Migratory Chondrogenic Progenitor Cells from Repair Tissue during the Later Stages of Human Osteoarthritis

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SUMMARY

The regeneration of diseased hyaline cartilage continues to be a great challenge, mainly because degeneration—caused either by major injury or by age-related processes—can overextend the tissue’s self-renewal capacity. We show that repair tissue from human articular cartilage during the late stages of osteoarthritis harbors a unique progenitor cell population, termed chondrogenic progenitor cells (CPCs). These exhibit stem cell characteristics such as clonogenicity, multipotency, and migratory activity. The isolated CPCs, which exhibit a high chondrogenic potential, were shown to populate diseased tissue ex vivo. Moreover, downregulation of the osteogenic transcription factor runx-2 enhanced the expression of the chondrogenic transcription factor sox-9. This, in turn, increased the matrix synthesis potential of the CPCs without altering their migratory capacity. Our results offer new insights into the biology of progenitor cells in the context of diseased cartilage tissue. Our work may be relevant in the development of novel therapeutics for the later stages of osteoarthritis.

INTRODUCTION

Articular cartilage is responsible for the smooth transmission of force from one bone to another, thereby allowing for painless skeletal movements. Severe cartilage injury due to age-related degeneration or excessive use rarely heals. It can result in osteoarthritis (OA), often with painful loss of joint function (Buckwalter and Mankin, 1998; Horton et al., 2006). Current therapeutic interventions serve primarily as short-term symptomatic relief, and joint replacement is ultimately required for almost all patients (Lohmander and Roos, 2007).

OA is the most common musculoskeletal disease in the elderly (Reginster, 2002). The world’s aging population is expected to make OA the fourth-leading cause of disability by the year 2020 (Woolf and Pfleger, 2003). This is our motivation for the further exploration of OA treatment options, including regenerative cell biological therapy.

In cartilage tissue, chondrocytes are embedded in a framework of collagens, mainly collagen type II, together with proteoglycans and glycoproteins. These act as link proteins to stabilize the collagen network (Kuettner, 1992). The maintenance of this extracellular matrix (ECM) is important for rigidity and helps the tissue resist compression and shear forces (Goldring and Goldring, 2007; Sandell, 2007). Disturbed cell-matrix interactions play a central role in OA pathogenesis (Goldring and Goldring, 2007; Kuettner, 1992; Poole, 1999; Sandell, 2007), leading to the loss of the superficial zone and eventually producing deep surface fissures and collagen fiber fibrillation (Goldring and Goldring, 2007; Poole, 1999; Sandell, 2007; Tesche and Miosge, 2005).

It seems to be the case that mesenchymal cells are involved in the regeneration processes that accompany cartilage tissue degradation. In early stages of the disease, Pridie drilling and microfracturing to open the bone marrow underneath the cartilage defect can sometimes encourage the formation of fibrocartilaginous repair tissue (Pridie, 1955; Simon and Jackson, 2006). In separate experiments, various populations of bone marrow stem cells have been shown to differentiate into cartilage-like tissue both in vivo and in vitro (Caplan, 2007; Gronthos et al., 2003; Johnstone et al., 1998; Kassem and Abdallah, 2008; Li et al., 2005; Mackay et al., 1998; Prockop et al., 2001; Tuan et al., 2003). After enzymatic digestion of OA tissue, cells with mesenchymal characteristics have been defined with the help of surface markers (Alsalam et al., 2004; Fickert et al., 2004; Hiraoka et al., 2006), and postnatal stem cells have been identified in the superficial zone of healthy cartilage (Downs whait et al., 2004). Nevertheless, no studies to date have identified a committed migratory chondrogenic progenitor cell population in repair tissue in the context of late-stage OA. With this study, our goal was to determine whether diseased adult cartilage harbors cells that feature progenitor cell characteristics and that specifically exhibit migratory capabilities. We believe that such cells may be a suitable starting point for the development of regenerative therapies for the treatment of OA. We isolated a progenitor cell population from human OA tissue, evaluated relevant stem cell characteristics, and assessed their multipotent differentiation capacity especially in the context of their chondrogenic lineage. We also evaluated their migratory
potential. Furthermore, with the help of RNA knockdown, we confirmed that it is the sox-9 and runt-related transcription factor 2 (runx-2) that regulate their chondrogenic differentiation. Since these cells show heterogeneity in these properties, and especially because of their chondrogenic and migratory potential, we term these cells chondrogenic progenitor cells (CPCs).

