Differentiation plasticity of chondrocytes derived from mouse embryonic stem cells

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Summary

Evidence exists that cells of mesenchymal origin show a differentiation plasticity that depends on their differentiation state. We used in vitro differentiation of embryonic stem cells through embryoid bodies as a model to analyze chondrogenic and osteogenic differentiation because embryonic stem cells recapitulate early embryonic developmental phases during in vitro differentiation. Here, we show that embryonic stem cells differentiate into chondrocytes, which progressively develop into hypertrophic and calcifying cells. At a terminal differentiation stage, cells expressing an osteoblast-like phenotype appeared either by transdifferentiation from hypertrophic chondrocytes or directly from osteoblast precursor cells. Chondrocytes isolated from embryoid bodies initially dedifferentiated in culture but later re-expressed characteristics of mature chondrocytes. The process of redifferentiation was completely inhibited by transforming growth factor β3. In clonal cultures of chondrocytes isolated from embryoid bodies, additional mesenchymal cell types expressing adipogenic properties were observed, which suggests that the subcultured chondrocytes indeed exhibit a certain differentiation plasticity. The clonal analysis confirmed that the chondrogenic cells change their developmental fate at least into the adipogenic lineage. In conclusion, we show that chondrocytic cells are able to transdifferentiate into other mesenchymal cells such as osteogenic and adipogenic cell types. These findings further strengthen the view that standardized selection strategies will be necessary to obtain defined cell populations for therapeutic applications.

Key words: Mouse embryonic stem cells, Chondrogenesis, Osteogenesis, Mesenchymal cells, Dedifferentiation, Redifferentiation

Introduction

The development of the vertebrate skeleton is a multistep process that involves lineage commitment of mesenchymal cells, migration of these cells to the sites of skeletogenesis, mesenchymal-epithelial interactions that result in cell condensations and differentiation of chondroblasts and/or osteoblasts. Although, many factors are known that have an important function during this process, it is still unclear how these processes are regulated (for a review, see Hall and Miyake, 2000). During endochondral ossification, proliferating chondroblasts differentiate into collagen-II-positive chondrocytes and further into hypertrophic chondrocytes expressing collagen X as a unique marker molecule (Sandell and Adler, 1999). Hypertrophic chondrocytes are thought to undergo apoptosis followed by cartilage matrix calcification (Zenmyo et al., 1996). Simultaneously vascular invasion occurs, and later osteoprogenitor cells differentiate into osteoblasts and deposit bone on the degraded matrix scaffold (Sandell and Adler, 1999). Alternatively, hypertrophic cells positioned between cartilage and osteogenic tissue may undergo differentiation to bone-producing osteoblasts (Bianco et al., 1998), which is often referred to as transdifferentiation (Descalzi et al., 1992; Roach et al., 1995). There is circumstantial evidence that both routes are followed, and the fate of hypertrophic chondrocytes depends on the microenvironment (Riminucci et al., 1998) (reviewed by Cancedda et al., 1995). Transdifferentiation seems to be quite common for cells of mesenchymal origin and has not only been observed for chondrocytes converted into osteocytes (Descalzi et al., 1992) but also for adipocytes converted into osteocytes (Bennett et al., 1991; Roach et al., 1995) or chondrocytes converted into adipocytes (Heermeier et al., 1994; Quarto et al., 1997), which indicates that mesenchymal cells exhibit a high differentiation plasticity.

Differentiation processes of chondrogenic cells can be studied by in vitro differentiation of mouse embryonic stem (ES) cells (Kramer et al., 2000). In vitro differentiation of ES cells closely recapitulates early embryonic developmental processes, and this system has been used to study differentiation of many cell types, such as cardiogenic, skeletal muscle, haematopoietic, neurogenic, epithelial, vascular smooth muscle, adipogenic and chondrogenic cells (reviewed by Keller, 1995; Rathjen et al., 1998; Guan et al., 1999; Wobus et al., 2001). This model system offers the possibility of tracing differentiation from the pluripotent stem cell to terminally differentiated cell types. ES cells have the potential to develop into cells of all three primary germ layers owing to their origin from the inner cell mass of blastocysts

(Evans and Kaufman, 1981; Martin, 1981). ES cell differentiation has also been discussed as an experimental avenue to generate cells for transplantation. ES-cell-derived cardiomyocytes, neuronal cells and insulin-secreting cells have been transplanted into animals and were able to integrate specifically (Klug et al., 1996; Brüstle et al., 1999; McDonald et al., 1999; Arnhold et al., 2000; Liu et al., 2000; Soria et al., 2000). Because ES cells differentiate spontaneously into a heterogeneous population of cells, it is necessary to use selection strategies to obtain pure cultures of a specific cell type. However, it is questionable how pure these populations are, because they originate from pluripotent and developing cells and therefore may contain cells of different developmental states.

Here, we used the ES cell model to study terminal differentiation of chondrocytes and to analyze their differentiation plasticity. We show that chondrocytes differentiated from mouse ES cells via embryoid bodies (EBs) progress further into hypertrophic chondrocytes, which later expressed markers of calcifying cells and present evidence that ES-derived chondrogenic cells continue to display a particular differentiation plasticity.

Materials and Methods

ES cell culture and differentiation of EBs

ES cells of line BLC6 (Wobus et al., 1988) were cultivated on a feeder-layer of Mitomycin-C-inactivated mouse embryonic fibroblasts in cultivation medium consisting of Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Eggenstein, Germany) supplemented with 15% fetal calf serum (FCS, selected batches, Life Technologies, Eggenstein, Germany), 2 mM L-glutamine (Life Technologies, Eggenstein, Germany), 5×10^{-5} M β-mercaptoethanol (Serva, Heidelberg, Germany), non-essential amino acids (Life Technologies, Eggenstein, Germany, stock solution diluted 1:100) as described previously (Wobus et al., 2002). For differentiation, aliquots of 20 μl differentiation medium (with 20% FCS instead of 15%) containing 800 cells were cultivated in 'hanging drops' for 2 days and subsequently in suspension on bacteriological Petri dishes for additional 3 days as described previously (Kramer et al., 2000). The 5 day (5 d) old EBs were plated onto gelatin (0.1%) coated 6 cm tissue culture plates for Alcian blue staining and total RNA isolation and onto gelatin (0.1%) coated two-well (21.3×20 mm/well) Lab-Tek chamber slides (Nunc, Wiesbaden, Germany) for indirect immunostaining and in situ hybridization. Alcian blue staining was performed using standard methods (Romeis, 1989). For a quantitative estimation, digital images from EB outgrowths after Alcian blue staining were done and the size of stained areas was measured using the Scion Image software (Scion Corporation, Frederick, MD).

Experiments were done at least in triplicate, and data analysis was performed using the Sigma Plot 5.0 software (Jandel, Corte Madeira, CA). For statistical analysis the Student's *t*-test was used.

Isolation and differentiation analysis of chondrocytes

For isolation of chondrogenic cells, EBs were cultivated as described above. Chondrogenic cells developed in dense, matrix-bordered areas called nodules. These nodules were cut off the EB outgrowths with a microscalpel under sterile conditions and collected for collagenase (0.1%) treatment for 50 minutes at 37°C to obtain single cell suspensions. Cells were centrifuged for 5 minutes at 180 ***g***, resuspended in differentiation medium and plated at high density onto gelatin- or collagen-II- (SIGMA, Taufkirchen, FRG) coated 6 cm tissue culture plates (at a density of $1\text{-}2\times10^5$ cells) or two-well (21.3×20 mm/well) Lab Tek chamber slides (at a density of 2.1×10^4 cells/well) for total RNA isolation, indirect immunostaining and Sudan III-staining, respectively.

