Characterisation of the cells in repair tissue following autologous chondrocyte implantation in mankind: a novel report of two cases.

Abstract

Aims: Autologous chondrocyte implantation (ACI) is used worldwide for the treatment of cartilage defects. This study has aimed to assess for the first time those cells that are contained within human ACI repair tissues several years post-treatment. We have compared the phenotypic properties of cells from within the ACI repair to adjacent chondrocytes and subchondral bone derived-mesenchymal stem cells (MSC).

Materials and methods: Two patients undergoing arthroplasty of their ACI-treated joint were investigated. Tissue and cells were isolated from the repair site, adjacent macroscopically normal cartilage and MSC from the subchondral bone and characterised for their growth kinetics, morphology, immunoprofile and differentiation capacity.

Results: ACI repair tissue appeared fibrocartilaginous and ACI repair cells were heterogeneous in morphology and size when freshly isolated, becoming more homogeneous, resembling chondrocytes from adjacent cartilage, after culture expansion. The same weight of ACI repair tissue resulted in less cells than macroscopically normal cartilage. During expansion ACI repair cells proliferated faster than MSC but slower than chondrocytes. ACI repair cell immunoprofiles resembled chondrocytes, but their differentiation capacity matched MSC.

Conclusion: This novel report demonstrates that human ACI repair cell phenotypes resemble both chondrocytes and MSC but at different stages of their isolation and expansion *in vitro*.

Keywords: Autologous chondrocyte implantation; histochemical analysis; repair cell characterisation, phenotype, morphology, growth kinetics, immunoprofile, differentiation potential.

Introduction

There is a huge interest worldwide in the development of tissue engineering and cell based therapies for the treatment of cartilage defects. Autologous chondrocyte implantation (ACI) is a procedure that has been used for more than 20 years for the treatment of cartilage injury and osteoarthritis[1-3]. Our centre has provided cells for over 400 ACI procedures since its inception, of which 81% were a success, as indicated by a postoperative increase in Lysholm score[4]. What happens to the culture expanded chondrocytes after implantation and the contribution that they make to the repair tissue compared to cells from surrounding tissues is still largely unknown[5-7]. Few preclinical studies have labelled and tracked transplanted chondrocytes in ACI models. Those that have, show that varying proportions of the cells injected form the cellular component of the tissue at the site of ACI. In these studies transplanted cells have been shown to contribute in part to the formation and integration of repair tissues. However, numerous unlabelled cells also form a major constituent, which suggests that cells of unknown origin migrate to ACI-treated lesions and combine with transplanted cells as part of the healing process[6-7].

The purpose of this study is to describe for the first time the phenotype of those cells that are contained within the tissue at the site of ACI in humans several years after treatment. Characterisation of the cells that are present at the site of ACI and hence, that are likely to produce and remodel the repair tissue, is critical to our understanding of the biological process in ACI. In previous studies we have only been able to assess the quality of ACI repair tissues in the clinic via magnetic resonance imaging (MRI) and histological analyses of small regions

(<1mm diameter cores)[8-11]. We have obtained two rare samples which have provided us with the opportunity to isolate and examine the behaviour and phenotypic properties of ACI repair cells in culture, in comparison to both chondrocytes in the adjacent cartilage and MSC from the subchondral bone. Observing ACI repair cells in culture will help to provide novel information on the cellular component of ACI repair tissues which we can then compare to histological analyses and clinical outcome. In addition, by analysing the properties of ACI repair cells in contrast to the phenotypes of cells isolated from neighbouring tissues (e.g. cartilage and bone) we may begin to elucidate ACI repair cell origin. Herein we describe the analysis of repair tissues and cells from two former ACI patients that have returned to our clinic for arthroplasty of their ACI-treated joints several years post-ACI.

Materials and methods

Patient information

Following Local Research Ethical Committee approval and with informed consent, tissues obtained from two patients undergoing joint replacement surgery were included in this study (one knee – Patient 1 and one hip – Patient 2). Both were males aged 49 and 44 years at the time of arthroplasty, which was 11 and 5 years, respectively, after previous ACI treatment (Table 1).

Histological analysis

For histological examination decalcified wax-embedded tissue sections from the region bridging macroscopically normal cartilage (MNC) and ACI repair tissues (with subchondral bone) from Patients 1 and 2 were examined using haematoxylin and eosin (H&E) and toluidine blue stains as described previously[12]. For H&E histological examination, sections were flooded with Mayer's haemalum (VWR International Ltd, Poole, UK) for 1 minute, drained, and washed in tap water for 5 minutes. Slides were then flooded with 1% eosin aqueous solution (BDH, Poole, UK) (in distilled water) for 30 seconds, briefly washed in tap water and dehydrated through a series of isopropanol (Genta Medical, York, UK) concentrations in distilled water and xylene, for 5 minutes each. Following dehydration, the tissue sections were mounted under glass coverslips (Cell Path Ltd, Newtown, UK) with Pertex mounting medium (Histolab Products AB, Gothenburg, Sweden) and allowed to air dry. Glycosaminoglycan (GAG) content was assessed by metachromasia by flooding sections with 1% aqueous toluidine blue (BDH) solution for 30 seconds and rinsed in tap water.