**RESULTS**

**Cartilage-Derived Cells from Late-Stage OA Exhibit Progenitor Cell Features**

In previous investigations (Bock et al., 2001; Koelling et al., 2006; Kruegel et al., 2008; Miosge et al., 2004; Tesche and Miosge, 2005) of the matrix composition of OA from patients undergoing total knee replacement (see Figure S1A available online), we found not only deep surface fissures and cell clusters but also breaks in the tidemark. These breaks were packed with blood vessels that had vascularized into the cartilage tissue (Figure 1A). Further analysis revealed certain elongated cells adjacent to round chondrocytes in the repair tissue that had been excised from the area adjacent to the main defect (Figure S1B). Our routine electron microscope evaluations exposed elongated cells with widened rough endoplasmic reticulum at their migration front crossing the tidemark and migrating into the cartilage matrix (Figure 1B) and confirmed the existence of these cells (Figures S1C and S1D). These phenomena—breaks in the tidemark and elongated migrating cells in deep zones—were not found in healthy cartilage.

To investigate whether cells are able to migrate out of cartilage tissue taken from the area adjacent to the main defect, we studied OA tissues from 262 patients (66.03% female). We placed excised repair tissue in a culture dish, and 5–10 days later we successfully observed migrating cells in 93.83% of the samples studied (Figure 1C). We did not observe the same behavior with healthy cartilage samples.

Population doubling (PD) calculations underline the nature of CPCs as a progenitor cell system. We derived a result of 60 PD, and we note that after 100 days of monolayer culture our CPCs had undergone 28–30 PD, with incrementally increasing time for each doubling event (Figure 1D). Taken together with increasing levels of p16INK in the heterogeneous population, this seems to indicate cell senescence. Similar PD values have been recorded for other stem cells, including human tendon stem cells (Bi et al., 2007). Immunocytochemistry showed that these cells were positive for STRO-1 and CD29 (Figure 1E), two markers that are related to stem cells. Dilution cloning generated 2%–8% clones derived from single cells of primary CPCs (Figure 1F). However, after 64.6 PD, these clones entered senescence. Therefore, we immortalized CPCs in order to generate sufficient numbers of cells from each individual
clone. Our goal was to further compare the characteristics of clonal CPCs and the heterogeneous population.

**CPCs Are Distinct from Cells of Osteoblastic Lineage and Chondrocytes**

Before we could further assess CPCs in terms of progenitor cell criteria, we compared the gene expression profile of CPCs to that of bone cells. For comparison purposes, we chose a heterogeneous population of an osteoblastic lineage, referred to here as osteoblast lineage cells. These cells were taken underneath the main OA defect. Chondrocytes were excised from healthy cartilage. We harvested these cells, expanded them to passage 1 (P1), and carried out real-time RT-PCRs. Chondrocytes exhibited high relative expressions of collagen type II; intermediate levels of sox-9, one of the major chondrogenic transcription factors; and low levels of collagen type I and runx-2, one of the major osteogenic transcription factors (Figure 2A). In contrast, CPCs exhibited intermediate levels of sox-9 and runx-2 as well as high levels of collagen type I (Figure 2B). The osteoblast lineage cells displayed high levels of collagen type I and runx-2 as well as the bone cell markers MEPE and sclerostin (Figure 2C). Immunocytochemistry confirmed this unique pattern. CPCs tested positive for sox-9, runx-2, and collagen type I. The same was seen for clonal CPCs, identified in Figure 2, IF8. Chondrocytes were positive for sox-9, aggrecan, and glycosaminoglycan (Alcian blue) staining and negative for runx-2. The opposite was true for the osteoblast lineage cells, which were also positive for osteocalcin staining (Figure 2D). Intracellular FACs analysis revealed that the CPC was positive for collagen type I and sox-9 but negative for collagen type II protein (Figure 2E). Osteoblast lineage cells were positive for collagen type I, β1, and α4 integrin (Figure 3A). Chondrocytes were positive for sox-9, β1, and α5 integrin (Figure 3B). CPCs tested positive for the integrins β1, α3, and α5 (Figure 3C).

We also used flow cytometry to examine the presence of surface antigens in our CPC population. We identified populations that were nearly homogeneous in respect of the following markers related to stem cells: CD13, CD29, CD44, CD73, CD90, and CD105. More than 15% were positive for STRO-1. CPCs were negative for the markers CD34, CD31, and
CD117, as well as for the leukocyte marker CD45, thus demonstrating an absence of contaminating hematopoietic cells (Figures 3D and 2F). Other markers assigned to bone marrow-derived stromal cells—for example, CD271, CD18, or CD31—were negative in our CPC populations. Similar marker profiles were seen for clonal CPCs. To test for cell culture artifacts, we also stained native OA tissue and confirmed the presence of CD73/CD29 double-positive cells in vivo (Figure 2G), allowing us to rule out cell culture artifacts as an explanation of our results.