To study the effect of the growth factor TGF-β3 on isolated chondrogenic cells, either 10 or 50 ng/ml of recombinant human TGF-β3 (R&D SYSTEMS, Wiesbaden, Germany), respectively, was added to the differentiation medium after replating of isolated chondrogenic cells derived from EBs, and differentiation was compared to control cultures without the growth factor. The concentration of TGF-β3 was in the range previously shown to induce chondrogenesis in cultures of human mesenchymal stem cells (Yoo et al., 1998; Mackay et al., 1998; Pittenger et al., 1999).

For clonal analysis of differentiation, 20, 200, 400, 1000 and 2000 chondrogenic cells isolated from EBs were plated onto each well of a four-well (20×8.5 mm/well) gelatin-coated Lab Tek chamber slide (Nunc, Wiesbaden, Germany), cultivated for 15 to 24 days and analyzed by immunostaining and Sudan III staining, respectively.

To study their differentiation capacity after replating, cell samples were isolated from chondrocyte cultures by trypsinization using 8×8 mm cloning cylinders (SIGMA, Taufkirchen, Germany). The cells were plated onto two-well (21.3×20 mm/well) gelatin-coated Lab Tek chamber slides (Nunc, Wiesbaden, Germany), cultivated for 18 to 24 days and analyzed by indirect immunostaining and Sudan III staining, respectively.

Sudan III staining

To detect adipocytic cells, the lipid stain Sudan III was used. Cells plated onto Labtek chamber slides were washed with PBS followed by staining for 3 minutes with a 0.2% solution of Sudan III (Sigma) in 70% ethanol.

Cryosections of isolated chondrogenic nodules

Chondrogenic nodules were isolated (see above), embedded in Tissue-Tek O.C.T. (Sakura Finetechnical, Tokyo, Japan) and frozen at –20°C. Cryosections (10 μm) were prepared using a cryostat (Leica, Bensheim, Germany) and placed onto Vectabond-coated slides. Sections were air-dryed, fixed in acetone for 10 minutes at –20°C and washed in PBS. Indirect immunostaining was performed after fixation as described below.

Detection of gene expression by semi-quantitative RT-PCR analysis

EBs (n=10) or isolated chondrocytes grown on 6 cm tissue culture plates were collected at different time points after plating, washed twice with phosphate-buffered saline (PBS), and total RNA was isolated using the RNeasy Mini-Kit (Qiagen, Hilden, Germany). The RNA concentrations were determined by measuring the absorbance at 260 nm. Samples of 500 ng of RNA were reverse transcribed using oligo-dT primer and Superscript II reverse transcriptase following the manufacturers recommendations (Life Technologies, Eggenstein, Germany).

Aliquots of 1 μl from the reverse transcriptase reactions were used for amplification of transcripts using primers specific for the analyzed genes and Vent DNA polymerase (New England Biolabs, Schwalbach, Germany) according to the manufacturer's instructions. Reverse transcriptase reactions were denatured for 2 minutes at 95°C, followed by 34-45 cycles of 40 seconds denaturation at 95°C, 40 seconds annealing at the primer-specific temperature (see below) and 50 seconds elongation at 72°C. Expression of the following genes was studied (oligonucleotide sequences are given in brackets in the order antisense-, sense-primer followed by the annealing temperature used for PCR, length of the amplified fragment and a reference): genes encoding collagen II [5′-AGGGGTACCAGGTTCTCCATC-3′, 5′-

CTGCTCATCGCCGCGGTCCTA-3′, 60°C, 432 bp (splice variant A) and 225 bp (splice variant B) (Metsäranta et al., 1991)], collagen X [5′-ATGCCTTGTTCTCCTCTTACTGGA-3′, 5′-CTTTCTGCTG-CTAATGTTCTTGACC-3′, 61°C, 164 bp (Elima et al., 1993)], osteocalcin [5′-ATGCTACTGGACGCTGGAGGGT-3′, 5′-GCG-GTCTTCAAGCCATACTGGTC-3′, 64°C, 330 bp (Desbois et al., 1994)], Cbfa-1 [5′-ATCCATCCACTCCACCACGC-3′, 5′-AAGGG-TCCACTCTGGCTTTGG-3′, 63°C, 371bp (Ducy et al., 1997)] and the 'house-keeping' gene *hypoxanthine guanine phosphoribosyl transferase* [*HPRT*, 5′-GCCTGTATCCAACACTTCG-3′, 5′-AGCG-TCGTGATTAGCGATG-3′, 63°C, 507 bp (Konecki et al., 1982)]. The latter was used as an internal standard. Electrophoretic separation of PCR products was carried out on 2% agarose gels. The fragments were analyzed by computer-assisted densitometry in relation to *HPRT* gene expression. RNA from limb buds or limbs of 16 day p.c. old mouse embryos were used as positive controls. Distilled water was always included as a negative control.

Indirect immunostaining

EBs or chondrogenic cells cultivated on chamber slides and rinsed twice with PBS were fixed for 5 minutes with methanol:acetone (7:3) at room temperature, washed three times with PBS again and incubated at 37°C for 30 minutes with 10% goat serum. Specimens were then incubated for 1 hour with the first antibody in a humidified chamber at 37°C. The following monoclonal antibodies (mAbs) diluted in PBS were obtained from the Developmental Studies Hybridoma Bank, University of Iowa, USA (designation of the mAb, the dilution used and a reference are given in brackets): Collagen II [II-II-6B3, 1:20 (Linsenmayer and Hendrix, 1980)], osteopontin [MPIIIB10$_1$,1:10 (Dorheim et al., 1993)], collagen X [X-AC9, 1:20 (Schmid and Linsenmayer, 1985)] and bone sialoprotein I + II [WVID1(9C5), 1:10 (Dorheim et al., 1993)]. To detect cytokeratins, anti-pan cytokeratin, diluted 1:100 (Sigma), for immunostaining of sarcomeric α-actinin mAb EA-53 (Sigma) diluted 1:20 and for collagen I, a polyclonal antiserum (Chemicon, Temecula, CA) diluted 1:100 were used. After rinsing three times with PBS, slides were incubated for 45 minutes at 37°C with either FITC- (1:200) or Cy3- (1:400) labeled anti-mouse IgG (Dianova, Hamburg, Germany), respectively. Slides were washed three times in PBS and briefly washed in distilled water. Specimens were embedded in Vectashield mounting medium (Vector, Burlinggame, CA) and analyzed with the fluorescence microscope Axioskop (Zeiss, Oberkochen, Germany).

Fluorescence in situ hybridization for *collagen X* mRNA

For fluorescence in situ hybridization of *collagen X* mRNA, a modified procedure of Yamada et al. (Yamada et al., 1994) was used. EBs (n=10) plated on chamber slides were rinsed twice with PBS and fixed with 4% (w/v) paraformaldehyde, 4% (w/v) sucrose in PBS for 20 minutes at room temperature. Prior to incubation at 70°C in 2×SSC for 15 minutes, cells were washed twice with PBS for 5 minutes. After rinsing once again with PBS followed by 2×SSC, the EBs were fixed for 5 minutes before washing with PBS and 2×SSC were repeated. The cells were subsequently dehydrated at room temperature for 2 minutes each in 50%, 70%, 95% and twice in 100% ethanol. Prehybridization was performed in a buffer containing 5×SSC, 5×Denhardt's, 50% formamide, 250 μg/ml yeast-t-RNA, 250 μg/ml denatured salmon sperm DNA and 4 mM EDTA in a humidified chamber at 45°C for 3 hours. For hybridization with digoxigenin-labeled sense and antisense probes against *collagen X* (1 ng/μl) the same buffer but without salmon sperm DNA was used. Hybridization was carried out at 45°C in a humidified chamber overnight. After hybridization, specimens were washed twice with 2×SSC for 15 minutes, once with 0.2×SSC for 15 minutes and twice with 0.1×SSC for 15 minutes at 45°C and rinsed in PBS. FITC-conjugated sheep F(ab) fragments against digoxigenin (Boehringer, Mannheim, Germany) diluted 1:800 in PBS were added. After incubation for 1 hour at 37°C, slides were washed three times in PBS and once in distilled water, embedded in Vectashield mounting medium and analyzed with the fluorescence microscope Axioskop (ZEISS, Oberkochen, Germany).

