalya 07070, Turkey

ARTICLE INFO

Article history:
Received 12 December 2013
Received in revised form 7 January 2014
Accepted 8 January 2014

Keywords:
Articular cartilage
Colony-forming cells
CD90
STRO-1
CD105
CD166
OCT-3/4

ABSTRACT

Recent studies have shown that adult human articular cartilage contains stem-like cells within the native structure. In this study, we aimed to determine the localization of putative stem cell markers such as CD90, STRO-1, OCT-3/4, CD105 and CD166 in adult human articular cartilage tissue sections and demonstrate the expression of these markers within the expanded surface zone colony-forming (CF) cells and evaluate their differentiation potential. Biopsy samples were either fixed immediately for immunohistochemical analyses or processed for *in vitro* cell culture. Immunohistochemical and flow cytometry analyses were performed by using CD90, STRO-1, OCT-3/4, CD105 and CD166 antibodies. Isolated colony-forming (CF) cells were further stimulated, by using the appropriate growth factors in their pellet culture, to obtain cartilage, bone and adipose lineages. We observed that the expression of the stem cell markers were in various zones of the human adult cartilage. Flow cytometry results showed that in CF cells the expression of CD90 and CD166 was high, while OCT-3/4 was low. We also determined that CF cells could be stimulated towards cartilage, bone and adipose lineages. The results of this research support the idea that the resident stem-like cells in adult human articular cartilage express these putative stem cell markers, but further experimental investigations are needed to determine the precise localization of these cells.

© 2014 Elsevier GmbH. All rights reserved.

Introduction

Articular cartilage is a highly organized avascular tissue comprising chondrocytes embedded within an extracellular matrix of collagens, proteoglycans and non-collagenous proteins. The chondrocytes degrade and synthesize the matrix proteins, which are essential for the tissue homeostasis. Articular cartilage has a highly organized structure composed of four zones: superficial zone, intermediate (or middle zone), radial zone (or deep zone) and calcified cartilage (or calcified zone) (Johnstone et al., 2013).

In some instances, traumatic lesions lead to chronic degeneration and may lead subsequently to osteoarthritis (OA). Owing to the limited inherent repair capacity of articular cartilage, damage to OA presents a major clinical challenge to clinicians (Nelson et al., 2010). The two most traditional methods to restore damaged articular cartilage are microfracture and autologous chondrocyte implantation (ACI), both of which are considered to have acceptable short-term results, with better clinical outcomes in younger and more active patients, but not in the long-term (Brittberg et al., 1994; Knutsen et al., 2004; Nelson et al., 2010; Triche and Mandelbaum, 2013). A disadvantage of the ACI procedure may be a significant loss of the cells from the perisutural region as a complication of the surgical procedure (Hunziker and Stahli, 2008). In addition, there are also problems related to chondrocyte de-differentiation during the expansion phase in monolayer culture and the poor integration of the implants into the surrounding cartilage tissue (Dell'Accio et al., 2001; Schnabel et al., 2002; Khan et al., 2009; Mobasheri et al., 2009).

Although surgical techniques to restore articular surfaces after cartilaginous injuries are still evolving, some experimental studies involve a new approach with the transplantation of chondrocytes and mesenchymal stem cells (MSC). Such a tissue engineering approach has the potential to stimulate the formation of a new articular surface (Singh et al., 2013). Regenerative medicine and more specifically tissue engineering, may provide novel treatment

Abbreviations: ACI, autologous chondrocyte implantation; CF, colony-forming; DMEM, Dulbecco's Modified Eagle's Medium; FBS, fetal bovine serum; HEPES, N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid; IBMX, 3-isobutyl-1-methylxanthine; ITS, insulin; MSC, mesenchymal stem cells; OA, osteoarthritis; PBS, phosphate buffered saline; TGF-β1, Transforming Growth Factor β1.

* Corresponding author at: Department of Histology and Embryology, Faculty of Medicine, Akdeniz University Campus, Antalya 07070, Turkey.
E-mail address: iustunel@akdeniz.edu.tr (I. Ustunel).

0065-1281/$ – see front matter © 2014 Elsevier GmbH. All rights reserved.
http://dx.doi.org/10.1016/j.acthis.2014.01.004

approaches to repair damage to skeletal structures caused by congenital, traumatic, or inflammatory conditions (Gao et al., 2007; Saha et al., 2013). Many of these approaches are still in early-phase clinical studies, gathering more information on the characteristics and differentiation of MSCs.

MSCs are multipotent progenitor cells traditionally found in the adult bone marrow stroma (Caplan, 1991; Prockop, 1997; Conget and Minguell, 1999). They can be expanded *in vitro* and, under appropriate conditions, can be differentiated into a variety of cell types, including osteocytes, chondrocytes, and adipocytes (Petersen et al., 1999; Pittenger et al., 1999; Brazelton et al., 2000; Mezey et al., 2000; Orlic et al., 2001; Alhadlaq and Mao, 2003, 2004; Poulsom et al., 2003). However, chondrocyte differentiated bone marrow stroma cells display an endochondral phenotype and form a calcified matrix, leading to failure in long-term defect repair (Shapiro et al., 1993; Muller and Kohn, 1999; Thornemo et al., 2005).

In previous studies in this field (Dowthwaite et al., 2004; Khan et al., 2009; Williams et al., 2010; McCarthy et al., 2012), a population of progenitor cells were isolated from bovine, equine and human articular cartilage. In these studies, articular cartilage progenitor cells were isolated using a previously described differential adhesion assay (Jones and Watt, 1993). High population doubling rates of these cells have shown that this method of isolation is indeed selecting for a cartilage progenitor population (Williams et al., 2010). These cells are able to form colonies and maintain their average telomere length (Khan et al., 2009; Williams et al., 2010; McCarthy et al., 2012). According to these studies results, these progenitor cells can be stem cell-like cells and located in the 2–3 cell layer of the superficial zone. They contribute to the limited regenerative capacity of the articular cartilage and are responsible for appositional growth of articular cartilage (Archer et al., 1994; Hayes et al., 2001; Dowthwaite et al., 2004).