To further characterize CPCs, we performed microarray analysis and compared the gene expression profiles of CPCs to chondrocytes taken from healthy donors and to osteoblast lineage cells taken from the bone underneath the main OA defect. For the surface antigens CD13, CD44, and CD73, higher expression values were found in CPCs compared with chondrocytes. CD29, CD90, and CD105 exhibited lower values (Figure 3D). CPCs revealed an mRNA pattern that was different from osteoblast lineage cells, in which the typical bone cell markers were upregulated (GEO database GSE10575). For CD13, CD44, CD73, and CD90, the results were consistent with the FACS analysis (Figure 3E). For CD29 and CD105, the FACS was positive, while we identified negative fold changes of runx-2 and collagen type I. Again, clonal CPCs exhibited a similar differentiation potential of CPCs. Error bars are means ± SD.

CPCs Are Multipotent

The multidifferentiation potential is the most important defining characteristic of stem cells. Therefore, we attempted to differentiate CPCs. After adipogenic differentiation, cells exhibited LPL and PPAR, staining as well as oil red O-positive fat vacuoles as an indication of terminal adipogenic differentiation (Figure 4A). RT-PCR confirmed the presence of low levels of sox-9 and collagen type I, alongside high levels of PPAR, and LPL. After osteogenic differentiation, cells tested positive for osteocalcin, osteopontin, and osteonectin. Furthermore, they exhibited alkaline phosphatase staining and, after prolonged cultivation, they showed Alizarin red-positive nodules, demonstrating calcification (Figure 4B). We detected low relative mRNA levels for sox-9 and collagen type II, as well as high levels for runx-2 and collagen type I. The same differentiation pattern was seen for clonal CPCs. However, we were most interested in the chondrogenic differentiation potential of CPCs. Even without any chondrogenic supplementation, we observed that cells that had been transferred to a 3D-alginate culture after 3 weeks regained a round chondrocyte-like phenotype. They exhibited collagen type II mRNA, as revealed by light and ultrastructural in situ hybridization (ISH), as well as collagen type II protein (Figure 4C). We also identified high levels of sox-9 and collagen type II mRNA and low levels of runx-2 and collagen type I. Again, clonal CPCs exhibited a similarly high chondrogenic differentiation potential. Control experiments were performed with immortalized fetal chondrocytes (T/C-28) and with isolated healthy chondrocytes, which did not differentiate and died soon after the start of the test (data not shown).

The Influence of TGFβ3 and BMP-6, ECM Components and Surface Antigens on CPCs

After we had identified the influence of a 3D environment in encouraging CPCs toward the chondrogenic lineage, we...
Decided to investigate the role of the chondrogenic mediators TGF-β3 and BMP-6. We cultured CPCs in 3D with and without TGF-β3/BMP-6. The amount of collagen type II protein and the extent of fiber formation, as observed using electron microscopy, were enhanced with TGF-β3/BMP-6 as compared to controls (Figure 5A). Collagen type II was increased, while collagen type I, runx-2, and MMP-13 mRNAs were reduced. Surprisingly, neither mediator had any effect on the expression of sox-9 (Figure 5B).

We cultured CPCs on collagen types I, II, or IV or on fibronectin or Matrigel. CPCs exhibited multiform cell shapes that were clearly different from the uniform ellipsoid shapes of these cells on plastic alone. We concluded that ECM components, such as collagen type IV, fibronectin, or laminin-111 (a major component of the basement membrane extract in Matrigel), alter the cell shape of CPCs. We again found differences on samples cultured on or within Matrigel. CPCs embedded within Matrigel showed elongated tube-like structures (Figure 5C), underlining the importance of a 3D environment, as already discussed. The expression level of the matrix components was also influenced by the nature of the ECM, as was the expression of markers related to stem cells such as CD29 and CD73. Matrigel allowed for a similar expression level of CD73 and a higher level of CD29 compared with controls on plastic or compared with CPCs grown on fibronectin or on collagen type IV (Figure 5D). Growing CPCs on human collagen type II enhanced their chondrogenic potential. To investigate the role of ECM components, we performed an in vitro adhesion assay for ECM molecules and found that CPCs would adhere to collagen types I, II, and IV or to fibronectin (Figure 5E).

The heterogeneity of the CPCs affects their differentiation potential. We found a reduced osteogenic differentiation potential for CPCs that expressed different levels of the various surface markers. Without the use of any osteogenic stimulation, we detected lower levels of runx-2 and collagen type I for CPCs that tested 99% positive for CD44, CD73, or CD105 as compared with heterogeneous CPCs (Figure 5F). CPCs that expressed CD73 or CD105 and that were grown in osteogenic media exhibited decreased levels of collagen type I, again indicating a reduced osteogenic differentiation potential (Figure 5G).