Hybridization probe

To obtain a hybridization probe for RNA in situ hybridization, we amplified a fragment of *collagen X* cDNA from RNA isolated from 16 day p.c. mouse limb buds following the protocol of RT-PCR as described above. The fragment was blunt-end cloned into the plasmid vector pCR-BluntII-TOPO using the TOPO II cloning kit according to the manufacturers protocol (Invitrogen, Groningen, NL) and the sequence was verified by sequencing. Digoxigenin-labeled RNA probes of either sense or antisense orientation of *collagen X* were synthesized from linearized plasmids of the cloned *collagen X* cDNA fragment by in vitro transcription using the T7- or SP6-RNA polymerase following the protocol supplied by the manufacturer (Boehringer, Mannheim, Germany).

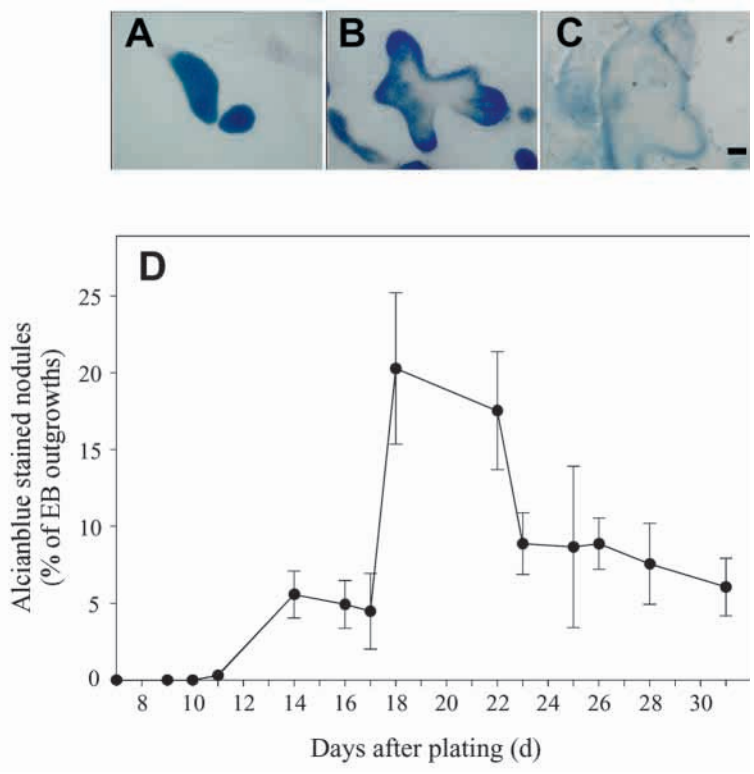

Fig. 1. Alcian-blue-stained areas loose their stainability during differentiation of ES cells in vitro. Alcian blue staining demonstrating differentiation of chondrocyte nodules in ES-cell-derived EBs at 5+21d (A), 5+26 d (B) and 5+31 d (C). The size of the stained areas was measured during EB cultivation from 5+7 d up to 5+31 d (D). Eleven days after plating (5+11 d) the first stained areas were detected, their number and size increased during cultivation up to 5+18 d and decreased during later stages. Mean values±s.e.m. are shown (n=10). The decrease in the size of Alcian-blue-stained areas was caused by a loss of stainability as demonstrated for nodules from 5+21d, 5+26 d and 5+31 d (A-C) EBs. Representative areas of EB outgrowths are shown. Bar, 100 μm.

Results

ES cells differentiate into hypertrophic chondrocytes and osteogenic cell types in vitro

In EB outgrowths derived from ES cell line BLC6, chondrogenic cells appeared as intensively Alcian-blue-stained areas of different size, called nodules (Fig. 1A). The number and size of these nodules increased during EB cultivation accompanied by a reduced intensity of Alcian blue staining (Fig. 1B). Eventually, the nodules lost their stainability (Fig. 1C). For a quantitative estimate, the size of the Alcian-blue-stained portions of nodules was determined in relation to the total EB outgrowths using digital images from EBs after staining (Fig. 1D). Eleven days after plating (5+11 d), the first stained areas were detected and their number and size increased during cultivation up to day 5+18. During later stages the percentage of Alcian-blue-stained regions decreased (Fig. 1D).

The disappearance of Alcian blue staining was accompanied by differential expression of marker genes, which are indicative of hypertrophic and/or calcifying chondrocytes as demonstrated by semi-quantitative RT-PCR analysis (Fig. 2). Transcription of the prechondrogenic splice variant of *collagen II* (*collagen IIA*) was detected throughout EB cultivation and the level did not change significantly over time (Fig. 2A), whereas mRNA transcription level of the adult or chondrocyte-specific splice variant of *collagen II* (*collagen IIB*) increased significantly (t-test=0.0002) from 5+7 d to 5+17 d (Fig. 2A). The gene encoding collagen X, specifically upregulated during chondrocyte hypertrophy in vivo, was expressed throughout EB cultivation, although a significant (t-test=0.02) increase of the mRNA level was detected from day 5+7 to 5+17 (Fig. 2B). The osteoblast-specific *osteocalcin* mRNA showed a significant (t-test=0.004) shift from 5+10 d to 5+22 d and thereafter was continuously transcribed on a high level up to 5+30 d (Fig. 2C). The mRNA of the osteoblast-related transcription factor Cbfa-1 was present in substantial amounts at the day of EB plating and, except for two phases of downregulation at 5+4 d and 5+22 d, showed an overall increase up to 5+30 d (Fig. 2D).

To demonstrate differentiation at the cellular level we performed RNA in situ hybridization and immunostaining for marker molecules of hypertrophic and calcifying chondrocytes as well as osteogenic cells (Fig. 3). *Collagen X* mRNA as well as the matrix proteins osteopontin and bone sialoprotein were detected in nodules of 5+16 d to 5+30 d EB outgrowths (Fig. 3A,C,E). Besides these positively stained cells organized in nodules we also found clusters of single cells expressing these marker (Fig. 3B,D,F). Apparently, two types of osteogenic cells appeared in EB outgrowths: (i) osteogenic cells derived from hypertrophic and calcifying chondrocytes organized in nodules and (ii) a second population differentiating as single cell cluster.

Chondrocytes isolated from EBs dedifferentiated but re-express chondrogenic markers

To test whether chondrocytes isolated from ES-cell-derived nodules are phenotypically stable in culture, nodules were cut from 5+16 d EB outgrowths using a microscalpel, dissociated into single cells using collagenase and replated. Because the nodules were surrounded by a rigid membraneous structure of extracellular matrix proteins, intact nodules could be isolated, virtually avoiding contamination by neighboring cells. As a test for the developmental stage, cryosections from isolated nodules were immunostained for chondrocyte marker proteins. The isolated nodules showed expression of collagen II, osteopontin and bone sialoprotein I and II and collagen X (Fig. 4A-D), and cells inside of the nodules showed the typical round-shaped morphology of hypertrophic chondrocytes (Fig. 4E-H). Single cell suspensions obtained from such nodules by