In our previous study, we demonstrated the presence of stem cell-like cells in adult rat articular cartilage with the expression of CD-105 and CD-166 (Ozbey et al., 2010). We hypothesized that adult human articular cartilage has cells positive for putative stem cell markers such as CD90, STRO-1, OCT-3/4, CD105, CD166 and they have differentiation potential. In this study, we aimed to determine the localization of putative stem cell markers such as CD90, STRO-1, OCT-3/4, CD105 and CD166 in adult human articular cartilage sections by using immunohistochemistry and to analyze their phenotypical properties and differentiation potential using flow cytometry and *invitro* cell culture techniques.

Materials and methods

Patients and biopsy samples

Six patients aged 20–40 were included in the study ($n = 6$). These patients underwent arthroscopic meniscus tear surgery at the Department of Orthopedics and Traumatology of Akdeniz University Faculty of Medicine Hospital. These individuals had neither acute nor chronically apparent articular cartilage pathology of any kind. Biopsies, approximately 3 mm in size, were taken from the lateral margin of the femoral condyles, from a non-load bearing area of the joint. Biopsy samples were divided into two pieces. One piece was fixed immediately in 10% neutral buffered formalin at room temperature for approximately 24 h. Tissues were washed in tap water for 4 h before dehydration through a graded series of ethanol and embedding in paraffin wax. Transverse sections were cut on a rotary microtome (RM2125 RT, Leica Microsystems, Nussloch, Germany). The other piece of tissue was used for *invitro* cell culture experiments.

All studies were performed according to the declaration of Helsinki and International Association for the Study of Pain guidelines and all the procedures were approved by a local ethics committee of Akdeniz University (#5-380; 26.12.2006). Written informed consent was obtained from each patient, and extensive precautions were taken to preserve the privacy of the participants donating tissue.

Detection of CD90, STRO-1, OCT-3/4, CD105 and CD166 expression on articular cartilage tissue sections by immunofluorescence

For immunohistochemical labelling, 5 μm-thick tissue sections were mounted on Superfrost®Plus slides (Thermo Scientific, Menzel-Gläser, Braunschweig, Germany). After deparaffination in xylene, sections were rehydrated through a decreasing gradient of ethanol. To unmask antigens, an antigen retrieval procedure was performed by treating the samples in boric acid (2.1 g boric acid + 100 ml distilled water, pH 7.2–7.4, Merck) overnight at 56 °C. A universal blocking reagent (TA-125UB; Ultra UV Block, Lab Vision Corporation, Cheshire, UK) was applied for 3 min at room temperature in order to block non-specific binding. Sections were then incubated with primary antisera: rabbit polyclonal anti-CD90 at 1:100 dilution (sc-9163; Santa Cruz, CA, USA), mouse monoclonal anti-Stro-1 at 1:100 dilution (MAB1038; Minneapolis, USA), mouse monoclonal anti-Oct3/4 at 1:100 dilution (sc-5279; Santa Cruz, CA, USA), rabbit polyclonal anti-CD105 at 1:100 dilution (sc-20632; Santa Cruz, CA, USA) and goat polyclonal anti-CD166 at 1:100 dilution (sc-8548; Santa Cruz, CA, USA) overnight at +4 °C. Primary antibody bindings were detected using fluorescent conjugated secondary antibodies: Alexa Fluor donkey anti-mouse IgG at 1:500 dilution (A10037; Life Technologies, Invitrogen, Eugene, Oregon, USA), Alexa Fluor donkey anti-rabbit IgG at 1:500 dilution (A21207; Life Technologies, Invitrogen, Eugene, Oregon, USA) and Alexa Fluor donkey anti-goat IgG at 1:500 dilution (A11055, Life Technologies, Invitrogen, Eugene, OR, USA) for 30 min at room temperature. All dilutions and washes between stages were performed using phosphate-buffered saline (PBS; pH 7.4) and all steps were performed at room temperature unless otherwise stated.

Negative control staining was performed by replacing the primary antibodies with the appropriate non-immune IgG at the same concentration. Sections were mounted under Vectashield with DAPI (1 μg mL^{-1}; VectaLabs, USA) and photomicrographs were taken with fluorescent optics (BX61 microscope, Olympus Corp., Tokyo, Japan) fitted with a DP30BW digital camera (Fluorescence filters, U-MNIBA3, U-MNUA2, U-MNIGA3).

Labelling intensities and localization of CD90, STRO-1, OCT-3/4, CD105 and CD166 were evaluated in a semi-quantitative fashion including evaluation of the labelling intensity according to zones and represented as follows: 0 = negative; (+) = weak positive; + = positive; ++ = intense staining; and +++ = very intense staining. Two observers, blinded to the experimental groups performed the semi-quantitative evaluation and the inter-individual variation was 9%.

Chondrocyte isolation from articular cartilage and differential adhesion assay

Chondrocytes were isolated by sequential pronase (11459643001; Roche, Indianapolis, USA; 70 U ml^{-1}, 1 h at 37 °C) and collagenase (C0130, Sigma–Aldrich, St. Louis, MO, USA) 300 U ml^{-1}, overnight at 37 °C) digestions. Isolated cells were used for fibronectin adhesion assay as described previously (Dowthwaite et al., 2004). Six well plates were coated with 10 mg ml^{-1} fibronectin (FN; F1141 Sigma–Aldrich, Gillingham, Dorset, UK) in 0.1 M phosphate buffered saline (PBS, pH 7.4) containing 1 mM $MgCl_2$ and 1 mM $CaCl_2$ (PBS+) overnight at 4 °C.

Isolated chondrocytes (4000 cells ml^{-1}) were seeded onto the coated plates for 20 min at 37 °C in Dulbecco's Modified Eagle Medium (DMEM; 10566-024, Invitrogen, Eugene, OR, USA). After 20 min, media and non-adherent cells were removed. Fresh DMEM containing Penicillin 10,000 mg ml^{-1}/Streptomycin 10,000 U ml^{-1} (15140-122; Invitrogen), 0.1 mM Sodium L-ascorbate (A4034; Sigma–Aldrich), 0.5 mg ml^{-1} L-glucose (G6152; Sigma–Aldrich), 100 mM N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid (HEPES; 15630-106, Invitrogen), 1 mM sodium pyruvate (11360-039, Invitrogen), 2 mM L-glutamine (25030-024, Invitrogen) and 10% fetal bovine serum (FBS; 10106-169, Invitrogen) (DMEM+) were added to the remaining adherent cells.