Knockdown of runx-2 Enhances the Chondrogenic Potential of CPCs

In CPCs that express both sox-9 and runx-2, we confirmed that TGF-β3/BMP-6 had no effect on sox-9 but downregulated runx-2. Therefore, we tested whether the knockdown of runx-2, one of the major osteogenic transcription factors, would influence the chondrogenic potential of CPCs. We administered interfering RNA for runx-2 and collagen type I to CPC samples and cultured them either on plastic or in 3D. The knockdown of collagen type I RNA in the 3D culture resulted in the reduction as expected of collagen type I mRNA as compared with control experiments. Similar results were observed with knockdown of runx-2 (Figure 6A). However, when cultured on plastic, the effect of the runx-2 knockdown was not observed, possibly due to the influence of the 2D culture conditions. Similarly, the collagen type I level did not decrease. The simultaneous knockdown of runx-2 and collagen type I showed an efficiency that was equivalent to that of the single runx-2 knockdown (Figure 6B). When we investigated the effect of the runx-2 knockdown (Figure 6C) on CPCs in 3D culture in the context of chondrogenic markers, we recorded a 92% reduction in runx-2, and we were surprised to observe a greater than 100% increase in the expression of sox-9. This was in line with an increase in two chondrogenic markers, namely collagen type II and aggrecan, and a decrease in their degrading potential.
enzymes, MMP-13 and ADAMTS-5 (Figure 6D). The clonal CPCs behaved in a similar manner in terms of runx-2, sox-9, and aggrecan expression. The reciprocal experiment, namely the knockdown of sox-9, revealed a strong downregulation of aggrecan and a significant reduction in runx-2 mRNA (Figure 6E). These results indicate a degree of crosstalk between these two regulators in CPCs.

The Migration Potential of CPCs

To further investigate the possible behavior of CPCs in vivo, and to evaluate their cell biological potential for a possible future regenerative OA therapy, we performed an in vitro migration assay. We also performed a similar assay for clonal CPCs (Figure 6F). We transfected CPCs with GFP and enriched a greater than 99% GFP-positive population (Figure 7A). To further test their migration capacity, GFP-positive CPCs were placed on the surface of late-stage OA tissue. CPCs not only migrated in vitro, they also populated diseased tissue ex vivo. The standard deviations indicate that certain slower- and faster-migrating CPCs do exist in the heterogeneous populations (Figure 7B). To investigate the influence of the runx-2 knockdown on the migration capacity, GFP-positive CPCs were transfected with the runx-2 siRNA. Using the ApoTome technique, an extensive analysis of ex vivo migration revealed both control CPCs and knockdown CPCs approximately 1200–1400 μm deep in the repair tissue after 2 days (Figure 7C). The same results were recorded for clonal CPCs (Figure 7D). CPCs were viable for up to 3 weeks; ki67 staining of GFP-positive CPCs deep in the OA tissue indicated their ability to proliferate (Figure 7E).
DISCUSSION

The physiological repair mechanisms associated with diseased cartilage are few, and all are generally overridden by matrix destruction that results in less-functional fibrocartilaginous, collagen type I-rich scar-like tissue (Sandell, 2007; Tesche and Miosge, 2005). The discovery of migratory CPCs that are able to grow from diseased cartilage tissue and that exhibit a high chondrogenic potential will open new possibilities for the future treatment of OA.

Based on a number of different criteria, we have successfully identified and isolated a migratory progenitor cell population from late-stage human OA tissue. We show that CPCs adhere to and are influenced by ECM components and that downregulation of runx-2 enhances their chondrogenic potential. Furthermore, we demonstrate that CPCs migrate into diseased cartilage tissue explants. As in the case of other stem/progenitor cells, no single marker can identify these cells. However, a combination of RT-PCR, immunocytochemistry, and FACS analysis has been successfully used to characterize CPCs. Our results demonstrate that these cells are distinct from chondrocytes, osteoblast lineage cells, and mesenchymal stem cells. They exhibit certain markers of bone marrow stromal cells—for example, CD44, CD73, and CD105—but they are clearly different from such cells. CPCs exhibit a low level of STRO-1 expression, indicating that they are best understood as progenitor cells. However, we note that even stem cells that are strongly positive for STRO-1/CD106 do sometimes fail to generate colonies (Gronthos et al., 2003).

In addition, our results demonstrate that CPCs are viable as cells of the chondrogenic lineage if they are simply placed in 3D surroundings. Culturing CPCs on Matrigel sustained the expression of markers of relevance to stem cells, such as CD29 and CD73. This speaks to the profound role of ECM components on CPCs and once again underlines the importance of the ECM in stem cell biology (Bi et al., 2007; Fuchs et al., 2004). Recent reports have highlighted the possibility that dedifferentiated chondrocytes may regain stem cell characteristics in culture (Barbero et al., 2003; Dell’Accio et al., 2003; Liu et al., 2007) and that stem cells have separately been found in fibroblast-like cell populations (Sudo et al., 2007). Even adult fibroblasts have been transformed into stem cells via the transfection of oct3/4, sox2, c-myc, and klf4 (Takahashi and Yamanaka, 2006). These results indicate that the nature of the stem cell (Jaenisch and Young, 2008), its niche, and its relation to progenitor cells all have yet to be clarified. This observation also holds true for the CPCs presented here. Clonal primary CPCs reach senescence after 60PD and heterogeneous populations exhibit increasing p16INK levels, suggesting that CPCs should be considered progenitor cells rather than true stem cells.