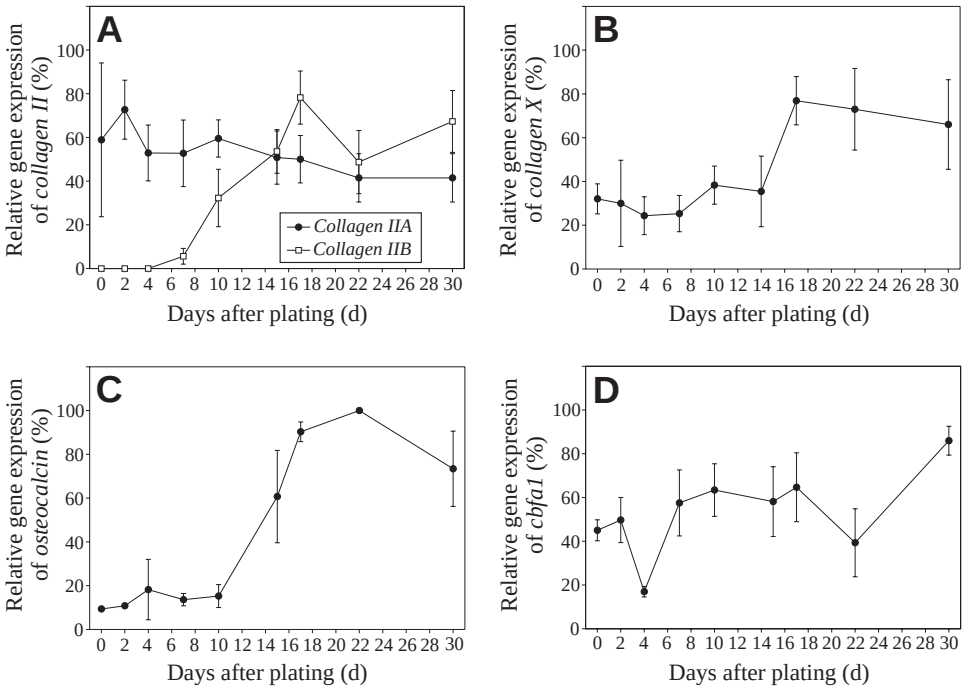

Fig. 2. Genes characteristically expressed during chondrocyte hypertrophy and calcification were found to be differentially expressed during ES cell differentiation in vitro. Genes encoding extracellular matrix proteins of cartilage tissue such as collagen II (A), collagen X (B), osteocalcin (C) and the osteoblast-specific transcription factor *Cbfa-1* (D) were expressed in EBs from different cultivation stages from 2 (5+2 d) up to 30 days (5+30 d) after plating as demonstrated by RT-PCR. The primers used to amplify *collagen II* transcripts detected two splice variants (A), the juvenile variant *A* and the adult variant *B*. The transcriptional levels relative to those of the house-keeping gene *HPRT* are presented. Mean values±s.e.m. derived from independent experiments (n=4) are shown.

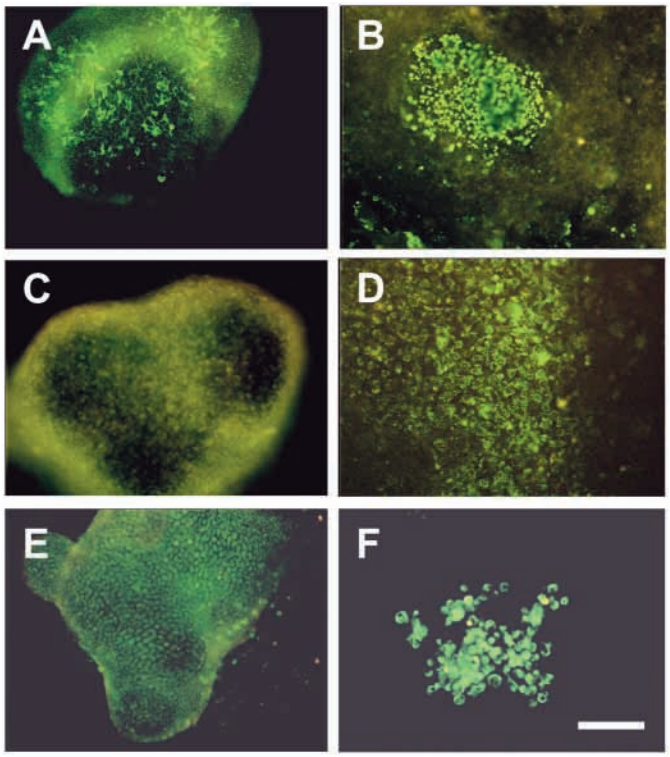

Fig. 3. Cells that express markers characteristic for hypertrophic and/or calcifying chondrocytes differentiated in EB outgrowths. *Collagen X* mRNA (A,B), osteopontin protein (C,D) and bone sialoprotein I + II (E,F) were expressed in cells organized in nodules (A,C,E) or as single cell clusters (B,D,F) in EB outgrowths. Representative areas of EB outgrowths at 5+23 d (A), 5+16 d (B) and 5+30 d (C-F) are shown. Bar, 100 μm.

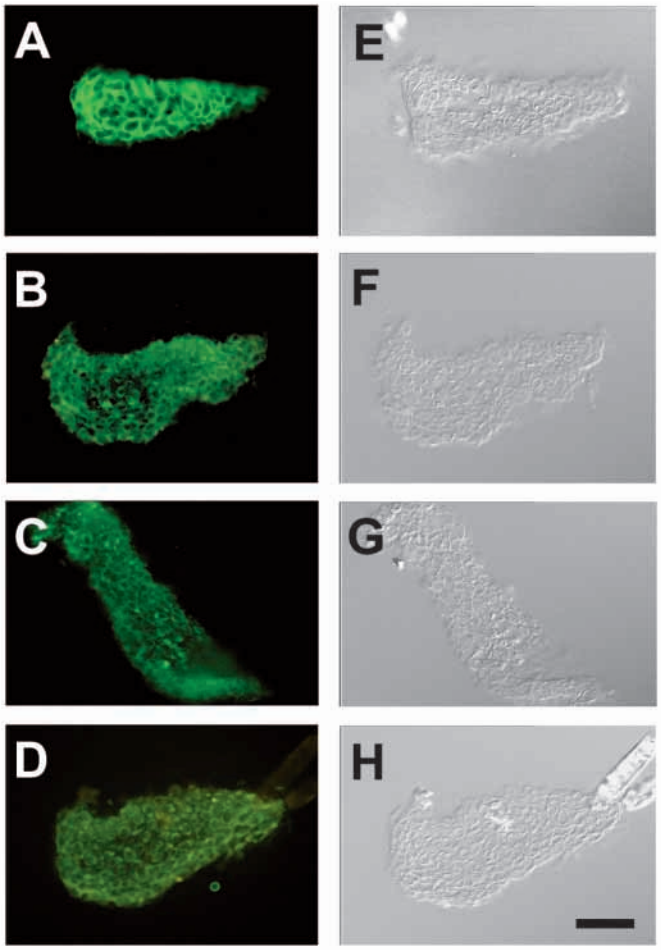

Fig. 4. Chondrogenic nodules isolated from EBs showed expression of marker proteins characteristic for mature and hypertrophic cartilage. Nodules were cut from 5+16 d EBs, and 10 μm cryosections were analyzed by immunostaining for collagen II (A), osteopontin (B), bone sialoprotein I + II (C) and collagen X (D). Interference contrast microscopy pictures (E-H) demonstrate the typical round-shaped morphology of chondrocytes. Bar, 100 μm.

dissociation with collagenase were plated onto gelatin- and collagen-II-coated dishes to test whether different substrates may influence the differentiation characteristics and marker gene expression profiles of the cells in culture. During cultivation, the expression of chondrogenic differentiation markers was analyzed using semi-quantitative RT-PCR and immunostaining (Fig. 5). mRNA levels of late-phase marker genes such as the adult splice variant of *collagen II* (*collagen IIB*) and *collagen X* were significantly downregulated during the first four days after isolation of chondrogenic cells from EBs (Fig. 5IA,B). However, two weeks after isolation, the transcriptional levels of *collagen IIB* and *collagen X* increased again (Fig. 5IA,B) indicating redifferentiation of chondrocytes. Freshly isolated cells showed a fibroblast-like morphology (Fig. 5IIA) and poor expression of collagen II (Fig. 5IIB), whereas expression of collagen I, characteristically expressed in dedifferentiated chondrocytes was prominent (Fig. 5IIC). When cultivated for four days, the dedifferentiated cells formed monolayers of fibroblastoid cells including areas of compact and distinct cellular entities (Fig. 5IID) consisting of cells positively stained for collagen II (Fig. 5IIE), whereas collagen I expression decreased (Fig. 5IIF). During further cultivation the size of these cell formations increased and they developed into dense aggregates (Fig. 5IIG) expressing collagen II (Fig. 5IIH) but not collagen I (Fig. 5III). Together, these results are in line with the observation that the isolated chondrogenic cells initially undergo transient dedifferentiation and later redifferentiate into mature chondrocytes.