Identification and isolation of colony-forming cells

Cells were cultured in 5% CO_2 at 37 °C (Thermo Fisher Scientific HERAcell 150, Franklin, MA, USA). Cells were observed microscopically for 15 days. Between day 10 and 15, colonies of over 32 cells were detected (Jones and Watt, 1993; Dowthwaite et al., 2004). Colonies were selected and isolated using sterile cloning rings (C7983; Sigma–Aldrich, UK). They were then expanded in fresh DMEM containing Penicillin 10,000 μg ml^{-1}/Streptomycin 10,000 U ml^{-1}, 0.1 mM Sodium L-ascorbate, 0.5 mg ml^{-1} L-glucose, 100 mM HEPES; 1 mM sodium pyruvate, 2 mM L-glutamine and 10% FBS (DMEM+). Colonies were cultured until they reached confluence in 3rd passage in 175 cm^2 flasks and medium was changed every 3 days (F7677, Sigma–Aldrich, St. Louis, MO, USA).

Flow cytometric analysis of colony-forming cells for CD90, STRO-1, OCT-3/4, CD105, CD166 and CD105/CD166

Expanded clonal cell lines were trypsinized and washed in CellWASH™ solution (349524, BD Biosciences, San Jose, ABD) and then incubated for 30 min at 4 °C in the dark with conjugated antibodies to CD90-FITC (Ab11155, Abcam, Cambridge, UK), OCT-3/4-FITC (IC1759F; R&D Systems, Minneapolis, USA), CD105-FITC (Ab53318; Abcam, Cambridge, UK), CD166-RPE (393-050; Ancell, Bayport, MN, USA) and non-conjugated antibody STRO-1 (MAB1038; R&D Systems, Minneapolis, USA). STRO-1 labelled cells were centrifuged at 7000 × *g* for 90 s. Supernatants were removed and cells were incubated for 20 s at 4 °C in the dark with conjugated secondary antibody (11-5890-82; eBioscience, San Diego, CA, USA). Labelled cells were washed three times in Cell WASH solution (554723; BD, CA, USA) and were analyzed using a FACSCanto II Flow Cytometer (BD, California, USA) and FACSDiva software (BD, San Jose, CA, USA). The appropriate IgG controls were run in parallel (555909, BD Pharmingen, San Jose, CA, USA; 553990; BD Pharmingen, CA, USA). A minimum of three clones ($n = 6$) were analyzed for each antibody.

Immunolocalization of CD90, STRO-1, OCT-3/4, CD105, CD166 and CD105/CD166 in colony-forming cells

Monolayer cultures of colony-forming cells were fixed and subjected to immunofluorescence for antibodies CD90, STRO-1, OCT-3/4, CD105 and CD166. The cells were washed repeatedly in PBS (3 × 5 min) and fixed in 95% ice-cold ethanol for 10 min. The cells were incubated with primary antibodies diluted in PBS (2 mg ml^{-1}), rabbit polyclonal anti-CD90 at 1:100 dilution (sc-9163; Santa Cruz, CA, USA), mouse monoclonal anti-Stro-1 at 1:100 dilution (MAB1038; Minneapolis, USA), mouse monoclonal anti-Oct3/4 at 1:100 dilution (sc-5279; Santa Cruz, CA, USA), rabbit polyclonal anti-CD105 at 1:100 dilution (sc-20632, Santa Cruz, CA, USA), goat polyclonal anti-CD166 at 1:100 dilution (sc-8548; Santa Cruz, CA, USA), overnight at +4 °C. They were next washed three times in PBS and were incubated with appropriate FITC-conjugated and TR-conjugated secondary antibodies (2 mg ml^{-1}) diluted in PBS; Alexa Fluor donkey anti-mouse IgG at 1:500 dilution (A10037, Life Technologies, Invitrogen, Eugene, OR, USA), Alexa Fluor donkey anti-rabbit IgG at 1:500 dilution (A21207; Life Technologies, Invitrogen, USA), Alexa Fluor donkey anti-goat IgG at 1:500 dilution (A11055; Life Technologies, Invitrogen, USA). Cells were washed in PBS and mounted in Vectashield with DAPI (1 μg mL^{-1}; Vector Labs, USA). Cells were finally analyzed and photographed using a fluorescence microscope.

Immunolabelling intensities and localization of CD90, STRO-1, OCT-3/4, CD105, CD166 and CD105/CD166 were scored in a semi-quantitative fashion that included the intensity of labelling of colony cells, categorized as: 0 = negative; (+) = weak positive; + = positive; ++ = intense immunostaining and +++ = very intense. Two observers blinded to the experimental groups performed the semi-quantitative evaluation and the inter-individual variation was 8%.

Differentiation of colony-forming cells

Chondrogenesis

Colony-forming cell clones were pelleted at a concentration of 0.5×10^6 cells per 1 ml media, and were then centrifuged at 2000 × *g* for 5 min in 1.5 ml micro-centrifuge tubes to form spherical pellets and were grown in chondrogenic medium; DMEM, 10 mM insulin, Transferrin, Selenium Solution (ITS; 10 μg ml^{-1} insulin, 5.5 μg ml^{-1} transferrin, 5 ng ml^{-1} selenium; 41400-045, Gibco, Paisley, UK), 100 mg ml^{-1} Gentamicin (15750-060, Invitrogen), 50 μg ml^{-1} sodium L-ascorbate, 1 mg ml^{-1} L-glucose, 2 mM L-glutamine, 10 mM HEPES, 10 nM dexamethasone (D8893, Sigma–Aldrich), 2% FBS and 10 ng ml^{-1} Transforming Growth Factor β1 (TGF-β1; PHG9204, Invitrogen). The chondrogenic medium was replaced every other day and pellet cultures were maintained for a total of 21 days. At 21 days, chondrogenic cell pellets were fixed with 10% neutral buffered formalin and then were processed for wax embedding. Sections 8 μm thick were cut and subjected to a standard histological protocol for alcian blue staining (Williams et al., 2009) for proteoglycan detection.