Slides were left to air dry before mounting under glass coverslips with Pertex mounting medium.

For collagen type II immunolocalisation, dewaxed and rehydrated sections were pre-treated with 0.1% (w/v) hyaluronidase and 0.2% (w/v) trypsin (Sigma-Aldrich, Poole, UK) for 1 hour at 37^0C. Sections were then washed in phosphate buffered saline (PBS) and incubated for 2 hours at room temperature in a humidified chamber with 10μg/ml of primary mouse monoclonal collagen type II antibody (clone CIIC1, Developmental Studies Hybridoma Bank, University of Iowa) in PBS. Parallel sections were incubated with a non-specific, isotype-matched antibody (IgG1- Dako, Glostrup, Denmark) instead of the primary antibody at the same concentration as a negative control. After incubation with the primary antibodies all sections were washed in PBS before incubation for 1 hour with a secondary biotinylated antibody at 50μg/ml (Vector ABC System, Vector Laboratories, Peterborough, UK) according to manufacturers instructions. To eliminate endogenous peroxidase activity sections were blocked with 3% (v/v) hydrogen peroxide (H_2O_2) in methanol (BDH) for 30 minutes. Collagen type II immunopositivity was finally visualised by testing for bound peroxidise, which is detected by incubation with a substrate of diaminobenzidine tetrahydrochloride (DAB), activated by H_2O_2. The sections were then dehydrated before mounting under glass coverslips with Pertex mounting medium as described previously.

Chondrocyte and ACI repair cell isolation and culture

Approximately 300mg of MNC and ACI repair tissues were harvested from the medial femoral condyle (MFC) of Patient 1 and the femoral head (FH) of Patient

2. Cells were isolated and cultured as described previously for chondrocytes[13]. MNC and ACI repair tissues were dissected into ~2mm^3 pieces and placed into 25cm^2 tissue culture flasks (Falcon 250 ml Polystyrene Tissue Culture Flask, BD Biosciences, UK). The weight of each tissue type was recorded and cells released by enzymic digestion. Dulbecco's Modified Eagle's Medium (DMEM)/F12 (Life Technologies, Paisley, UK) containing 0.8mg/ml type XI collagenase (Sigma-Aldrich) was added to each of the flasks which were then incubated at 5% (v/v) CO_2 for 20 hours at 37^{o}C.

Following this incubation, each tissue digest was passed through a 70μm cell strainer (BD Biosciences); cells were recovered by centrifugation at 750g for 10 minutes to form a cell pellet. Cells were plated out in DMEM/F12, supplemented with 10% foetal bovine serum (FBS) (Life Technologies), 50μg/ml ascorbic acid (AA; Sigma-Aldrich) and 1% (v/v) penicillin and streptomycin (P/S; Life Technologies) at a seeding density of 5 x 10^5 cells per cm^2. After 5 days, non-adherent cells were removed and the adherent cell population was cultured in monolayer in DMEM/F12 10% FBS medium supplemented with AA and P/S. Cells were routinely passaged at 70% confluence by trypsinisation (0.05% v/v Trypsin-EDTA) and re-seeded at 5 x 10^3 cells/ cm^2.

Human bone marrow stem cell (MSC) isolation and culture

The underlying bone from the MFC of Patient 1 and the FH of Patient 2 was perfused with DMEM/F12 (Life Technologies) supplemented with 10% FBS and P/S. Mononuclear cells isolated and MSC cultured as described previously[14]. Mononuclear cells isolated by density gradient centrifugation (Lymphoprep,

Fresenius Kabi Norge, AS) were plated out in DMEM/F12, supplemented with 20% FBS and P/S at a seeding density of 20 x 10^6 cells per 25cm^2 tissue culture flask. After 24 hours, non-adherent cells were removed and the adherent cell population was cultured in monolayer in DMEM/F12 10% FBS medium supplemented with P/S. Cells were routinely passaged at 70% confluence by trypsinisation (0.05% v/v Trypsin-EDTA) and re-seeded at 5 x 10^3 cells/ cm^2. Viability was assessed at each passage by trypan blue exclusion (Sigma-Aldrich).