Consistent with the importance of the ECM, 3D culture conditions seem to be sufficient for the chondrogenic differentiation of CPCs. However, their chondrogenic potential can also be enhanced. OA chondrocytes are known to express less sox-9. Its overexpression has been shown elsewhere to restore the ECM of OA tissue (Cucchiarini et al., 2007). Recently, asporin (a small leucine-rich proteoglycan) has been shown to bind TGFβ to reduce chondrogenesis (Nakajima et al., 2007). Our work has shown that TGFβ3 together with BMP-6 does not alter the level of sox-9. However, both of
these additives together reduce expression of runx-2 in CPCs. TGFβ3, and to a lesser extent BMP-6, serves as a chemoattractant for CPCs in vitro. Runx-2 has been shown to be increased in OA cartilage (Wang et al., 2004) and to induce MMP-13 as a means of promoting OA (Kamekura et al., 2006). We observed that the chondrogenic potential of CPCs was even more dramatically enhanced via the direct knockdown of runx-2. This elevated sox-9, aggrecan, and collagen type II expression of these cells. In contrast, the sox-9 knockdown reduced aggrecan and runx-2 expression. This possible cross-talk between runx-2 and sox-9 in CPCs needs to be further investigated.

The expression of the collagen receptor DDR-2 has been linked to elevated MMP-13 expression (Xu et al., 2007), and increased levels of DDR-2 have been correlated with the degree of cartilage degeneration (Sunk et al., 2007). Our microarray data indicate that CPCs exhibit decreased expression of DDR-1 and the integrin repertoire, together with increased expression of MMPs. Indeed, in our ex vivo experiments, CPCs were found to migrate into the diseased cartilage tissue, and the knockdown of runx-2 did not alter their migration capacity.

Recent studies have described cells with mesenchymal stem/progenitor characteristics that have been isolated from OA tissue following tissue digestion and prolonged culture on plastic (Alsalameh et al., 2004; Fickert et al., 2004; Hiraoka et al., 2006). Our identified cell population was derived from repair tissue, selected based on cell-migratory capacity. We consider that our CPCs are committed toward the chondrogenic lineage. We found evidence that CPCs migrate from the bone marrow through breaks in the tidemark. However, in cartilage tissue, they retain their runx-2 and collagen type I expression. The exact origin of the CPC remains to be elucidated. Whether they are derived from the various mesenchymal stem cell populations that have already been identified or whether they are transdifferentiated chondrocytes that are associated with the late stages of OA remains an open question. However, because CPCs migrate into and populate diseased cartilage tissue ex vivo, CPCs may be an ideal starting point for the exploration of OA regenerative therapy options.

There are several key limitations that must be overcome before a CPC-based regenerative therapy can be conceptualized. First, one issue is the differing chondrogenic potential of the CPC and its clones across different patients. Individual cells from the heterogeneous population may serve to influence one other. Furthermore, it remains unclear whether CPCs can be permanently transfected to reduce runx-2 levels and exhibit a chondrogenic potential that can be sustained over time. Second, we have yet to confirm that CPCs will produce an ECM that supports a more cartilage-like repair tissue with a higher physical resistance to mechanical stress than the fibrocartilaginous tissue that develops during the course of OA. Identifying the optimal conditions to manipulate CPCs such that they can exhibit sustained chondrogenic capacities will be crucial. We hope ultimately to work toward utilizing these cells—which are already present in diseased tissue—for the development of OA-relevant regenerative therapies.
Tissue Sources and Preparation

Adult osteoarthritic cartilage, without signs of rheumatoid involvement, was obtained from the knee joints of patients (ages 65–75 years) suffering from late-stage OA after total knee replacement. The patients met the American College of Rheumatology classification criteria (Altman et al., 1986) and gave their written informed consent consistent with relevant ethics regulations. The histopathological classification of OA cartilage confirmed the presence of late-stage OA (Pritzker et al., 2006). For light microscopy, tissue samples were processed for immunohistochemistry as described elsewhere (Bock et al., 2001). For ultrastructural investigations, 8 mm² cartilage samples from the upper intermediate zones were dissected from areas adjacent to the main defect at a maximum distance of 0.5 cm. Thereafter, all tissue samples were fixed and embedded in the hydrophilic resin LR-Gold (London Resin Company, Reading, UK). Subsequently, semithin (1 μm) and ultrathin sections (80 nm) were prepared; the ultrathin sections were collected on formvar-coated grids as described elsewhere (Kruegel et al., 2008).