Osteogenesis

For osteogenic differentiation, pellets were suspended in DMEM, 10% FBS, 10 mM β-glycerophosphate (G9891; Invitrogen), 10 nM dexamethasone, 0.1 mM sodium L-ascorbate, 10 mM HEPES, 100 mg ml^{-1} Gentamicin and 100 mg ml^{-1} sodium pyruvate and 50 μg/ml L-proline (P0380, Sigma–Aldrich). Pellets were cultured for 21 days with a medium change every other day. At 21 days, osteogenic cell pellets were fixed with 10% neutral buffered formalin and were then processed for wax embedding. Sections were cut and subjected to standard histological protocols for alizarin red staining (Williams et al., 2010) for calcium detection.

Adipogenesis

For adipogenic differentiation, pellets were suspended in DMEM, 10% FBS, 10 nM dexamethasone, 200 μm indomethacin (I7378, Sigma–Aldrich), 0.5 mM 3-isobutyl-1-methylxanthine (IBMX; I7018, Sigma–Aldrich), 10 mM HEPES, 100 mg ml^{-1} Gentamicin, 100 mg ml^{-1} sodium pyruvate, ITS; 10 μg ml^{-1} insulin, 5.5 μg ml^{-1} transferrin and 5 ng ml^{-1} selenium. Pellets were cultured for 21 days with a medium change every other day. Adipogenically-induced pellets were frozen in 'Cryomatrix' (6769006, Thermo Scientific™ Shandon™ Cryomatrix™), and 8 μm cryosections were cut in a cryostat (Cryotome SME Cryostat Microtome, Thermo Shandon). Cryosections were stained with routine method for oil red O in 60% isopropanol for 15 min to visualize lipid accumulations.

Table 1
Immunofluorescence labelling intensities in articular cartilage sections: Immunofluorescence labelling intensities were categorized into zonal regions and represented as follows: 0: negative; (+): weak positive; +: positive; ++: intense; and +++: very intense.

Regions of articular cartilage	CD90 (Thy1)	Oct-3/4	CD105	Stro-1	CD166
Superficial zone	++	(+)	+++	(+)	++
Middle zone	+	0	++	(+)	++
Deep zone	++	0	+	++	+++

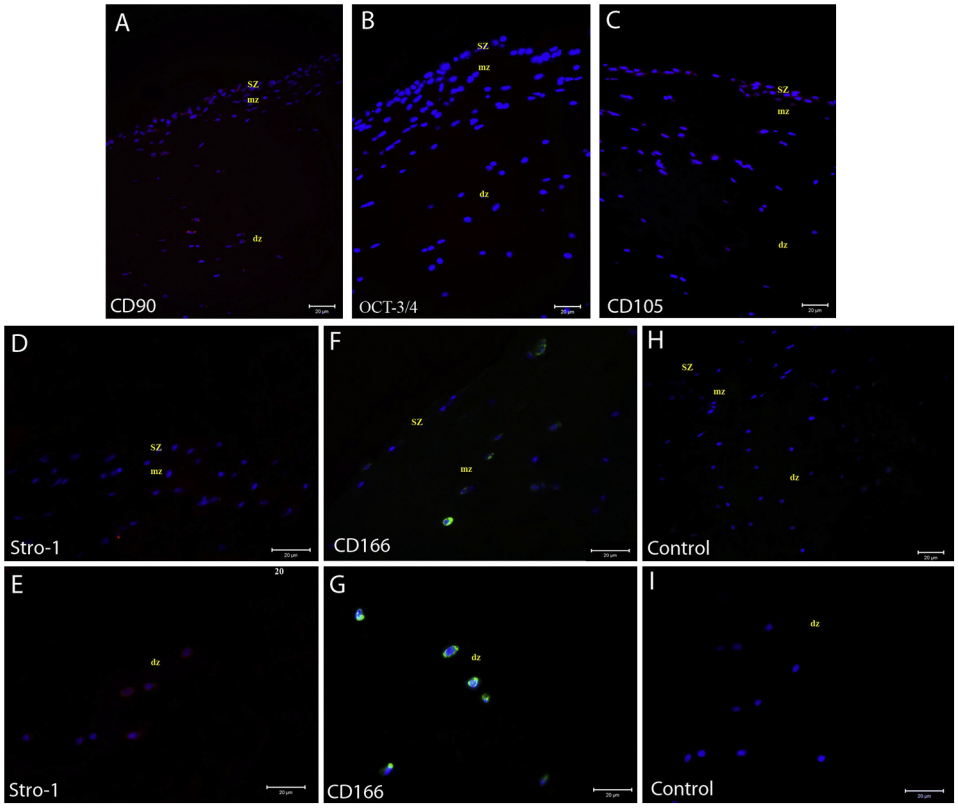


Fig. 1. Immunofluorescence staining of putative stem cell markers on adult human articular cartilage. The expression of CD90 (A) was mainly intensely positive in superficial and deep zones, OCT-3/4 (B) had a weak signal in the superficial zone, CD105 (C) was very intense in the superficial zone, intense in middle zone and positive in the deep zone, an intense positive expression for STRO-1 was observed mainly in the deep zone (D, E), and CD166 was expressed intensively through all zones of the articular cartilage (F, G). Control sections were negative (H, I). Scale bar = 20 μm.

Results

Immunohistochemical detection of CD90, STRO-1, OCT-3/4, CD105 and CD166 on adult human articular cartilage tissue sections

The labelling and intensity of the reaction for CD90, STRO-1, OCT-3/4, CD105 and CD166 on adult human articular cartilage zones are summarized in Table 1. Positive labelling for CD90 was intense in the superficial and deep zones of the articular cartilage while there was a positive labelling in the middle zone (Fig. 1A). The labelling for Oct-3/4 was weak positive in the superficial zone chondrocytes and was not apparent in the middle and deep zone chondrocytes (Fig. 1B). Positive labelling for CD105 was observed as very intense in the superficial zone, intense in the middle zone and positive in the deep zone (Fig. 1C). A weak positive signal for STRO-1 in the superficial and middle zones and an intense signal in the deep zone were observed (Fig. 1D and E). The positive signal for CD166 was intense in the superficial and middle zones and, very intense in the deep zone cells of the articular cartilage (Fig. 1F and G). No positive signal was observed in negative control sections of all studied proteins (Fig. 1H1 and I).