Microscopy, image capture and analysis

Histological sections were viewed using bright light and polarized light microscopy (Leitz Diaplan, Wetzlar, Germany) and digitized images were captured with a Nikon digital camera (DS-Fi1, Nikon, Kingston-upon-Thames, UK). Cultures were viewed using phase contrast microscopy (Nikon Eclipse TS100) and digitized images were captured with a Hamamatsu digital camera (C4742-95, Bridgewater, NJ). The mean cell area was determined from passage 0-3 for the three cell populations using IPLab software (Version 3.6, Nikon). For each cell type, results from at least 5 separate images per culture were combined.

Growth kinetics

Culture doubling time was calculated for each cell population (from passage 0-3) using the following formula: doubling time (DT) = (t2-t1) x ln(2)/ ln(n2/n1), where t1 = the time of cell seeding, t2 = the time of cell harvest and n = the matching cell numbers at these time points.

Immunoprofiling

Immunoprofiling via flow cytometry was used to assess culture expanded cells (at passage 2) using a FACScan flow cytometer (BD Biosciences). A profile typical of MSC was targeted [15]. In brief, cells were blocked for one hour in a buffer of 10% normal human Ig (Grifols, Cambridge, UK). Cells were then incubated with mouse anti-human monoclonal primary antibodies against CD14, CD19, CD31, CD34, CD45, CD73, CD90, CD105 and HLA-DR (all phycoerythrin-conjugated) (Immunotools, Friesoythe, Germany) for 30 minutes. Matched cell populations were also exposed to isotype-matched IgG negative control antibodies (Sigma-Aldrich). Immunoprofiles were produced using Cell Quest software (BD Biosciences).

Multipotency assays

Established protocols [16-18] were used to assay the differentiation potential of cells at passage 2 for adipogenic, osteoblastic and chondrogenic lineages. In brief, for 21 days, cell cultures were exposed to appropriate conditions for: (i) adipogenic differentiation via monolayer culture in DMEM/F12 10% FCS, 1% ITS-X (Life Technologies), dexamethasone, 3-isobutyl-1-methylxanthine and indomethacin (Sigma-Aldrich); (ii) osteoblastic differentiation via monolayer culture in DMEM/F12 10% FCS, ascorbate 2-phosphate, dexamethasone and β-glycerophosphate (Sigma-Aldrich); (iii) chondrogenic differentiation via micro-mass pellet culture in DMEM/F12, 1% ITS-X, ascorbate 2-phosphate (Sigma-Aldrich), dexamethasone (Sigma-Aldrich) and TGF-β1 (PeproTech Ltd., London, UK).

182 At the 21 day time point, adipogenic differentiation potential was examined
183 via oil red-O visualisation of lipid formation, alkaline phosphatase activity was
184 used to assess osteoblast differentiation and for chondrogenic differentiation,
185 toluidine blue staining was used to detect the presence of GAGs in micro-mass
186 pellets.

187 **Statistical analysis**

188 The Kruskal-Wallis non-parametric ANOVA and post-hoc Bonferroni pair-wise
189 comparison tests were used to assess significant differences between the size
190 (area coverage) of each cell type isolated from the same joint e.g. Chondrocytes,
191 ACI repair cells and MSC between passages 0-3.

192

Results

Histological analysis

Tissue sections from the region bridging MNC and ACI repair tissues (Figure 1A) were examined via H&E staining (Figure 1B) which demonstrated a disorganized cellular distribution throughout ACI repair tissues in both patients compared to neighbouring MNC. There appeared to be good integration between the repair cartilage and the neighbouring MNC and the underlying bone. The surface integrity and smoothness of ACI repair tissues differed between patients; in the knee (patient 1) the repair surface was rough, whereas the hip repair tissue (patient 2) was smooth but undulating compared to neighbouring MNC which was smooth and flat in both patients.

Viewing the same section under polarized light (Figure 1C) revealed illuminated areas of scattered light indicative of fibrocartilage throughout the repair region and in particular at the border with MNC. Toluidine blue staining (Figure 1D) indicated that the GAG content of ACI repair tissues was lower than that in neighbouring MNC, particularly in the surface zone. For both patients collagen type II staining (Figure 1E) was similarly weaker in the surface zone of ACI repair tissues.

Morphological analyses

For both patients freshly isolated chondrocytes and MSC had a uniform fibroblast-like morphology with their size (surface area coverage) increasing after expansion. MSC appeared consistently larger at each passage compared to chondrocytes. In addition, for MSC cultures numerous intracellular stress fibres were clearly visible at passage 3. In contrast, the morphologies of freshly isolated

ACI repair cells were more heterogeneous. ACI repair cells possessed a combination of early and later passage chondrocyte and MSC-like morphologies after initial seeding but became homogeneous after sub-culture. There were no obvious or significant differences between ACI and chondrocyte cultures (Figures 2A and 2B).

