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Chondrocytes derived from mouse embryonic stem cells

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Abstract

Our knowledge of cellular differentiation processes during chondro- and osteogenesis, in particular the complex interaction of differentiation factors, is still limited. We used the model system of embryonic stem (ES) cell differentiation *in vitro* via cellular aggregates, so called embryoid bodies (EBs), to analyze chondrogenic and osteogenic differentiation. ES cells differentiated into chondrocytes and osteocytes throughout a series of developmental stages resembling cellular differentiation events during skeletal development *in vivo*. A lineage from pluripotent ES cells via mesenchymal, prechondrogenic cells, chondrocytes and hypertrophic chondrocytes up to osteogenic cells was characterized. Furthermore, we found evidence for another osteogenic lineage, bypassing the chondrogenic stage. Together our results suggest that this *in vitro* system will be helpful to answer so far unacknowledged questions regarding chondrogenic and osteogenic differentiation. For example, we isolated an as yet unknown cDNA fragment from ES cell-derived chondrocytes, which showed a developmentally regulated expression pattern during EB differentiation. Considering ES cell differentiation as an alternative approach for cellular therapy, we used two different methods to obtain pure chondrocyte cultures from the heterogenous EBs. First, members of the transforming growth factor (TGF)-β family were applied and found to modulate chondrogenic differentiation but were not effective enough to produce sufficient amounts of chondrocytes. Second, chondrocytes were isolated from EBs by micro-manipulation. These cells initially showed dedifferentiation into fiboblastoid cells in culture, but later redifferentiated into mature chondrocytes. However, a small amount of chondrocytes isolated from EBs transdifferentiated into other mesenchymal cell types, indicating that chondrocytes derived from ES cells posses a distinct differentiation plasticity.

Introduction

Many bones of the mammalian body develop via endochondral ossification. During this process mesenchymal cells aggregate, form condensations and develop into mature chondrocytes. These cells undergo further differentiation into hypertrophic cells, which either develop into osteocytes or undergo apoptosis and are replaced by osteoblasts resulting in bone formation. Several transcription factors are known to be involved in early determination of mesenchymal cells into the chondrogenic lineage. For example, the basic helix-loop-helix (bHLH) protein scleraxis (Cserjesi et al., 1995) is expressed during early embryogenesis in mesenchymal cells. Other transcription factors which play an important role during early stages of chondrogenesis are the high-mobility-group (HMG) box protein Sox9 (Wright et al., 1995) and the paired-box-gene Pax-1 (Deutsch et al., 1988; Wallin et al., 1994). The temporally and spatiallay regulated expression of homeobox-genes is crucial for correct cartilage morphogenesis (Gruss and Kessel, 1991; Morgan and Tabin, 1994). During early steps of chondrogenic development, cell aggregation plays an important role. N-cadherin, a calcium-dependent cell adhesion molecule, seems to be a mediator of mesenchymal cell

condensation (Oberlender and Tuan, 1994; DeLise and Tuan, 2002) and interactions between epithelial and mesenchymal cells mediated by signaling molecules, such as growth factors of the TGF-beta family are important for the initiation of condensations (for review see Hall and Miyake, 2000).

During chondrocyte maturation characteristic matrix components are expressed in a regulated fashion. Chondrogenic cells express collagen II, cartilage oligomeric matrix protein (COMP, Hedbom et al., 1992) and proteoglycans such as aggrecan (Walcz et al., 1994). Collagen II is the major protein of cartilage tissue. Chondrogenic precursor cells express the juvenile splice variant *collagen IIA*, whereas adult chondrocytes express *collagen IIB* (Sandell et al., 1991, 1994). Later, hypertrophy of chondrocytes is accompanied by the expression of collagen X (Schmid and Linsenmayer, 1985; Castagnola et al., 1988). It is still unclear whether hypertrophic cells transdifferentiate into osteoblasts or whether they undergo apoptosis and are replaced by immigrating osteoblasts (Descalzi et al., 1992; Roach et al., 1995; Cancedda et al., 2000). During bone formation the composition of the matrix is changing. Osteoblasts produce matrix proteins such as osteonectin (Termine et al., 1981), osteopontin (Mark et al., 1988), bone sialoprotein (Bianco et al., 1991) and osteocalcin (Hauschka and Wians, 1989; Desbois et al., 1994). The expression of these osteogenic markers is induced by ectopic expression of Cbfa-1 in non-osteoblastic cells (Ducy et al., 1997), a transcription factor shown to play an important role during osteoblast determination (Komori et al., 1997; Otto et al., 1997).