Flow cytometric analysis of colony-forming cells for CD90, STRO-1, OCT-3/4, CD105, CD166 and CD105/CD166

Percent expression of stem cell markers for colony-forming cells expanded until 3rd passage, cell cultures were determined as follows; CD90, 96.4 ± 1.4; STRO-1, 13.4 ± 3.1; OCT-3/4, 1.5 ± 0.5; CD105, 42.3 ± 9.8; CD166.78.4 ± 59; and CD105/CD166, 27.3 ± 10.6 (Fig. 2A and B).

Table 2
Immunofluorescence labelling intensities in colony forming cells. The intensity of the labelling of CD90, Stro-1, Oct-3/4, CD105, CD166 and CD105/CD166 were categorized and presented as follows: 0 = negative; (+) = weak positive; + = positive; ++ = intense; and +++ = very intense.

	CD105	CD166	CD105/CD166	CD90 (Thy1)	Stro-1	Oct-3/4
Colony-forming cells	+++	+++	+++	++	+++	(+)

Immunolocalization of CD90, STRO-1, OCT-3/4, CD105, CD166 and CD105/CD166 in colony-forming cells

CD90, STRO-1, OCT-3/4, CD105, CD166 and CD105/CD166 reaction intensities of colony-forming cells were evaluated semi-quantitatively and summarized in Table 2.

Although positive signal for CD90, STRO-1, CD105, CD166 and CD105/CD166 were observed in many colony-forming cells, the signal for OCT-3/4 was observed only in a few cells (Table 2; Fig. 3A–L). No positive signal was observed in negative control sections of all studied proteins (Fig. 3M).

Differentiation of colony-forming cells

Stimulations of colony-forming cells towards chondrogenesis, osteogenesis and adipogenesis were successfully achieved. Differentiation towards chondrogenesis was detected by alcian blue staining (Fig. 3N and P), differentiation towards osteogenesis was detected by alizarin red staining of the bone matrix (Fig. 3R and S) and differentiation towards adipogenesis was detected by the staining of Oil-Red-O (Fig. 3T and U).

Discussion

This study shows the localization of CD90, STRO-1, OCT-3/4, CD105 and CD166 on adult human articular cartilage tissue sections, expression of these markers within the expanded surface zone colony-forming cells and their differentiation potential with *in vitro* cell-culture techniques. This supports the work of Khan et al. (2009), Williams et al. (2010) and McCarthy et al. (2012) who showed that bovine, equine and human chondroprogenitors retain their chondrogenic phenotype after extended expansion in monolayer culture. The localization of colony-forming cells from entire cartilage tissue is an important step towards the eventual usage of these cells for therapeutic approaches.

We have previously identified the presence of progenitor/stem cells in adult rat articular cartilage by using stem cell markers as CD-105, CD-166 (Ozbey et al., 2010) and have also demonstrated immunolocalization of Notch and its ligands *in vivo* and *invitro* in humans using immunocytochemistry (Ustunel et al., 2008). Previous studies have suggested that CD105 and CD166 as possible candidates of cartilage stem cell markers, but recent research has demonstrated that a sub-population of cartilage stem cells cannot be isolated from these markers alone, as they are widely expressed by mature chondrocytes in the native tissue (Alsalameh et al., 2004; de la Fuente et al., 2004; Dowthwaite et al., 2004; Diaz-Romero et al., 2008).

Previous studies have demonstrated that adult human articular cartilage might contain cells possessing progenitor cell features (Archer et al., 1990; Dowthwaite et al., 2004; Williams et al., 2010). With this study, we advance our knowledge one step forward. The presence of adult human stem cell-like cells in the articular cartilage was confirmed by: (a) expression of stem cells markers such as CD90, STRO-1, OCT-3/4, CD105 and CD166 in adult human articular cartilage tissue sectionsnull; (b) expansion of isolated surface zone colony-forming cells in *in vitro* culture till 3rd passage; (c) at the end of 3rd passage, the expanded cells were demonstrated to express stem cells markers such as: CD90, STRO-1, OCT-3/4, CD105 and CD166; (d) these colony-forming cells were able to be stimulated towards different lineages under appropriate growth factors.

Detection of stem cell markers, such as CD90, STRO-1, OCT-3/4, CD105 and CD166 in various zones of the adult cartilage, suggest