Multilineage differentiation of ES cell-derived chondrocytes

The majority of the cells in cultures derived from chondrocyte nodules redifferentiated into chondrogenic condensations. After prolonged cultivation times, up to 14-20 days, occasionally other cell types appeared. Cells carrying large lipid droplets were found. Staining with Sudan III revealed that these cells were adipogenic cells (Fig. 6A). Moreover, muscle cells staining positively for sarcomeric α-actinin (Fig. 6B), and large flattened cells staining positive for pan cytokeratins characteristic of epithelial cells (Fig. 6C) were detected. These additional cell types formed colonies. The number of these colonies and the amount of cells were low in comparison to the number of chondrogenic condensations and the number of chondrocytes, respectively.

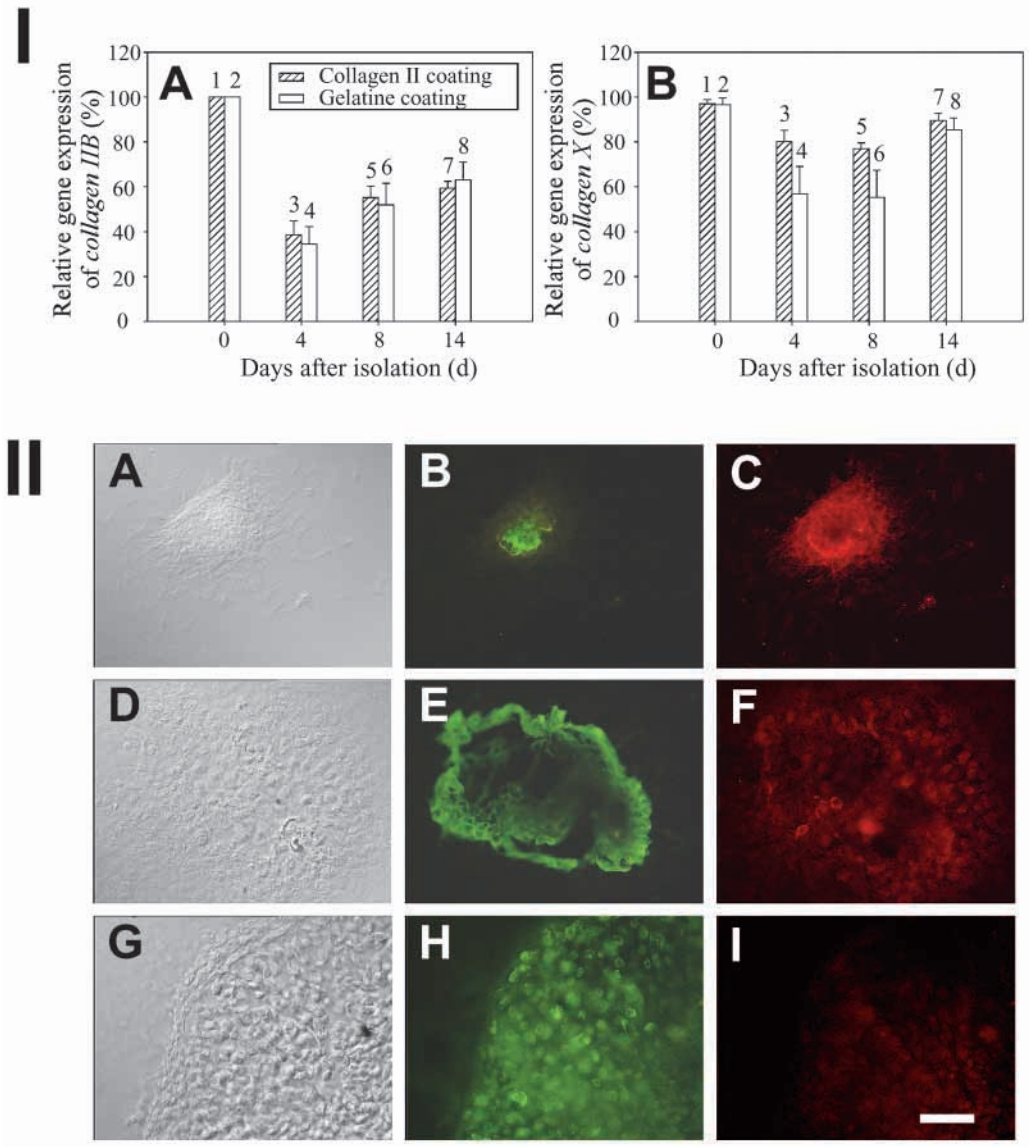

Fig. 5. Expression of marker genes (I) and proteins (II) during cultivation of chondrogenic cells isolated from EBs, indicating initial dedifferentiation followed by redifferentiation after prolonged cultivation. In chondrocyte cultures obtained from 5+16 d EB outgrowths mRNA levels of chondrocyte marker genes such as the adult splice variant *B* of *collagen II* (IA) and *collagen X* (IB) declined 4 days after isolation and were upregulated again up to 14 days after isolation. Coating of tissue culture plastic either with collagen II or gelatin did not cause significant differences. The level of transcription in relation to *HPRT* is shown. Significant differences (**P* ≤0.05; ***P*≤0.01; ****P*≤0.001) in the mRNA levels for *collagen IIB*: 1-3, 2-4=***; 3-7=**; 4-8 and 3-5=* and for *collagen X*: 1-3, 2-4, 5-7=**. Mean values±s.e.m. from independent experiments (*n*=8) are shown. The isolated chondrogenic cells initially showed a fibroblastic morphology after one day in culture (IIA), started to form condensations after 4 days (IID) and eventually showed a round-shaped morphology after 8 days (IIG). These morphological alterations were accompanied by increasing expression of collagen II (IIB,E,H) and loss of collagen I expression (IIC,F,I) after one (IIB,C), four (IIE,F) and eight (IIH,I) days. Representative areas are shown. Bar, 100 μm.

Representative samples of approximately 2.1×10^4 cells plated to form a cell monolayer and analyzed after 14 days of culture contained (per 1000 cells) 9.1 chondrogenic condensations of 50-60 chondrocytes, 0.67 colonies of 20-40 epithelial cells, 0.43 colonies of 3-6 skeletal muscle cells and 0.19 colonies of 5-30 adipocytes, representing 51.4%, 2.3%, 0.29% and 0.25%, respectively, of the analysed cells (Table 1). The rest of the cells showed a fibroblastic morphology.

To exclude the possibility that the observed mesenchymal cell types originated from contaminating progenitor cells, two different approaches were used. First, various areas of the redifferentiated cell monolayers were isolated using cloning cylinders and again tested for differentiation, and second, single clones of cells isolated from nodules 16 days after EB plating were analyzed. Cells recovered from the redifferentiated monolayers after cultivation for up to 18 days still showed a fibroblastic morphology and we were not able to detect any differentiated cell by immunostaining for collagen II, sarcomeric actinin or cytokeratins. After 24 days

Table 1. Proportion of mesenchymal cell types differentiating in cultures of dedifferentiated chondrocytes isolated from EBs

Cell type	Proportion of cells	Chondrogenic condensations or cell colonies per 1000 cells plated
Chondrocytes	51.4%	9.1
Epithelial cells	2.3%	0.67
Skeletal muscle cells	0.29%	0.43
Adipocytes	0.25%	0.19
Fibroblastoid cells	45.76%	–

Chondrogenic cells isolated from EBs were plated at high density and formed a cell monolayer of fibroblastoid cells. After 14 days of cultivation, chondrocytes formed cell condensations, and additional mesenchymal cell types developed in colonies. The number of differentiated cells and the number of cell colonies and chondrogenic condensations were determined after specific staining (see Materials and Methods), and their proportion calculated in relation to the total number of cells plated.