Growth kinetics

Chondrocytes and ACI repair cells proliferated at similar rates for both patients (from passages 0-3), with DTs of 2 weeks between passages 0-1, decreasing to 4-6 days between passages 1-2 and 2-3. In contrast, the DT of MSC was markedly higher at 8-14 days between passages 1-2 and 2-3 (Figure 3A). At passage 3 chondrocyte cultures produced harvests of $1.4x10^8$, ACI repair cells $4.3x10^7$ and MSC $9.0x10^6$ (Figure 3B). Viability for all cells was >98% at each passage.

Immunoprofiles

MSC from both patients' were CD14, CD19, CD31, CD34, CD45 and HLA-DR negative and CD73, CD90 and CD105 positive; this matches previously published MSC immunoprofiles[15]. Chondrocytes and ACI repair cells from both patients had MSC-like immunoprofiles apart from some positivity for CD14; for patient 1 CD14 was detected on ~90% of chondrocytes and ~50% of ACI repair cells, whereas for patient 2 the reverse pattern of positivity was observed between chondrocytes (~50% immunopositive) and ACI (~90% immunopositive) (Figure 4).

Differentiation potential

Chondrocytes, ACI repair cells and MSC from both patients differentiated along all three mesenchymal cell lineages tested but to varying degrees, as delineated by lipid accumulation, alkaline phosphatase activity and toluidine blue GAG staining. Chondrocytes from patient 1 produced a large frequency of clustered globular lipids, whereas chondrocytes from patient 2 showed more diffuse staining of smaller lipids throughout. ACI and MSC staining for lipid accumulation was similar in pattern for both patients, hence, a few unilocular lipid clusters (which may be indicative of committed adipocytes) were seen in both (Figure 5A). Chondrocytes from both patients showed intense uniform staining for alkaline phosphatase activity, whereas ACI repair cells and MSC demonstrated a more heterogeneous pattern of staining (Figure 5B). All pellet cultures showed the presence of some GAGs via toluidine blue staining. Chondrocyte fractions showed the most intense toluidine blue staining, ACI repair cell and MSC pellets showed weaker GAG staining for both patients (Figure 5C).

Discussion

These samples have provided a unique opportunity to study both the histology of the complete area of ACI repair tissue compared to neighbouring MNC as well as examining the phenotype of ACI repair cells compared to chondrocytes from adjacent cartilage and MSC from the underlying subchondral bone, several years post-ACI. For these analyses, tissues and cells were harvested from two former ACI patients at the time of arthroplasty. It is debatable whether the patients included in this study and hence, the tissues and cells examined here should be considered as ACI successes or failures. These individuals were 38 and 39 years of age at the time that they received ACI and although their treated joints ultimately failed, ACI prolonged the life of their natural joints for 11 and 5 years, respectively. For patients of such a young age this may have important implications for later life as joint replacement is accompanied by some loss of function which restricts activities[19]. In addition, joint replacements will probably not last for the full life of younger patients. Hence, the initial surgery may need revision which is a more complicated and expensive operation with lower success rates than primary arthroplasty[20]. As such, ACI therapy which increases the life-span of these young patients' joints may understandably be considered a successful intervention even though their treated joints eventually failed.

Histologically, we have shown that ACI repair tissues resembled those previously described[8-10]; cells were numerous but disorganised and matrices appeared predominantly fibrocartilaginous. Repair tissues were well integrated

with adjacent MNC and subchondral bone. The surface of repair tissue for patient 1 was rough compared to that of patient 2, perhaps because patient 1 received a periosteal graft, which may exhibit hypertrophy, compared to the collagen membrane which was used for patient 2[21]. There were no obvious histological signs that may have explained joint failure in these patients. However, there is limited evidence that histology is a reliable indicator of clinical outcome for ACI patients[22]. In this study we have some preliminary data for ACI repair cell characterisation, which may represent a promising additional prognostic marker in future analyses. An understanding of the ACI repair cell phenotype will help to elucidate ACI repair tissue formation and remodelling processes. In addition, we may be able to use this data to begin to 'unpick' the origin of ACI repair cells and hence, to determine if ACI tissues contain any of those cells initially implanted at ACI stage II. In preclinical studies transplanted cells are known to persist in ACI repair zones for up to 14 weeks in large animal models[6]. Alternatively, the site of ACI repair may contain a completely different cell type (e.g. synovium, bone, or bone marrow-derived MSC) that have migrated and integrated into ACI zones from surrounding tissues[23-25].