Cell Isolation and Culture

Standard explant cultures were performed using 8–15 mm² tissue specimens taken from areas adjacent to the main defect. After 10 days, outgrown cells were harvested and 10⁶ cells/cm² transferred to a monolayer culture in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum (GIBCO, lot number 41F2061K), supplemented with penicillin/streptomycin (50,000 U/50μg/ml) and L-glutamine (10 mM), and cultured under standard conditions.

To test the influence of ECM components, explant cultures were grown in 6-well plates coated overnight at 37°C with 10 μg/cm² of human fibronectin (MP Biomedicals Inc.), collagen type IV (bovine, Sigma), or collagen type II (human adult knee cartilage, RayBiotech, Norcross, USA). Growth factor-reduced Matrigel was handled according to the manufacturer’s instructions.

As controls, chondrocytes from healthy cartilage and osteoblast lineage cells derived from the bone underneath the OA defect were harvested after digestion for 6 hr at 37°C with the aid of collagenase I (152 U/ml; Invitrogen, Karlsruhe, Germany), collagenase II (280 U/ml; Biochrom, Berlin, Germany), and dispase (15 U/ml; Invitrogen). To eliminate cell-matrix residues, digested material was filtered through a 40 μm mesh sieve (BD Falcon, Heidelberg, Germany) and cultured as described above. Immortalized human fetal chondrocytes (T/C-28) were donated by Mary Goldring, Hospital for Special Surgery, New York (Goldring et al., 1994). PDs were calculated as described elsewhere (Bi et al., 2007).

Cloning and Immortalization

We performed dilution cloning with 10 cells/ml and 100 μl/well on a microtiter plate with three individual CPC populations. Single cells were grown in conditioned medium taken from the heterogenous cells and supplemented with 10% ECS.

Lentivirus expressing hTERT was produced as described elsewhere (Docheva et al., 2009). Plasmid pLent6/s/V-HTERT was cotransfected with pLP1, pLP2, and pLP/VSVG helper plasmids (ViraPower lentiviral expression system, Invitrogen, Karlsruhe, Germany) and cultured as described above. Immortalized human fetal chondrocytes (T/C-28) were donated by Mary Goldring, Hospital for Special Surgery, New York (Goldring et al., 1994). PDs were calculated as described elsewhere (Bi et al., 2007).

Multipotent Differentiation

A number of 10³ CPC/cm² samples in 75 cm² flasks were encouraged toward the osteogenic lineage by applying NH OsteoDiffMedium (Miltenyi Biotec) containing dexamethasone, ascorbic acid, and 1-glycerophosphate. For adipogenesis, we used NH AdipoDiffMedium containing hydrocortisone, isobutylmethylxanthine, and indomethacin. For chondrogenic differentiation, a 3D-alginate culture was initiated when 70% confluence in the monolayer was reached. CPCs in P1 were harvested, counted, and tested for their viability by trypan exclusion. Forty thousand CPCs per bead were gelated in low-viscosity alginate with or without human recombinant 2.5 ng/ml TGFβ3 (Sigma) and 40 ng/ml BMP-6 (R&D Systems) for 3 or 6 weeks.

Cytchemistry

The use of alkaline phosphatase (BC, Sigma), Alizarin red S, and oil red O staining has been previously described in detail (Ponce et al., 2008).

Antibodies and Immunocytochemistry

The anti-human bone alkaline phosphatase (AP) antibody, a mouse anti-human monoclonal IgG, was purchased from Metra Biosystems (Palo Alto, CA). John D. Brunzell from the University of Washington provided the lipoprotein lipase (LPL)-specific (S2D) antibody. The PPAR, (E8, sc-7273), runx-2 (M70, sc10758), and sox-9 (H90, sc-2095) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The osteocalcin (OC) antibody was a rabbit polyclonal antibody (BTI, MA). Monoclonal anti-type I collagen (M38), an anti-type II collagen antibody (CIC31), osteonectin (ON, AON-1), osteopontin (OP, MP1IIIB10), and STR-1 antibodies were obtained from the Developmental Studies Hybridoma Bank, University of Iowa. The kit-8 anti-biody was purchased from Dako, and the anti-aggreican (SC5) antibody was purchased from http://www.antibodies-online.de (Germany). We used phycoerythrin (PE)- or fluorescein isothiocyanate (FITC)-coupled primary antibodies against human integrins s2 (FITC), s3 (PE), s4 (PE), s5 (PE), s6 (FITC), and b1 (FITC) as well as CD13 (PE), CD14 (FITC), CD18 (PE), CD24, CD29 (FITC), CD31 (PE), CD34 (FITC), CD44 (FITC), CD45 (FITC), CD73 (PE), CD90 (FITC), CD105 (FITC), CD106 (FITC), CD117 (FITC), CD166 (BD PharMingen), and CD271 (FITC, Miltenyi Biotec). As secondary antibodies, a goat anti-mouse, a goat anti-rabbit-FITC IgG (Dianova, Hamburg, Germany), and anti-mouse-PE/FITC monoclonal immunoglobulin isotype controls were purchased from BD PharMingen. For the ISH, the anti-DIG antibody (sheep-IgG) was obtained from Quartett (Berlin, Germany). For immunoblotting, we used a goat anti-mouse antibody coupled to alkaline phosphatase and a pan-ii-actin antibody (Dako, Hamburg, Germany) to determine the amount of protein loaded onto the gels.