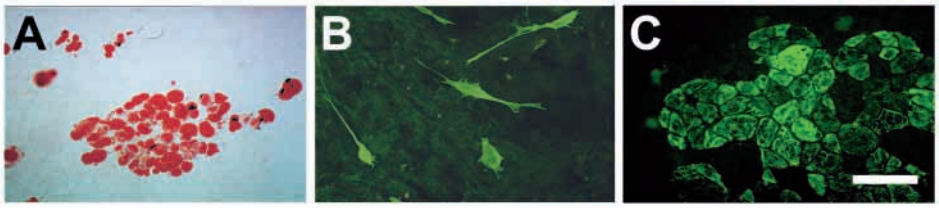

Fig. 6. Additional mesenchymal cell types developed in cultures of chondrogenic cells isolated from EBs. Adipocytes (A), skeletal muscle cells (B) and epithelial cells (C) were detected in cultures of isolated chondrocytes after 12-14 days as demonstrated by histochemical staining with Sudan III (A) and immunostaining for sarcomeric actinin (B) or a mixture of cytokeratins (C), respectively. Bar, 100 μm.

of cultivation, differentiating cell types appeared. In seven samples of 8×10^3 cells the majority of the cells (94.76%) still appeared to be fibroblastoid but we found per 1000 cells 2.37 chondrogenic condensations of 10-30 chondrocytes, 0.75 colonies of 1-10 epithelial cells, 0.62 colonies of 2-4 skeletal muscle cells and 4.12 colonies of 2-3 adipocytes, representing 3.6%, 0.45%, 0.19% and 1%, respectively, of the analysed cells (Table 2). Four samples showed differentiation into all analyzed cell types. The remaining three samples showed poor differentiation into chondrocytes, skeletal muscle cells and adipocytes or epithelial cells and adipocytes or no differentiation at all. These results show that the capacity of the isolated dedifferentiated chondrocytic cells for redifferentiation was remarkably reduced after replating, but other cell types were still observed in the cultures.

We analyzed single clones by plating diluted suspensions of cells isolated from nodules 16 days after EB plating. Cells (n=20, 200, 400, 1000 and 2000) were plated onto chamber slides generating up to 55 clones per slide. After 15 days in culture, most of the cells appeared to be morphologically fibroblastoid. Histo- and immunochemical staining showed that only rarely could some single differentiated cells be found in some of the clones. 24 days after cell plating more differentiated cells appeared. Among 422 clones of approximately $1\text{-}3\times10^2$ cells, we did not find any clone that was entirely composed of a single differentiated cell type as judged by their morphology. Histochemical analysis and immunostaining showed that almost 90% of the clones analyzed by Sudan III staining contained adipocytes and approximately one third of those immunostained for collagen II carried chondrocytes, whereas skeletal muscle cells and particularly epithelial cells were only rarely found (Table 3). The number of differentiated cells per positive clone was very low with mean values from 32 chondrocytes to 1.6 epithelial cells. In total, a number of 2627 differentiated cells was found, from which 49% were collagen-II-positive chondrocytes, 48% Sudan-III-positive adipocytes, 2.7% sarcomeric actinin-positive skeletal muscle cells and only 0.3% cytokeratin-positive epithelial cells.

TGF-β3 inhibited chondrogenic differentiation in chondrocyte cultures

TGF-β3 is able to promote chondrogenic differentiation in cultures of human mesenchymal stem cells (Mackay et al., 1998). We therefore analyzed a potentially similar effect in our mouse system by application of TGF-β3 (10 ng/ml and 50 ng/ml) to cultures of chondrogenic cells isolated from EBs. For both experimental conditions, semi-quantitative RT-PCR analysis showed that at day 8 and even more prominently at day 14 after replating, chondrogenic cells treated with TGF-β3 still showed signs of dedifferentiation in contrast to untreated control cultures. A significant decrease in the mRNA levels of the gene encoding the prechondrogenic *collagen II* splice variant (Fig. 7A) and the late-phase marker genes *collagen IIB* (Fig. 7B), *collagen X* (Fig. 7C) and *osteocalcin* (Fig. 7D) was observed in TGF-β3 treated cultures at day 14 after replating in comparison to untreated control cells. This effect was found in the presence of 10 ng/ml TGF-β3 as well as 50 ng/ml, the latter being somewhat stronger.

Table 2. Proportion of mesenchymal cell types after replating of chondrocytes initially isolated from EBs

Cell types	Proportion of cells	Chondrogenic condensations or cell colonies per 1000 cells plated
Chondrocytes	3.6%	2.37
Epithelial cells	0.45%	0.75
Skeletal muscle cells	0.19%	0.62
Adipocytes	1%	4.12
Fibroblastoid cells	94.76%	–

Using cloning cylinders, cells were isolated from monolayer cultures of chondrocytes initially isolated from EBs, and after 24 days of culture they were analyzed for differentiated chondrocytes and other mesenchymal cell types by specific staining (see Materials and Methods). The number of differentiated cells and the number of cell colonies and chondrogenic condensations were determined and their proportion calculated in relation to the total number of cells plated.

Table 3. Proportion of mesenchymal cell types differentiating in clones of dedifferentiated chondrocytes isolated from EBs

Cell types	Percentage of analyzed clones (n=422)	Mean number of differentiated cells per chondrocyte-derived clone
Chondrocytes	33.6%	32
Epithelial cells	5.7%	1.6
Skeletal muscle cells	14.6%	5.9
Adipocytes	89.1%	9.6

Chondrocytes were isolated from EBs and analyzed as single cell clones of approximately $1\text{-}3\times10^2$ cells at 24 days after plating as diluted cell suspensions. The number of chondrogenic cells and other mesenchymal cell types were determined after specific staining (see Materials and Methods). The clones were found to be heterogeneous with varying numbers of the analyzed cell types.