In vitro systems used to analyze chondrogenic and osteogenic differentiation include primary cultures of chondrocytes derived from adult or embryonic cartilage, cell lines of mesenchymal origin (Ahrens et al., 1993; Laitinen et al., 1999; Lou et al., 1999) or mesenchymal stem cells derived from bone marrow (Pittenger et al., 1999). *In vitro* differentiation of pluripotent mouse embryonic stem (ES) cells via cell aggregates, so called embryoid bodies (EBs), is an alternative system comprising differentiation from the early pluripotent state up to terminal differentiation stages. ES cells are pluripotent because they are derived from the inner cell mass of blastocysts (Evans and Kaufman, 1981; Martin, 1981). *In vitro*, ES cells differentiate spontaneously into cell types of all three primary germlayers (Doetschman et al., 1985; Rathjen et al., 1998) recapitulating temporally regulated patterns of embryonic cellular differentiation (for reviews see Guan et al., 1999; Rohwedel et al., 2001). As we have shown recently, ES cells differentiate into chondrocytes *in vitro* (Kramer et al., 2000). We were able to characterize successive steps of chondrogenic and osteogenic differentiation during ES cell differentiation *in vitro* from early mesenchymal and prechondrogenic cells up to mature chondrocytes undergoing hypertrophy, and finally forming osteogenic cells (Hegert et al., 2002).

To enhance ES cell-derived chondrocyte differentiation we tested members of the transforming growth factor (TGF)-β family and found that BMP-2 and BMP-4 activated chondrogenic differentiation (Kramer et al., 2000). However, we did not obtain cell amounts, which could be sufficient for transplantation approaches. Alternatively, we isolated and collected chondrocytes from EBs by micro-dissection. Initially, these cells dedifferentiated in culture but later redifferentiated into mature chondrocytes (Hegert et al., 2002) and thus, showed a relatively stable phenotype compared to progressively dedifferentiating chondrogenic cells derived from primary cultures (Von der Mark et al., 1977). However, chondrocytes released from murine EBs transdifferentiated into other mesenchymal cell types, especially of the adipogenic lineage (Hegert et al., 2002). This indicates that it will be necessary to develop more sophisticated selection strategies for the isolation of differentiated cells from ES cells for therapeutic application.

In this article we summarize the developmentally regulated process of ES cell-derived chondrogenic and osteogenic differentiation *in vitro*, its modulation by external growth factors and the differentiation plasticity of cells after release from EBs. Finally, we present preliminary results on the isolation of as yet unknown genes, which may have an important function during ES cell-derived chondrogenesis.

Materials and methods

ES cell cultivation and differentiation of ES cells via EBs

ES cells of line D3 (Doetschman et al., 1985) or BLC6 (Wobus et al., 1988) were cultivated on a feeder layer of primary mouse embryonic fibroblasts (Wobus et al., 1991) in Dulbecco‘s modified Eagle’s medium (Invitrogen, Karlsruhe, Germany) supplemented with 15% fetal calf serum (FCS, selected batches, Invitrogen, Karlsruhe, Germany), 2 mM L-glutamine (Invitrogen,

Karlsruhe, Germany), 5×10^{-5} M β-mercaptoethanol (Serva, Heidelberg, Germany), non-essential amino acids (Invitrogen, Karlsruhe, Germany; stock solution diluted 1:100) to keep the cells in the undifferentiated stage as described previously (Wobus et al., 2002). The embryonic fibroblasts were growth inactivated by treatment with Mitomycin C (Serva, Heidelberg, Germany). In addition, leukaemia inhibitory factor (LIF; 5 ng ml^{-1}; Invitrogen, Karlsruhe, Germany) was supplemented to media for the maintenance of the ES cell pluripotency. 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 (0–2 d) and subsequently in suspension on bacteriological petri dishes for additional 3 days (2–5 d) as described (Kramer et al., 2000). The 5 day (5 d) old EBs were plated onto gelatin (0.1%)-coated 6 cm tissue culture plates for histochemical staining and total RNA isolation, and onto gelatin (0.1%)-coated 2 well Lab-Tek chamber slides (Nunc, Wiesbaden, Germany) for indirect immunostaining and *in situ* hybridization. The influence of growth factors on ES cell-derived chondrogenic differentiation was investigated as described (Kramer et al., 2000).

Analysis of differentiation by histochemical staining

Alcian blue (Sigma, Taufenkirchen, Germany) staining was performed using standard methods to detect cartilage-associated proteoglycans. To detect adipocytic cells, the lipid stain Sudan III (Sigma, Taufenkirchen, Germany) was used, as recently described (Hegert et al., 2002). For alkaline phosphatase (AP)-staining a kit including all solutions was used (Sigma, Taufenkirchen, Germany) according to the manufacturers recommendations.