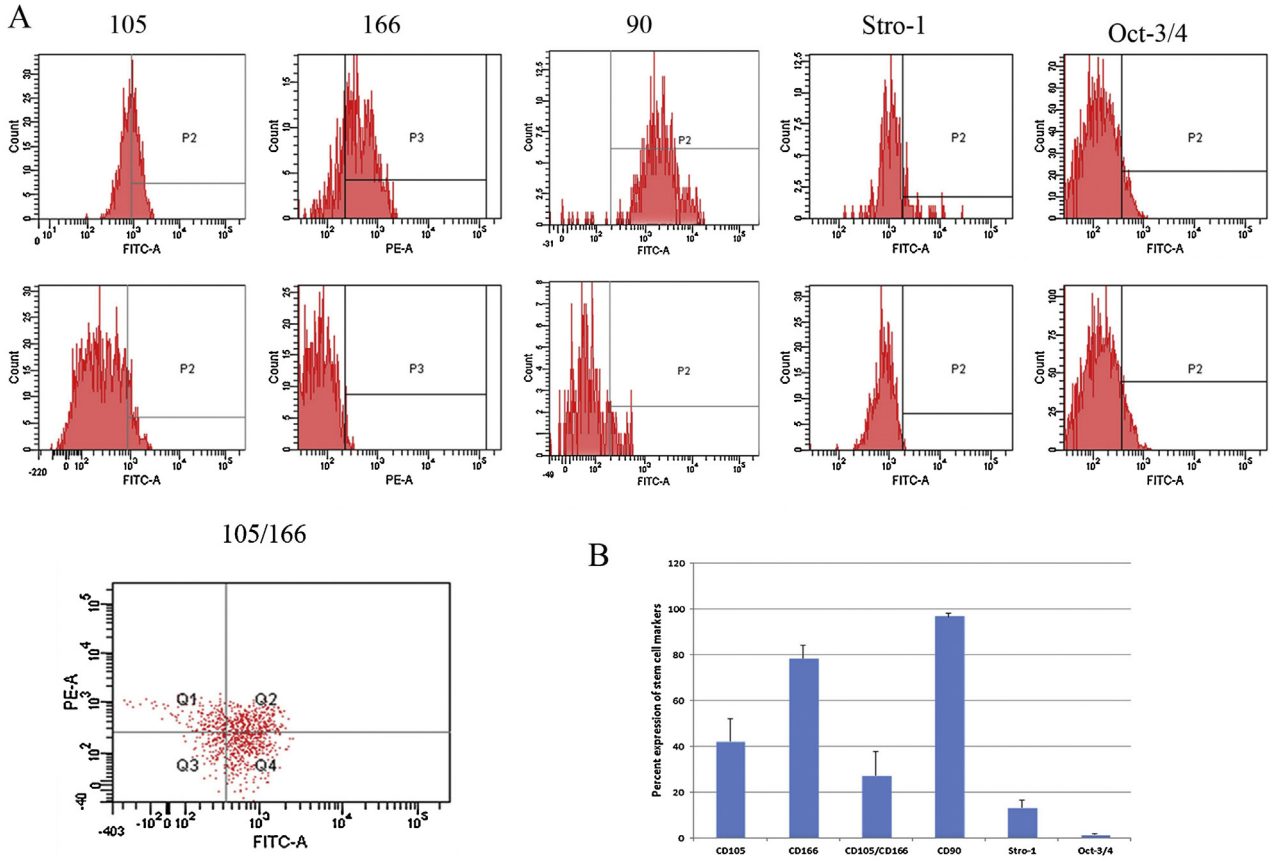


Fig. 2. Flow cytometric analysis of colony-forming cells for the putative stem cell surface markers. (A) Representative figures showing the expression of CD105, CD166, CD105/CD166, CD90, Stro-1 and Oct 3–4 (top panels) and their respective isotype controls (bottom panels). (B) Mean (±STD) expression levels of the markers ($n = 6$).

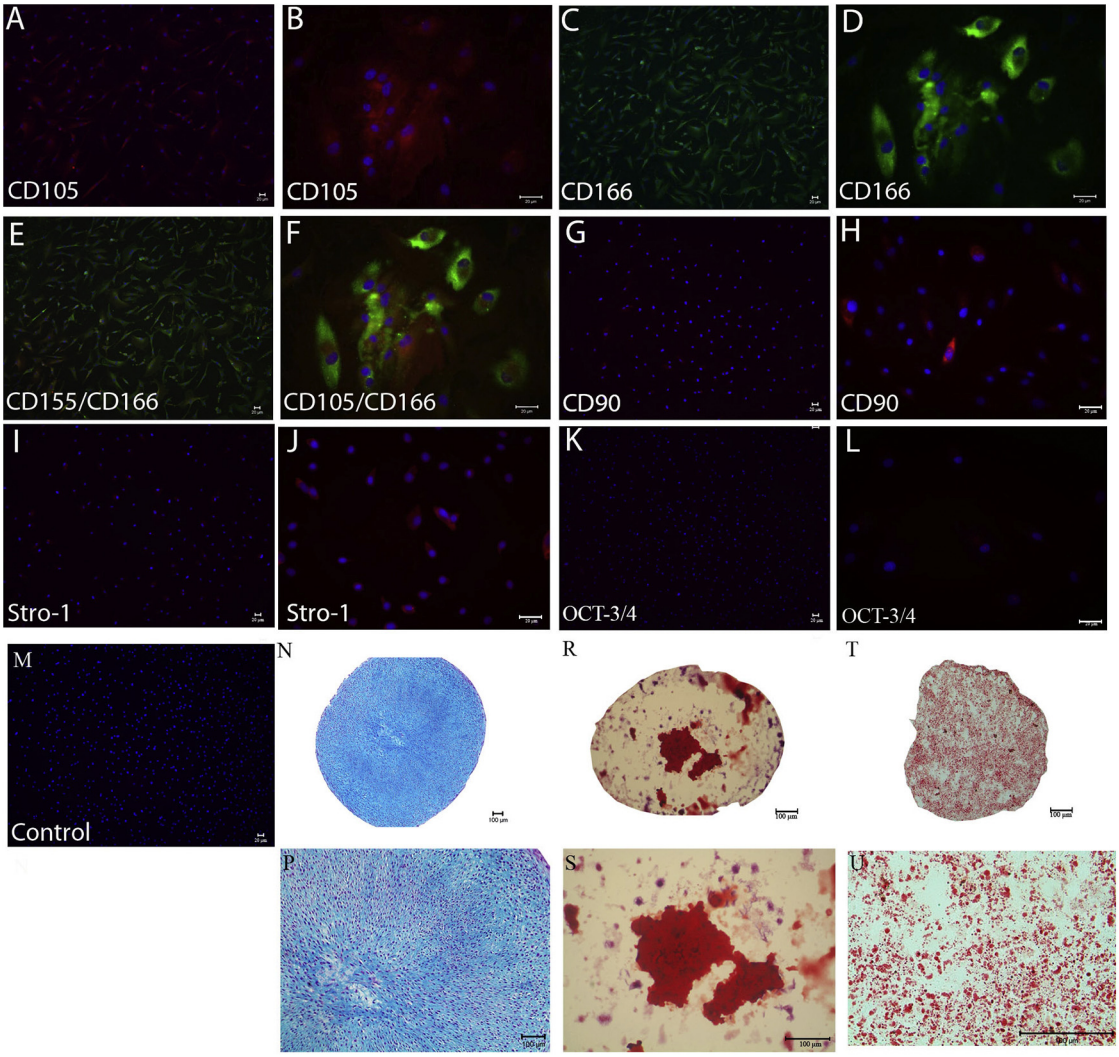


Fig. 3. Immunofluorescence staining of putative stem cell markers on in vitro expanded cartilage stem cells. The expression of CD105 (A, B), CD166 (C, D), CD90 (G, H), Stro-1 (I, J), Oct-3/4 (K, L) and co-expression of CD105/CD166 (E, F), control staining section (M). The differentiation of in vitro expanded cartilage stem cells to chondrogenesis (N–P), osteogenesis (R, S) and adipogenesis (T, U).

that the stem/progenitor cells exist not only in the superficial zone, but also in other zones, dissimilar to what we have previously demonstrated in rats. While the locations of CD105 expressing cells are similar between rats and humans, CD166 expressing cells are located in all zones in humans, in contrast to those of rats (Ozbey et al., 2010). A similar study by Hayes et al. (2003) described the surface zone chondrocytes of bovine articular cartilage express Notch 1. The same group has also shown that Notch 1 is expressed by murine chondrocytes at the articular surface before birth, but that expression becomes restricted to deeper layers after birth (Hayes et al., 2003). Zonal distribution of stem cell markers shows different localization according to the species and the development process.