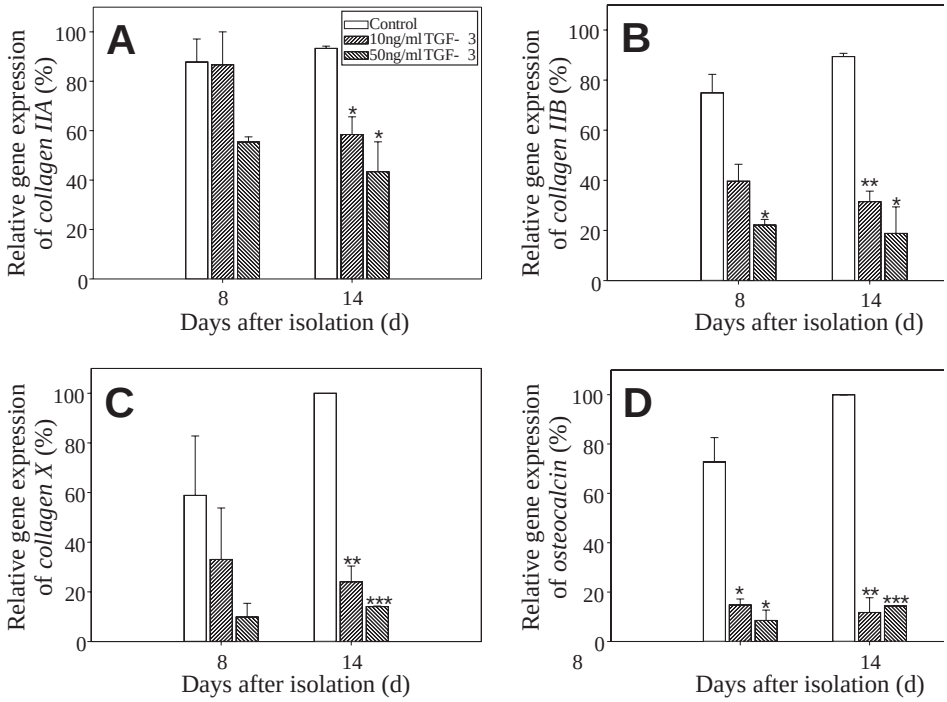

Fig. 7. Application of TGF-β3 resulted in downregulation of chondrogenic marker genes in cultures of chondrogenic cells isolated from EBs. Semi-quantitative RT-PCR demonstrated that the transcription levels of the juvenile splice variant *A* (A) as well as the adult splice variant *B* (B) of *collagen II*, *collagen X* (C) and *osteocalcin* (D) significantly decreased after application of TGF-β3 at two different concentrations of 10 and 50 ng/ml to cultures of chondrogenic cells isolated from EBs. The mRNA levels in relation to those of *HPRT* are shown. Mean values±s.e.m. derived from independent experiments (n=2) are presented. Significant differences (in comparison to untreated control cells): *P≤0.05; **P≤0.01; ***P≤0.001.

Immunostaining for collagen I as a marker of dedifferentiation and collagen II as a marker of differentiated chondrocytes confirmed that the TGF-β3-treated cells continued to display a fibroblast-like morphology and did not recover a chondrogenic phenotype 8 days after replating (Fig. 8D,G). The cells lost expression of collagen II (Fig. 8E,H) but showed prominent collagen I expression (Fig. 8F,I). By contrast, untreated cultures formed chondrogenic condensations (Fig. 8A), expressed collagen II (Fig. 8B) and showed only trace amounts of collagen I (Fig. 8C).

Discussion

ES cells recapitulate the process of endochondral ossification

We have recently shown that ES cells differentiate into chondrocytes in vitro (Kramer et al., 2000). Here, we show that these cells develop further into hypertrophic and calcifying chondrocytes. First evidence for terminal differentiation of chondrocytes derived from ES cells came from the observation that the nodules lost Alcian blue staining, indicating that matrix components were altered. In addition, the nodules

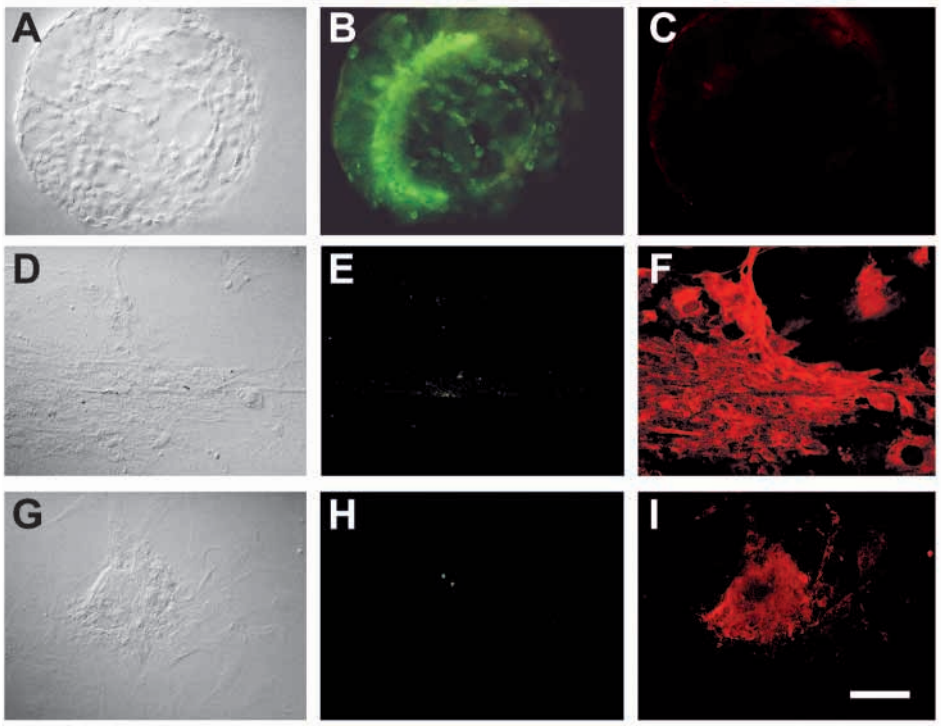

Fig. 8. Chondrogenic cells isolated from EBs were kept in a dedifferentiated state by treatment with TGF-β3. After 8 days in culture, initially dedifferentiated chondrogenic cells isolated from EBs formed cell condensations (A) and showed expression of collagen II (B) but only trace amounts of collagen I (C) as demonstrated by immunostaining. Treatment with TGF-β3 with either 10 ng/ml (D-F) or 50 ng/ml (G-I) resulted in inhibition of cell condensations (D,G) and collagen II expression (E,H) but increased expression of collagen I (F,I). Representative areas are shown. Bar, 100 μm.

expressed proteins characteristic for hypertrophic and calcifying chondrocytes such as collagen X, osteopontin and bone sialoprotein (Schmid and Linsenmayer, 1985; Mark et al., 1988; Gerstenfeld and Shapiro, 1996). The expression pattern of collagen marker genes during the process of endochondral ossification in vivo (Perälä et al., 1997) was nicely recapitulated during EB cultivation. RT-PCR analysis showed that the juvenile splice variant of *collagen II* was already expressed at a high level at the day of EB plating, whereas the adult splice variant of *collagen II* was upregulated later at day 7 after plating. Finally, at a terminal stage, expression of *collagen X*, the marker of hypertrophic chondrocytes (Schmid and Linsenmayer, 1985), increased. Similarly, during development in vivo, the juvenile splice variant of *collagen II* is already upregulated during early embryonic stages from day 9.5 up to 12.5 p.c., whereas the adult splice variant of *collagen II* is activated in mature chondrocytes (Sandell et al., 1991; Ng et al., 1993; Sandell et al., 1994) beginning at day 12.5 p.c. and followed by expression of *collagen X* at day 14.5 p.c. (Perälä et al., 1997). Furthermore, we found that mRNA expression of *Cbfa1*, encoding a transcription factor essential for osteoblast differentiation during endochondral as well as intramembranous ossification (Komori et al., 1997; Otto et al., 1997; Ducy et al., 1997) (reviewed by Komori and Kishimoto, 1998) was upregulated during EB differentiation, although it already showed significant expression during early stages when chondrocytic markers were still absent. This observation is in line with in vivo results demonstrating moderate expression of *Cbfa1* already at day 9.5 p.c. in the notochord, later in mesoderm derivatives destined to become skeletal components and strong expression around day 12.5 p.c. throughout the developing skeleton (Otto et al., 1997). Thus, the model system of ES cell differentiation recapitulates the complete process of chondrogenic differentiation from pluripotent cells up to terminally differentiated chondrocytes and osteogenic cells (Fig. 9).

It has been reported that differentiation of chondrocytes into osteoblasts occurs in vivo and in vitro (Moskalewski and Malejczyk, 1989; Descalzi et al., 1992; Ishizeki et al., 1997). This hypothesis is in accordance with our results demonstrating that hypertrophic chondrocytes organized in nodules start to express osteogenic markers. In line with our results it was shown recently that ES cells are able to differentiate into bone nodules in vitro (Buttery et al., 2001). Interestingly, besides those osteogenic cells differentiated in nodules, we observed clusters of single cells expressing osteogenic markers. It is conceivable that these cells are derived from an alternative osteogenic lineage of cells. We tested for the expression of *osteocalcin*, an osteoblast-specific marker (Hauschka and Wians, 1989; Ducy et al., 1996) and found that *osteocalcin* was already upregulated 10 days after EB plating, which is earlier than *collagen X*, the marker of hypertrophic chondrocytes. This indicates that at least some osteoblasts may have bypassed the chondrocytic state (Fig. 9). The hypothesis that ES-cell-derived osteoblasts are differentiated from two different types of precursors agrees with the in vivo situation where skeletal elements are formed either by replacing a cartilaginous template as most bones of the skeleton or directly from mesenchyme such as the flat bones of the skull (Erlebacher et al., 1995).