Isolation of chondrocytes from EBs

For isolation of chondrogenic cells, EBs were cultivated as described above. Chondrogenic cells developed in areas, so called nodules. These nodules were cut off the EB outgrowths with a microscalpel under sterile conditions and collected for collagenase (0.1%) treatment, as described (Hegert et al., 2002; Kramer et al., in press). The dissociated cells were resuspended in differentiation medium and replated at high density onto gelatin- or collagen II- (Sigma, Taufenkirchen, Germany) coated 6 cm tissue culture plates or 2 well chamber slides for total RNA isolation, indirect immunostaining and Sudan III-staining, respectively. Clonal analysis was performed as described (Hegert et al., 2002).

Semiquantitative RT-PCR analysis

The expression of cartilage-associated genes in EB outgrowth was analysed by RT-PCR. For semiquantitative analysis a 'house keeping gene' was used as an internal standard. EBs were collected at several stages after plating of EBs. Total RNA was isolated using the RNeasy Mini-Kit (Qiagen, Hilden, Germany). RNA was reverse transcribed using oligo-dT primer and Superscript II reverse transcriptase (Invitrogen, Karlsruhe, Germany), and amplified using Taq DNA Polymerase (Roche Diagnostics, Mannheim, Germany) and oligonucleotide primer specific for the analysed genes as described (Kramer et al., 2000; Hegert et al., 2002).

Immunostaining and mRNA in situ hybridization

The formation of cartilage-associated proteins in EB outgrowths of different cultivation stages was analysed by indirect immunostaining and fluorescence *in situ* hybridization for *scleraxis*- (Kramer et al., 2000) and *collagen X* mRNA (Hegert et al., 2002), respectively.

Non-radiocative differential display analysis

Total RNA was extracted from undifferentiated murine ES cells, embryonic fibroblasts (feeder layer cells) and chondrocytes isolated from EBs at 5+16 d using the RNeasy Mini-Kit (Qiagen, Hilden, Germany). All additional material was obtained from Invitrogen (Karlsruhe, Germany), unless otherwise marked. RNA was reverse transcribed using a T_{11}GC-tailing primer and Superscript II RNase H^- reverse transcriptase. The mix for the cDNA synthesis contained 500 ng total RNA, 2.5 μM T_{11}GC-primer, 1$\times$ first strand buffer, 10 nM dithiotreitol (DTT), 500 μM desoxynucleotides (dNTPs), 0.001% bovine serum-albumine (BSA), 50 U RNase OUT, about 25% trehalose and 200 U Superscript II reverse transcriptase. The reaction was carried out for 90 min at 50 °C, followed by incubation at 95 °C for 3 min. The resulting cDNA pool was amplified using random-primer as well as the T_{11}GC-primer. The mix for the PCR contained 1x PCR puffer, 1 mM DTT, 0.01% BSA, 2.5 μM T_{11}GC-primer, 0.2 mM dNTP-mix (Amersham, Freiburg, Germany), 1.5 mM $MgCl_2$, 0.5 U Taq polymerase, 0.5 μM 10mer-random primer (GC 1, 2, 3 and 4;

Operon, Hilden, Germany). 0.5 μl cDNA were added. Incubation at 95 °C for 3 min was followed by 43 cycles, each of them consisting of denaturation at 95 °C for 40 sec, annealing at 40 °C for 60 sec and elongation at 72 °C for 90 sec. Finally, the reaction was incubated for 5 min at 72 °C. The resulting PCR-products were separated by a horizontal 15% polyacrylamid gel-electrophoresis (ETC, Kirchentellinsfurt, Germany) for 1 h at 7 mA/200 V, followed by 3 h at 11 mA/500 V. After separation the fragments were silver-stained. To this end, the polyacrylamid-gel was fixed for 30 min in 10% acetic acid, washed three times, each 2 min, in aqua dest. and stained for 30 min with a solution of 0.1% $AgNO_3$ in 0.037% formaldehyde. A developer solution (235 mM Na_2CO_3, 0.037% formaldehyde and 0.002% Na_2S2O_3) was added for about 5 up to 15 min. Development of bands was finished by treatment with stop-solution (266 mM glycine, 17 mM EDTA). Finally, the gel was incubated for at least 2 h in 434 mM EDTA and air-dried. Bands of amplified transcripts expressed in chondrocytes but not in ES cells or feeder cells were isolated from the gel with a scalpel. The gel-slices were transferred into reaction tubes containing aqua dest. and heated twice for 1 min at 95 °C. Subsequently, the DNA-fragments were eluted by incubation for 1 h at room temperature. The eluted fragments were reamplified using again T_{11}GC-primer and random primer. The PCR mix for this reaction contained 1× PCR-puffer, 6.25 mM $MgCl_2$, 1.25 μM T_{11}GC-primer, 0.5 μM 10mer-random primer (either GC 1, 2, 3 or 4; Operon, Hilden, Germany), 200 μM dNTP-mix (Amersham, Freiburg, Germany) and 0.5 U Taq polymerase. After initial heating for 2 min at 95 °C PCR was performed with 40 cycles (45 sec at 95 °C, 1 min at 40 °C and 90 sec at 72 °C). The differentially expressed fragments were cloned into the pGEM-T Vector System (Promega, Mannheim, Germany) and finally sequenced (Agowa, Berlin, Germany).