It is likely that the anatomical location (i.e. knee versus hip) and the patch used at ACI (i.e. periosteum versus Chondro-Gide®) will have influenced the quality and extent of the repair tissue observed for Patient 1 compared to Patient 2. However, there were no discernible differences observed in the phenotypes of ACI repair cells isolated from Patient 1 or Patient 2. This suggests that the dissimilarities between these examples of ACI i.e. the type of joint treated and

patch used might not have contributed significantly to the tissue regeneration seen (or the cells involved) in these ACI treated joints. We have shown that freshly isolated cells from ACI repair tissues appeared to contain a mixture of chondrocyte and MSC morphologies, but that MSC-like cells disappear over time in culture. Our growth kinetics data supports the theory that chondrocytes may have outgrown MSC *in vitro*. In addition, the immunoprofiles of chondrocytes and ACI repair cells were similar after sub-culture, both demonstrated some CD14 positivity, a marker found on freshly isolated chondrocytes[26,27] compared to a complete absence of CD14 on MSC at the same passage. It is unlikely that the source of CD14 positive cells that were cultured from ACI repair tissues represent MSC which have migrated from surrounding tissues e.g. MSC from synovium and bone or bone marrow as these MSC do not express CD14 in an undifferentiated state[15,28]. It is conceivable that these cells instead either represent a proportion of the chondrocytes that were originally transplanted at ACI or that have migrated from adjacent cartilage. Alternatively, an MSC population may have homed to the injured region and differentiated *in vivo* towards a chondrogenic lineage[29,30].

Interestingly, our multipotency studies showed a marked contrast between passaged chondrocyte and ACI repair cell differentiation potential. Chondrocyte populations differentiated along adipogenic, osteogenic and chondrogenic lineages in a strongly positive and uniform manner as articular cartilage itself contains a multipotent progenitor cell population [31-35]. In contrast, ACI repair cells appeared heterogeneous and on the whole, differentiated along each

mesenchymal lineage tested to a lesser extent, akin to MSC isolated from the same joint. However, we acknowledge that culture expansion may change the characteristics of ACI repair cells. It is therefore difficult to ascertain exactly how the immunoprofile and differentiation potential of culture-expanded ACI repair cells relate to their *in vivo* characteristics. None the less, this study demonstrates that although cultured ACI repair cells possess many phenotypic characeristics of chondrocytes e.g. similar morphologies, growth kinetics and immunoprofiles, they do not possess the ability to differentiate, importantly in this setting, into chondrocytes with physiologically relevant properties. For example, ACI repair cells do not appear to synthesise GAG rich matrices to the same extent as chondrocytes when chondrogenically induced *in vitro*. This finding coincides with our *in vivo* histological evidence which clearly shows that ACI repair tissues possess a lower GAG content in comparison to neighbouring MNC.

A limitation of this study is that it is based on only two human samples, making the findings a little difficult to interpret. We hope to expand and corroborate the findings of these pilot experiments by increasing the sample size of donors and expanding the molecules and markers to be investigated in longer term studies. For example, immunohistochemical analyses to determine the presence of type X collagen as a marker of hypertrophy in repair tissues and gene expression studies of isolated repair cells for osteogenic and chondrogenic associated molecules using RT-qPCR would provide valuable additional data. Nonetheless, this pilot study has provided a rare opportunity to carry out studies which are normally only possible in animals, but even then at much shorter time

347 points. The information obtained is therefore completely novel and likely to be
348 more relevant to the human patient than results from animal studies.

349

Conclusions

This study presents the first data on human ACI repair cell phenotypes in culture, several years after ACI treatment. Cells isolated from ACI repair tissue appeared to contain a mixture of chondrocytes and MSC morphologies at initial seeding, but became more like chondrocytes with regard to morphology, proliferation and immunoprofile at later passage. However, the differentiation potential of expanded ACI repair cells was reduced for each mesenchymal lineage tested compared to chondrocytes and notably so for chondrogenic potential which is considerably relevant in this setting. These findings indicate that ACI repair cells are composed of a mixture of cells with features resembling both chondrocyte and MSC phenotypes. This suggests that ACI tissues contain both chondrocytes (either originally implanted or integrated from surrounding cartilage *in vivo*) and also MSC that have infiltrated the treated region from synovium or subchondral bone. A better understanding of the source of cells which contribute to the repair tissue in ACI, especially when associated with the best clinical outcome, will provide valuable information to help improve the ACI technique in the clinic. For example, we may be able to select the most effective cells prior to implantation or to augment the migration of desirable endogenous cells from the nearby tissues. In this way we can make step changes and improvements in current cell therapy treatments of chondral defects.