For *in vitro* cell culture experiments, superficial zone cartilage stem cells were isolated through differential adhesion to fibronectin based on the mechanism that keratinocyte and cartilage stem cells have a higher quotient of α5β1 integrin (Jones and Watt, 1993; Salter et al., 1995; Chevalier et al., 1996). A study by Williams et al. (2010) has shown that a sub-population (0.7%) of cells within the total chondrocyte cell mass express CD49e (α5 integrin) and indeed the clonal cell lines obtained by adhesion to fibronectin express these markers (99%) when expanded. The expression of this marker may explain the high affinity of these progenitor cells for fibronectin. Characterization of superficial zone colony-forming cells was achieved by the expression of mesenchymal stem cell markers. The intensive expression of CD105, CD166, CD90, Stro-1 on *in vitro* cultured cartilage cells coincided with the expression of these markers in *invivo* cartilage cells. In previous studies, positive labelling for CD90 and CD166 was observed in the majority of superficial zone clonal cell lines with a low expression for Stro-1 and positive labelling for CD90 and CD166 observed in the majority of human full-depth clonal cell lines (Williams et al., 2010; McCarthy et al., 2012).

When we investigated the stem cell characteristics of the isolated superficial zone colony-forming cells by flow cytometry, we observed that these stem/progenitor cells express very high percentages of mesenchymal stem cell markers, such as CD90 and CD166 and very low percentages of embryonic stem cell markers such as Oct3/4. In another study, full-depth chondrocytes expressed CD105 and CD166 in over 95% (Williams et al., 2010). Furthermore, the expression of Stro-1 was lower than we expected (13.4 ± 3.1). According to these results, we speculate that these colony-forming cells include not only mesenchymal type stem cells, but also other cell types. Flow cytometry results supported the immunofluorescence data, where we observed that the expression of CD90, CD105 and CD166 were very intense in colony-forming

cells, which had been expanded until they reached confluence in *in vitro* cell culture.

As a consequence, we suggest that the superficial zone colony-forming cells in adult human articular cartilage may have progenitor cell features. Compared with other studies, according to flow-cytometry results, full-depth chondrocytes were shown to have higher expression rates for stem cell markers (Williams et al., 2010), other than superficial zone chondrocytes. These findings suggest that, not only the superficial zone of articular cartilage, but also deeper zones of articular cartilage (middle and deep zones) may have stem cell-like cells. Our immunohistochemical results are supported these findings. Positive labelling for CD90 and Stro-1 was intense and CD166 was very intense in the deep zone of the articular cartilage. These stem/progenitor cells were located in various zones of the cartilage. The stem cell characteristics of these colony-forming cells were further confirmed by the stimulation of these cells towards different lineages such as adipogenesis, osteogenesis and chondrogenesis. Considerably more work will need to be carried out to determine the precise localization of these progenitor cells in adult human articular cartilage. If the localization of these cells can be found, a new treatment method for articular cartilage injury may arise.

Acknowledgements

We thank Charles William Archer and his co-workers from the Connective Tissue Biology Laboratories, School of Biosciences, Cardiff University, Wales, UK and also from Swansea University, Wales, UK for their helpful comments in the preparation of technical procedures of this project and for critical reading of this manuscript. This study was supported by Akdeniz University Research Foundation in Antalya, Turkey, project number 2009.01.0103.008.

References

Alhadlaq A, Mao JJ. Tissue-engineered neogenesis of human-shaped mandibular condyle from rat mesenchymal stem cells. J Dent Res 2003;82:951–6.

Alhadlaq A, Mao JJ. Mesenchymal stem cells: isolation and therapeutics. Stem Cells Dev 2004;13:436–48.

Alsalameh S, Amin R, Gemba T, Lotz M. Identification of mesenchymal progenitor cells in normal and osteoarthritic human articular cartilage. Arthritis Rheum 2004;50:1522–32.

Archer CW, McDowell J, Bayliss MT, Stephens MD, Bentley G. Phenotypic modulation in sub-populations of human articular chondrocytes in vitro. J Cell Sci 1990;97(Pt 2):361–71.

Archer CW, Morrison H, Pitsillides AA. Cellular aspects of the development of diarthrodial joints and articular cartilage. J Anat 1994;184:447–56.

Brazelton TR, Rossi FMV, Keshet GI, Blau HM. From marrow to brain: expression of neuronal phenotypes in adult mice. Science 2000;290:1775–9.

Brittberg M, Lindahl A, Nilsson A, Ohlsson C, Isaksson O, Peterson L. Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation. New Engl J Med 1994;331:889–95.

Caplan AI. Mesenchymal stem cells. J Orthop Res 1991;9:641–50.

Chevalier X, Claudepierre P, Groult N, Zardi L, Hornebeck W. Presence of ED-A containing fibronectin in human articular cartilage from patients with osteoarthritis and rheumatoid arthritis. J Rheumatol 1996;23:1022–30.

Conget PA, Minguell JJ. Phenotypical and functional properties of human bone marrow mesenchymal progenitor cells. J Cell Physiol 1999;181:67–73.

de la Fuente R, Abad JL, Garcia-Castro J, Fernandez-Miguel G, Petriz J, Rubio D, et al. Dedifferentiated adult articular chondrocytes: a population of human multipotent primitive cells. Exp Cell Res 2004;297:313–28.

Dell'Accio F, De Bari C, Luyten FP. Molecular markers predictive of the capacity of expanded human articular chondrocytes to form stable cartilage in vivo. Arthritis Rheum 2001;44:1608–19.

Diaz-Romero J, Nesic D, Grogan SP, Heini P, Mainil-Varlet P. Immunophenotypic changes of human articular chondrocytes during monolayer culture reflect bona fide dedifferentiation rather than amplification of progenitor cells. J Cell Physiol 2008;214:75–83.