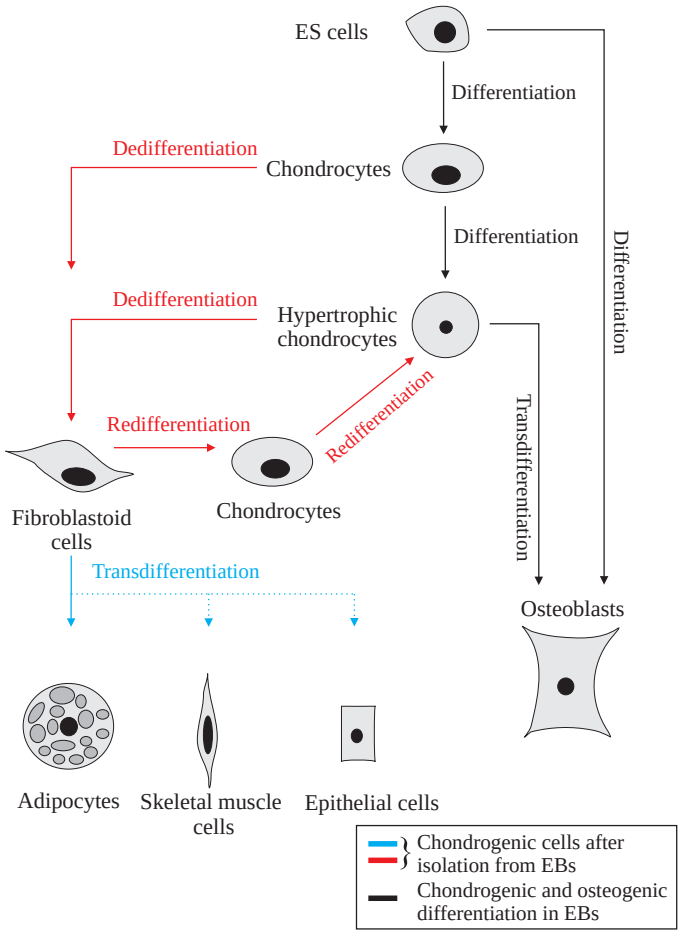

Fig. 9. ES-cell-derived chondrocytes display a differentiation plasticity. As shown in this paper ES cells differentiate in vitro into chondrocytes as well as osteocytes and into chondrocytes, which transdifferentiate into osteocytic cells. Chondrocytes isolated from EBs after initial dedifferentiation in culture, redifferentiate into chondrocytes and transdifferentiate mainly into adipocytes.

Differentiation plasticity of isolated chondrocytes

Dedifferentiation of primary cultures of chondrocytes isolated from mature cartilage is a well known process (von der Mark et al., 1977) that can be repressed by cultivation of chondrocytes in suspension, at high density or in agarose cultures (Castagnola et al., 1986; Bruckner et al., 1989). We prepared chondrocytes from EB nodules to test how these cells behave after release from their EB environment. In tissue culture, after initial dedifferentiation they showed rapid redifferentiation, indicating that they possess a high potential for regeneration. However, additional mesenchymal cell types such as adipocytes, epithelial cells and muscle cells appeared in the cultures. This may be explained either by the presence of contaminating mesenchymal progenitor cells in the chondrocyte cultures or by transdifferentiation of chondrocytes. The fact that we did not obtain clones of a single discrete cell type from the isolated chondrocytes excludes the possibility that the additional cell types were derived from distinct precursor cells. The additional cell types could, however, still be derived from mesenchymal progenitor cells,

which display a broader differentiation capacity. It is well known that such progenitor cells are able to differentiate at least into chondrocytes, osteocytes and adipocytes (Berry et al., 1992; Pittenger et al., 1999; Muraglia et al., 2000; Dennis et al., 2002). They share these characteristics with other cells or cell lines derived from mesenchymal tissues such as 10T1/2 (Taylor and Jones, 1979), RCJ 3.1 (Grigoriadis et al., 1988) or human trabecular bone-derived cells (Sottile et al., 2002). The cell lines 10T1/2 and RCJ 3.1 in addition are also capable of differentiating into myocytes. Recently it has been shown that clones of mesenchymal progenitor cells differentiate into a mixture of mesenchymal cell types (Muraglia et al., 2000). If such mesenchymal progenitor cells were present as a contamination in the chondrocyte cultures derived from the EBs we would expect a majority of the clones to redifferentiate into chondrocytes and rarely into clones with additional cell types. We found that adipocytes differentiated in most of the clones, whereas epithelial cells and myocytes were observed to only differentiate to a minor extent. This indicates that the adipocytes, at least, were not derived from contaminating mesenchymal stem cells. Furthermore, the chondrocytic cells isolated from EBs did not exhibit characteristics of mesenchymal progenitor cells such as expression of the stromal cell marker Stro-1 (data not shown) or inducibility of chondrocyte differentiation by TGF-β3 (Mackay et al., 1998). By contrast, we found that the cells expressed collagen I, a marker for dedifferentiated chondrocytes, and TGF-β3 kept the cells in a dedifferentiated state.

Cells reisolated from cultures of chondrocytes initially isolated from EBs showed a drastically reduced differentiation efficiency into chondrocytes whereas differentiation into adipocytes increased (Table 2). Also clonal growth of chondrocytes isolated from EBs resulted in a reduced differentiation into chondrocytes compared with high-density cultures and into enhanced differentiation into adipocytes. Probably, when the cells are plated at a low density they change differentiation into the adipocytic direction. In fact, it has been observed in several studies that differentiation of primary cultures of chondrocytes was affected by the density of cells (e.g. Castagnola et al., 1986; Bruckner et al., 1989). The observation that mesenchymal cells are able to transdifferentiate into other mesenchymal cell types together with the finding that mesenchymal differentiation is regulated by multiple inductive or repressive factors rather than a set of specific master genes resulted in a stochastic repression/induction model for mesenchymal cell differentiation (Dennis and Charbord, 2002). Stochastic activation and repression events affecting genes encoding transcription factors could account for the plasticity of differentiating mesenchymal cells. The chondrocytic cells released from the EBs are placed into a new environment with an altered composition of activating and repressive factors. This new environment could have induced additional mesenchymal cell types such as the adipocytes in the ES-cell-derived chondryocyte cultures (Fig. 9). For the epithelial cells and myocytes, however, we can not completely rule out the possibility that they were generated by contaminating precursor cells.

Consideration of ES-cell-derived chondrocytes for therapeutic applications

The regenerative capacity of articular cartilage is limited. Transplantation methods used to treat cartilage lesions rely mainly on primary cultures of chondrocytes that do not restore the original hyaline cartilage (Chen et al., 1999). ES cells are considered to be an alternative source for generating cells used in cell therapy for two reasons: first they can almost indefinitely divide in culture and second they are able to differentiate into cell types of all three germ layers. Furthermore, because ES cells recapitulate early embryonic developmental phases during in vitro differentiation it should be possible to isolate cells at different developmental stages, which could be beneficial for therapeutic approaches. However, as shown in this paper, chondrocytes derived from ES cells exhibit a certain differentiation plasticity after release from EBs probably because the correct combination of determining factors was missing. In line with these results it has been shown that differentiation, survival and maintenance of ES-cell-derived dopaminergic neurons was enhanced by survival-promoting factors present in the culture medium (Rolletschek et al., 2001). Together these results indicate that it crucially depends on the appropriate culture and selection strategies to obtain defined cell types from ES cells that show a stable phenotype to be used as cellular grafts.

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