Summary Points

Patient Information

- We have analysed tissues and cells isolated from regions of repair in two patients undergoing arthroplasty several years after cell therapy with autologous chondrocytes; these have been compared to tissues and cells derived from adjacent cartilage and bone.

Histological analysis

- ACI repair tissues were densely populated with cells but the extracellular matrix was disorganised and contained little GAG or collagen type II in surface zones compared to adjacent, macroscopically normal cartilage.

Morphological analyses

- Cells which were isolated freshly from ACI repair tissues had a mixture of chondrocyte and MSC-like morphologies.

Growth kinetics, Immunoprofiles and Differentiation potential

- Following culture expansion, cells isolated from ACI repair tissues resembled chondrocytes in terms of their growth and immunoprofile but their adipogenic, osteogenic and (importantly) chondrogenic differentiation capacity was markedly reduced in comparison to chondrocytes isolated from adjacent macroscopically normal cartilage.

Acknowledgements

We are grateful to Arthritis Research UK for supporting this work (Grants 19429 and 18480), to the Wellcome Trust for supporting Hannah Fox and to The John Charnley Laboratory, RJAH Orthopaedic Hospital, UK for the expansion and preparation of harvested chondrocytes used in the ACI procedures.

References

1. Brittberg M, Lindhall A, Nilsson A, Ohlsson C, Isaksson, O, Peterson L. Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation. *N. Eng. J. Med.* 331, 889-895 (1994).
2. Richardson JB, Caterson, B, Evans EH, Ashton BA, Roberts S. Repair of human articular cartilage after implantation of autologous chondrocytes. *J. Bone. Joint. Surg. (Br).* 81, 1064-1068 (1999).
3. Corpus KT, Bajaj S, Daley E *et al*. Long-term evaluation of autologous chondrocyte implantation: Minimum 7-year follow-up. *Cartilage*. 3, 342-350 (2012).
4. Bhosale AM, Kuiper JH, Johnson WEB, Harrison, PE, Richardson JB. Midterm to long-term longitudinal outcome of autologous chondrocyte implantation in the knee joint. *Am. J. Sports. Med.* 37, 131S-38S (2009).
5. Grande DA, Pitman MI, Peterson L, Menche D, Klein M. The repair of experimentally produced defects in rabbit articular cartilage by autologous chondrocyte transplantation. *J. Orthop. Res.* 7, 208-218 (1989).
6. Dell'Accio F, Vanlauwe J, Bellemans J, Neys J, De Bari C, Luyten FP. Expanded phenotypically stable chondrocytes persist in the repair tissue and contribute to cartilage matrix formation and structural integration in a goat model of autologous chondrocyte implantation. *J. Orth. Res.* 21, 123-131 (2003).
7. Miot S, Gianni-Barrera R, Pelttari K *et al*. In vitro and in vivo validation of human and goat chondrocyte labelling by green fluorescent protein lentivirus transduction. *Tiss. Eng. Part C. Methods.* 16, 11-21 (2010).

8. Roberts S, McCall IW, Darby AJ *et al*. Autologous chondrocyte implantation for cartilage repair: monitoring its success by magnetic resonance imaging and histology. *Arthritis. Res. Ther.* 5, R60-73 (2003).
9. Tins BJ, McCall IW, Takahashi T *et al*. Autologous chondrocyte implantation in knee joint: MR imaging and histologic features at 1-year follow-up. *Radiology.* 234, 501-508 (2005).
10. Sharma A, Wood LD, Richardson JB, Roberts S, Kuiper NJ. Glycosaminoglycan profiles of repair tissue formed following autologous chondrocyte implantation differ from control cartilage. *Arthritis. Res. Ther.* 9, R79-88 (2007).
11. Roberts S, Menage J, Sandell SJ, Evans EH, Richardson JB. Immunochemical study of collagen types I and II and procollagen IIA in human cartilage repair tissue following autologous chondrocyte implantation. *The Knee*. 16, 398-404 (2009).
12. Roberts S, Menage J. Microscopic methods for the analysis of engineered tissues. In: *Biopolmer methods in tissue engineering*. Hollander AP, Hatton PV (Ed). Humana Press, Totowa, NJ. 177-185. (2004).
13. Harrison PE, Ashton IK, Johnson WEB, Turner SL, Richardson JB, Ashton BA. The in vitro growth of human chondrocytes. *Cell. Tissue. Bank.* 1, 255-260 (2001).
14. Wright KT, El Masri W, Osman A *et al*. Bone marrow stromal cells stimulate neurite outgrowth over neural proteoglycans (CSPG), myelin associated glycoprotein and Nogo-A. *Biochem. Biophys. Res. Comm.* 354, 559-566 (2007).