Light Microscopy Immunofluorescence

Primary cells were transferred to P1 in 96-well plates. After 16 hr, they were fixed with 70% ethanol and incubated with 100 μl of primary antibody diluted 1:50 in PBS for 1 hr at RT in the dark. When necessary, we followed this procedure with a secondary fluorescence-coupled antibody diluted 1:500 for 20 min at RT. Two washing steps with PBS and DAPI staining were performed thereafter. The STRO-1 antibody was applied according to published protocols (Gronthos et al., 2003). Cells were examined using a fluorescence microscope, and pictures were captured with a Coolpix MDC camera (Nikon, Japan).

Electron Microscopy Immunocytochemistry

For ultrastructural analysis, CPC beads were fixed for 15 min in 4% paraformaldehyde and 0.5% glutaraldehyde in HEPES buffer supplemented with Ca²⁺ at 37°C. They were subsequently dehydrated in up to 70% ethanol also containing Ca²⁺ and embedded in the hydrophilic resin LR-Gold. A goat anti-mouse IgG sample was labeled with 16 nm gold particles as previously described (Gersdorff et al., 2005). For controls and further details, see the Supplemental Experimental Procedures.

Immunoblotting

Proteins were extracted using 5 M guanidine hydrochloride and protease inhibitors, precipitated in ethanol, washed in PBS, again precipitated, and finally dissolved in PBS containing 0.4% SDS. SDS-PAGE was performed with 6% acrylamide in the stacking gel and 12% in the separation gel. Gel loading was determined by β-actin staining. Proteins were blotted onto nitrocellulose membranes, washed, and blocked, and immunoreactions were performed by exposure to the antibodies for 2 hr, diluted 1:500 in PBS. The secondary goat anti-mouse antibody was diluted 1:1000 and incubated for 1 hr at RT. Visualization was achieved by applying NBT/BCIP coloring agent.

RNA Extraction from Cultured Cells, RT, and Probe Preparation

For ISH

A number of 10³ CPC/cm² samples in 75 cm² flasks were encouraged toward the osteogenic lineage by applying NH OsteoDiffMedium (Miltenyi Biotec) containing dexamethasone, ascorbic acid, and 1-glycerophosphate. For adipogenesis, we used NH AdipoDiffMedium containing hydrocortisone, isobutylmethylxanthine, and indomethacin. For chondrogenic differentiation, a 3D-alginate culture was initiated when 70% confluence in the monolayer was reached. CPCs in P1 were harvested, counted, and tested for their viability by trypan exclusion. Forty thousand CPCs per bead were gelated in low-viscosity alginate with or without human recombinant 2.5 ng/ml TGFβ3 (Sigma) and 40 ng/ml BMP-6 (R&D Systems) for 3 or 6 weeks.
manufacturer’s instructions (RNeasy Mini Kit, QIAGEN). RNA was reverse transcribed into collagen type II-specific cDNA. Afterwards, in vitro transcription of nonradioactive sense and antisense RNAs was performed (Miosge et al., 2004) with a digoxigenin labeling kit (Boehringer DIG-RNA labeling kit, Boehringer, Mannheim, Germany).

Light and Electron Microscopic ISH
The hybridization solution consisted of 50% formamide, 5× SSC, and 1 μg/μl yeast-RNA. For light microscopy, we used 50 ng Dig-labeled antisense probe per section. The reactions were carried out at 55°C for 20 hr. Posthybridization treatment consisted of washes with 2× SSC (2× 5 min) at 37°C. For electron microscopy, the probe concentration was 100 ng Dig-labeled antisense probe in 20 μl incubation solution per grid (37°C, 20 hr). Posthybridization steps included a washing procedure with 1× SSC (2× 10 min at 37°C), 0.1× SSC (5 min at 50°C), and 10 min PBS at RT (Kruegel et al., 2008). For controls and further details, see the Supplemental Experimental Procedures.

Quantitative Real-Time RT-PCR
Quantities of 5 μl of RealMasterMix (2.5×) (Eppendorf, Hamburg, Germany), 20 pmol of each primer, and 1 μg of cDNA were added to a final volume of 10 μl. Primers were designed with the help of primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and can be found at http://www.prothetik.med.uni-goettingen.de/miosge under "Forschung" and then under "Primer." For details, see the Supplemental Experimental Procedures.