Dowthwaite GP, Bishop JC, Redman SN, Khan IM, Rooney P, Evans DJ, et al. The surface of articular cartilage contains a progenitor cell population. J Cell Sci 2004;117:889–97.

Gao J, Yao JQ, Caplan AI. Stem cells for tissue engineering of articular cartilage. Proc Inst Mech Eng H 2007;221:441–50.

Hayes AJ, Dowthwaite GP, Webster SV, Archer CW. The distribution of Notch receptors and their ligands during articular cartilage development. J Anat 2003;202:495–502.

Hayes AJ, MacPherson S, Morrison H, Dowthwaite G, Archer CW. The development of articular cartilage: evidence for an appositional growth mechanism. Anat Embryol (Berl) 2001;203:469–79.

Hunziker EB, Stahli A. Surgical suturing of articular cartilage induces osteoarthritis-like changes. Osteoarthritis Cartilage 2008;16:1067–73.

Johnstone B, Alini M, Cucchiarini M, Dodge GR, Eglin D, Guilak F, et al. Tissue engineering for articular cartilage repair – the state of the art. Eur Cell Mater 2013;25:248–67.

Jones PH, Watt FM. Separation of human epidermal stem cells from transit amplifying cells on the basis of differences in integrin function and expression. Cell 1993;73:713–24.

Khan IM, Bishop JC, Gilbert S, Archer CW. Clonal chondroprogenitors maintain telomerase activity and Sox9 expression during extended monolayer culture and retain chondrogenic potential. Osteoarthritis Cartilage 2009;17:518–28.

Knutsen G, Engebretsen L, Ludvigsen TC, Drogset JO, Grontvedt T, Solheim E, et al. Autologous chondrocyte implantation compared with microfracture in the knee – a randomized trial. J Bone Joint Surg Am 2004;86A:455–64.

McCarthy HE, Bara JJ, Brakspear K, Singhrao SK, Archer CW. The comparison of equine articular cartilage progenitor cells and bone marrow-derived stromal cells as potential cell sources for cartilage repair in the horse. Vet J 2012;192:345–51.

Mezey E, Chandross KJ, Harta G, Maki RA, McKercher SR. Turning blood into brain: cells bearing neuronal antigens generated in vivo from bone marrow. Science 2000;290:1779–82.

Mobasheri A, Csaki C, Clutterbuck AL, Rahmanzadeh M, Shakibaei M. Mesenchymal stem cells in connective tissue engineering and regenerative medicine: applications in cartilage repair and osteoarthritis therapy. Histol Histopathol 2009;24:347–66.

Muller B, Kohn D. Indication for and performance of articular cartilage drilling using the Pridie method. Der Orthopade 1999;28:4–10.

Nelson L, Fairclough J, Archer CW. Use of stem cells in the biological repair of articular cartilage. Expert Opin Biol Ther 2010;10:43–55.

Orlic D, Kajstura J, Chimenti S, Jakoniuk I, Anderson SM, Li B, et al. Bone marrow cells regenerate infarcted myocardium. Nature 2001;410:701–5.

Ozbey O, Sahin Z, Acar N, Ustunel I. Distribution of CD105 and CD166 positive cells in the proximal epiphysis of developing rat humerus. Histol Histopathol 2010;25:1437–45.

Petersen BE, Bowen WC, Patrene KD, Mars WM, Sullivan AK, Murase N, et al. Bone marrow as a potential source of hepatic oval cells. Science 1999;284:1168–70.

Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, et al. Multilineage potential of adult human mesenchymal stem cells. Science 1999;284:143–7.

Poulsom R, Alison MR, Cook T, Jeffery R, Ryan E, Forbes SJ, et al. Bone marrow stem cells contribute to healing of the kidney. J Am Soc Nephrology 2003;14(Suppl. 1): S48–54.

Prockop DJ. Marrow stromal cells as stem cells for nonhematopoietic tissues. Science 1997;276:71–4.

Saha S, Kirkham J, Wood D, Curran S, Yang XB. Informing future cartilage repair strategies: a comparative study of three different human cell types for cartilage tissue engineering. Cell Tissue Res 2013;352(3):495–507.

Salter DM, Godolphin JL, Gourlay MS. Chondrocyte heterogeneity: immunohistologically defined variation of integrin expression at different sites in human fetal knees. J Histochem Cytochem 1995;43:447–57.

Schnabel M, Marlovits S, Eckhoff G, Fichtel I, Gotzen L, Vecsei V, et al. Dedifferentiation-associated changes in morphology and gene expression in primary human articular chondrocytes in cell culture. Osteoarthritis Cartilage 2002;10: 62–70.

Shapiro F, Koide S, Glimcher MJ. Cell origin and differentiation in the repair of full-thickness defects of articular cartilage. J Bone Joint Surg Am 1993;75:532–53.

Singh NK, Singh GR, Jeong DK, Lee SJ. Healing of full-thickness articular cartilage defects treated with cultured autologous chondrogenic satellite cells isolated from chondral stem cell niche in rabbits. J Surg Res 2013;183:629–38.

Thornemo M, Tallheden T, Sjogren Jansson E, Larsson A, Lovstedt K, Nannmark U, et al. Clonal populations of chondrocytes with progenitor properties identified within human articular cartilage. Cells Tissues Organs 2005;180:141–50.

Triche R, Mandelbaum BR. Overview of cartilage biology and new trends in cartilage stimulation. Foot Ankle Clin 2013;18:1–12.

Ustunel I, Ozenci AM, Sahin Z, Ozbey O, Acar N, Tanriover G, et al. The immunohistochemical localization of notch receptors and ligands in human articular cartilage, chondroprogenitor culture and ultrastructural characteristics of these progenitor cells. Acta Histochem 2008;110:397–407.

Williams R, Khan IM, Richardson K, Nelson L, McCarthy HE, Analbelsi T, et al. Identification and clonal characterisation of a progenitor cell sub-population in normal human articular cartilage. PLoS ONE 2010;5:e13246.

Williams R, Nelson L, Dowthwaite GP, Evans DJ, Archer CW. Notch receptor and Notch ligand expression in developing avian cartilage. J Anat 2009;215:159–69.