15. Dominici M, Le Blanc K, Mueller I *et al*. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy*. 8, 315-317 (2006).
16. Jaiswal N, Haynesworth SE, Caplan AI, Bruder S. Osteogenic differentiation of purified culture-expanded human mesenchymal stem cells in vitro. *J. Cell. Biochem.* 64, 295-312 (1997).
17. Johnstone B, Hering TM, Caplan AI, Goldberg VM, Yoo JU. In vitro chondrogenesis of bone marrow-derived mesenchymal progenitor cells. *Exp. Cell. Res.* 238, 265-272 (1998).
18. Pittenger MF, Mackay AM, Beck SC *et al*. Multilineage potential of adult human mesenchymal stem cells. *Science*. 384, 143-147 (1999).
19. Scott CE, Bugler KE, Clement ND, MacDonald D, Howie CR, Biant LC. Patient expectations of arthroplasty of the hip and knee. *J. Bone. Joint. Surg. (Br).* 94, 974-981 (2012).
20. Labek G, Thaler M, Janda W, Agreiter M, Stockl B. Revision rates after joint replacement: cumulative results from worldwide joint register data sets. *J. Bone. Joint. Surg.* 93, 293-297 (2011).
21. Gooding CR, Bartlett W, Bentley G, Skinner JA, Carrington R, Flanagan A. A prospective, randomized study comparing two techniques of autologous chondrocyte implantation for osteochondral defects in the knee: periosteum covered versus type I/III collagen covered. *Knee*. 13, 203-210 (2006).
22. Hanifi A, Richardson JB, Kuiper JH, Roberts S, Pleshko N. Clinical outcome of autologous chondrocyte implantation is correlated with infrared

spectroscopic imaging-derived parameters. *Osteoarthritis. Cartilage.* 20, 988-996 (2012).

23. Shapiro F, Koide S, Glimcher MJ. Cell origin and differentiation in the repair of full-thickness defects of articular cartilage. *J. Bone. Joint. Surg.* 75, 532-553 (1993).
24. De Bari C, Dell'Accio F, Tyzanowski P, Luyten FP. Multipotent mesenchymal stem cells from adult human synovial membrane. *Arthritis. Rheum.* 44, 1928-1942 (2001).
25. Kurth TB, Dell'Accio F, Crouch V, Augello A, Sharpe PT, De Bari C. Functional mesenchymal stem cell niches in adult mouse knee joint synovium in vitro. *Arthritis. Rheum.* 63, 1289-1300 (2011).
26. Diaz-Romero J, Nesic D, Grogan SP, Heini P, Mainl-Varlet P. Immunophenotypic changes of human articular chondrocytes during monolayer culture reflect bona fide dedifferentiation rather than amplification of progenitor cells. *J. Cell. Physiol.* 214, 75-83 (2007).
27. Lee HJ, Choi BH, Min B-H, Park SR. Changes in surface markers of human mesenchymal stem cells during the chondrogenic differentiation process in vitro. *Arthritis. Rheum.* 60, 2325-2332 (2009).
28. Rosengren S, Boyle DL, Firestein GS. Acquisition, culture, and phenotyping of synovial fibroblasts. *Methods. Mol. Med.* 135, 365-375 (2007).
29. Summers KL, O'Donnel JL, Hoy MS *et al*. Monocyte-macrophage antigen expression on chondrocytes. *J. Rheumatol.* 22, 1326-1334 (1995).

30. Steck E, Fischer J, Lorenz H, Gotterbarm T, Jung M, Richter W. Mesenchymal stem cell differentiation in an experimental cartilage defect: restriction of hypertrophy to bone-close neocartilage. *Stem. Cells. Dev.* 18, 969-978 (2009).
31. de la Fuente R, Abad JL, García-Castro J *et al*. Dedifferentiated adult articular chondrocytes: a population of human multipotent primitive cells. *Exp. Cell. Res.* 297, 313-328 (2004).
32. Alsalameh S, Amin R, Gemba T, Lotz M. Identification of mesenchymal progenitor cells in normal and osteoarthritic human articular cartilage. *Arthritis. Rheum.* 50, 1522-1532 (2004).
33. Dowthwaite GP, Bishop JC, Redman SN *et al*. The surface of articular cartilage contains a progenitor cell population. *J. Cell. Sci*. 117, 889-897 (2004).
34. Grogan SP, Miyaki S, Asahara H, D'Lima DD, Lotz MK. Mesenchymal progenitor cell markers in human articular cartilage: normal distribution and changes in osteoarthritis. *Arthritis. Res. Ther.* 11, R85-97 (2009).
35. Koelling S, Kruegel J, Irmer M *et al*. Migratory chondrogenic progenitor cells from repair tissue during the later stages of human osteoarthritis. *Cell. Stem. Cell.* 4, 324-325.