Microarray Analysis
Quality control and quantification of total RNA samples were conducted prior to the microarray experiments (Agilent 2100 Bioanalyzer, Agilent Technologien, Palo Alto, CA). We used equal amounts of total RNA from each of six patients’ CPCs and osteoblast lineage cells, chondrocytes, and T/C-28 cells. Array analysis was carried out by Atlas Biolabs GmbH Berlin using Affymetrix GeneChip HG_U133A Plus 2.0 (Affymetrix, Santa Clara, CA). Microarray experiments were performed according to the manufacturer’s protocols. Biotin-labeled cRNA, fragmented to an average size of 100–150 bp, was hybridized at 45°C for 16 hr and washed in an automated Affymetrix fluidic station. Hybridized cRNA was fluorescently labeled by adding streptavidin-phycocerythrin (Molecular Probes, Eugene, OR). Stained arrays were then scanned using a Hewlett-Packard Gene Array Scanner (Agilent Technologies, Palo Alto, CA). A complete list of genes present on the chip can be found at http://www.affymetrix.com/analysis/index.affx. Data were analyzed using Affymetrix Microarray Suite 5.0. Using the Affymetrix Data Mining Tool 3.0, a gene expression was evaluated. The entire data set is published in a MIAME compliant format in the GEO database GSE10575 (http://www.ncbi.nlm.nih.gov/geo/). We clustered the significantly increased and decreased genes according to the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (Kanehisa and Goto, 2000).

FACS Analysis
Cultured cells were suspended in PBS with the fluorescence-coupled antibodies listed above (1 μl/100 μl/10^6 cells) at RT for 1 hr in the dark. Two subsequent washing steps followed (10 min, 800 rpm). Cells were analyzed on a FACSscan (Becton Dickinson, Mountain View, CA), as described in detail elsewhere (Diaz-Romero et al., 2005). At least 10,000 living cells were analyzed. Data were evaluated with the aid of WinMDI2.9. For cell selection, we applied FACS Vantage SE (Becton Dickinson, Mountain View, CA). We performed analyses using the Cell Quest Pro 2000 software package.

siRNA Transfection
We harvested CPCs with a cell scraper, centrifuged them 10 min at 1000 rpm, washed the cell pellet, and resuspended it in 100 μl Human Chondrocyte Nucleofector Solution with 5 μl si-RNA at 5 nmol (Hs_runx-2, Hp_type I collagen validated siRNA and sox-9 ATGGGAGTAAACATAGTCTGA; Hs_SOX9_1 from QIAGEN). We then selected program U24 in the nucleofector (Amaxa) and immediately pipetted the solution into preincubated 6- or 24-well plates with DMEM + 20% FCS. After 16 hr, 50%–60% of the cells were dead, and the rest were attached to the plastic. We decanted the solution and added the standard medium. CPCs were harvested and analyzed after 96 hr.

GFP Transfection
CPCs (5 × 10^5) were transfected with 2.5 μg of the vector pmaxGFP (Amaxa Biosystems) in 100 μl Human Chondrocyte Nucleofector Solution using nucleofector program U24 (Amaxa). Immediately afterwards, the cells were transferred to DMEM supplemented with 20% FCS, incubated overnight, and then cultured under standard conditions. The transfection efficiency reached approximately 75%. FACS enriched the GFP-positive CPCs to greater than 99%.

Migration Assay
To test the migration capacity of CPCs with and without run-2 knockdown, GFP-labeled 5 × 10^4/cm^2 CPCs were seeded on the surface of an OA cartilage sample and maintained under standard culture conditions. After 2, 3, 9, and 14 days, 1 mm specimens were cut longitudinally and examined with the AxioImager using the ApoTome technique. The distance from the surface was measured with the digital imaging software AxioVision 4.6 (Zeiss, Jena). For the in vitro migration assay, we used a commercial two-chamber system with filters featuring 8 nm pores (CytoSelect, Cell Biolabs, San Diego, CA). Extinctions were measured at 560 nm.

Statistical Analysis
We reported representative data from at least three independent experiments and statistically tested our results using separate specimens. Analysis was performed using SPSS software (13.0) (SPSS, Chicago, IL). Results are reported as mean values and standard deviations (SD). After testing for normal distribution and variance homogeneity, we performed a one-way analysis of variance (ANOVA) and post-hoc pairwise comparison of mean values. The Pearson correlation coefficients were calculated to examine the relationships between the parameters. A p value of <0.05 was considered statistically significant.

SUPPLEMENTAL DATA
The Supplemental Data include one figure and Supplemental Experimental Procedures and can be found with this article online at http://www.cell.com/cell-Stem-Cell/supplemental/S1934-5909(09)00198-6.

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