Figure Legends

Figure 1. Histology of the ACI: adjacent macroscopically normal cartilage (MNC) interface. Dotted line shows the ACI treated areas. **A,** Samples received following arthroplasty, showing ACI repair regions at the centre of the medial condyle (patient 1, top) and femoral head (patient 2, bottom). Regions dissected for histology are indicated (boxed). **B,** Haematoxylin and eosin stained sections showing a disorganized cell distribution in ACI repair tissues (left) compared to typical hyaline cartilage cell organization (right). **C,** Polarized light illumination showing fibrocartilage (bright scatter) in ACI repair tissues (left), particularly evident at ACI: MNC interfaces (centre). **D,** Toluidine blue localization of GAGs demonstrates that there are fewer GAGs present in ACI repair regions (left) compared to MNC (right). A dramatic reduction in GAG staining intensity is seen in the surface zone of ACI repair tissues. **E,** Collagen type II immunolocalization demonstrates that ACI repair region matrices (left) contain less collagen type II compared to MNC (right). All calibration bars = 1mm.

Figure 2. Cell characterisation: Morphology. **A,** Freshly isolated cells are shown (top panels) compared to cells at passage 3 (bottom panels). Chondrocytes (C) and mesenchymal stromal cells (MSC) isolated from both patients have a uniform fibroblast-like morphology which increase in size (area coverage) with sub-culture. Freshly isolated ACI repair cells (ACI) show a heterogeneous morphology which becomes homogeneous at passage 3. All calibration bars = 200μm. **B,** Chondrocytes (black bars) and MSC (white bars) increase in size from passages 0-3. Chondrocytes are significantly smaller than MSC at every passage

quantified. At initial seeding ACI repair cells (grey bars) are significantly larger than chondrocytes, but similar in size to MSC. At passage 3 ACI repair cells are significantly smaller than MSC, but similar in size to chondrocytes (means +/- SEM Kruskal-Wallis ANOVA and post-hoc Bonferroni ***p <0.001).

Figure 3. Cell characterisation: Growth kinetics. **A,** The doubling times (DT) of chondrocytes and ACI repair cells decrease similarly through passages 0-3. In contrast, MSC DT increase between passages 1-3 (means +/- SEM from pooled patient data). **B,** After harvest at passage 3 chondrocyte cultures produced $1.4X10^8$ cells, ACI repair cultures $4.3X10^7$ cells and MSC cultures $9.0X10^6$ cells (means +/- SEM from pooled patient data).

Figure 4. Phenotypic characterisation: Immunoprofile and differentiation capacity (passage 2 cells). **A,** Flow cytometry analysis for CD-immunolabelling of chondrocytes, ACI repair cells and MSC. All cultures tested are CD19-ve, CD31-ve, CD34-ve, CD45-ve, CD73+ve, CD90+ve, CD105+ve and HLA-DR-ve at passage 2. C and ACI repair cells are also immunopositive for CD14 to varying degrees (patient 1 chondrocytes are ~50%+ve and ACI are ~90%+ve, patient 2 chondrocytes are ~90%+ve and ACI are ~50%+ve), where as MSC are CD14-ve. The green-lined histogram denotes immunopositivity (fluorescence intensity) for each indicated marker, whilst the purple histogram denotes immunolabelling with an isotype-matched control antibody.

Figure 5. Phenotypic characterisation: Differentiation capacity (passage 2 cells). **A,** The presence of lipid vesicles is increased in chondrocytes treated with adipogenic stimuli (but a different type of staining pattern between patients can be observed, as revealed with oil red-O), compared with ACI repair cells or MSC

559 (which had similar localised unilocular lipid staining in patches). **B,** Alkaline
560 phosphatase activity is markedly increased in chondrocyte cultures treated with
561 osteogenic stimuli compared with ACI repair cells or MSC. **C,** Toluidine blue
562 staining of chondrogenically induced pellet cultures shows more intense staining
563 in chondrocyte pellets compared to ACI repair cell or MSC pellets. Calibration
564 bars = 100μm, inset calibration bar = 200μm.

565 **Table 1.** Patient information: Demographics and ACI treatment received.

	Patient 1	***Patient 2***
Time since ACI	135 months (~11 years)	59 months (~5 years)
Site of ACI	Medial Femoral Condyle	Lateral aspect of Femoral Head
Size of defect	25 x 15mm	12 mm diameter
Cells received	Knee chondrocytes	Hip chondrocytes
Patch received	Periosteum	Chondro-Gide®

566