Results

ES cell-derived chondrogenic and osteogenic differentiation

ES cells of different lines were differentiated via EBs. Using a combination of mRNA *in situ* hybridization and immunostaining we were able to demonstrate that already around four days after plating of EBs (5+4 d) early mesenchymal cells appeared, which expressed *scleraxis*, a transcription factor expressed in pre-chondrogenic mesenchymal condensations, but only small amounts of collagen II, the major cartilage matrix molecule (Figure 1, I A–C). These *scleraxis*-positive cells later formed cellular condensations and expressed increasing amounts of collagen II approximately at 5+17 d (Figure 1, I D–F). *Scleraxis*-positive cells organized in condensations also expressed the mesenchymal cell adhesion molecule N-cadherin (Figure 1, I G–I). During further cultivation around 5+24 d the size of the cell formations increased and they developed into dense aggregates, so called nodules, which showed an increasing level of *scleraxis* and collagen II expression indicating the differentiation of mature chondrocytes (Figure 1, I J–L). Furthermore, the nodules were surrounded by a distinct membranous structure containing collagen II and showed expression of COMP as revealed by immunostaining (Kramer et al., 2000). Later, *collagen X* was expressed in the nodules, characteristic for differentiation of hypertrophic chondrocytes, while collagen II expression decreased (Figure 1, I M–O). Moreover, the shape of the nodules changed during terminal differentiation. The density of these areas increased and finally, the nodules disaggregated. Around 5+30 d the cells located in these nodules produced osteopontin and bone sialoprotein as revealed by immunostaining (Figure 1, I P–Q) indicating osteogenic differentiation.

During cultivation EBs were also analyzed by Alcian blue staining. The first Alcian blue stained nodules could be detected around 5+12 d and their number increased up to 5+22 d (Figure 1, II A). During terminal differentiation stages around 5+24 d the chondrogenic nodules lost their stainability for Alcian blue indicating that the composition of the extracellular matrix was altered. The described morphological changes were accompanied by a pattern of regulated chondrocytic and osteogenic marker gene expression as summarized here for the ES cell line BLC6 (Figure 1, II B). RT-PCR analysis revealed an early upregulation of the prechondrogenic marker *scleraxis*, followed by an expression maximum for the chondrocyte marker *collagen IIB*, *collagen X* and the osteoblast-specific genes *osteocalcin* and *cbfa-1* at later stages of EB cultivation (Figure 1, II B). Besides the osteogenic cells developing in cartilage nodules we found additional single cell clusters expressing the osteogenic markers in EB outgrowths, indicating the appearance of a second osteogenic lineage bypassing the chondrocytic stage (Hegert et al., 2002). Alkaline phosphatase staining confirmed these results.

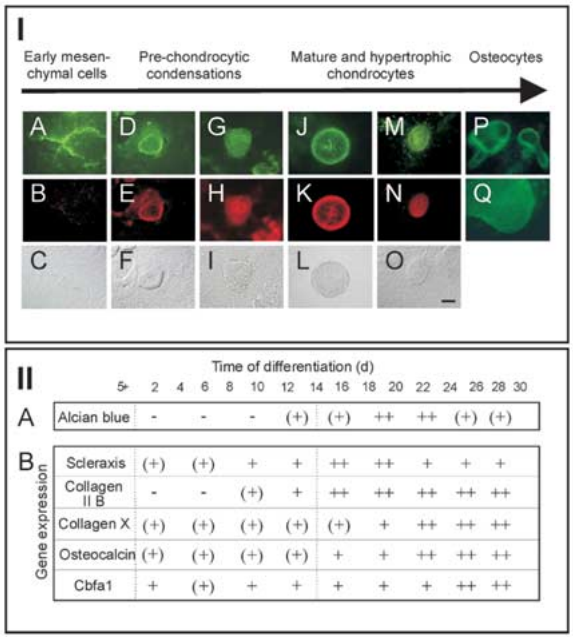

Figure 1. ES cell-derived chondrogenesis *in vitro* recapitulates cellular differentiation stages of endochondral skeletal development. During early EB differentiation stages mesenchymal cells were organized in streaks and expressed *scleraxis* (I; A). These cells only expressed small amounts of collagen II (I; B) and were morphologically indistinguishable from neighbouring cells (I; C). During further differentiation the *scleraxis*-expressing cells (I; D) start to express collagen II (I; E) and form aggregates (I; F). In addition to *scleraxis* (I; G) expression of N-cadherin (I; H) was detected by immunostaining in these aggregates (I; I) characteristic for prechondrogenic cells. Later, *scleraxis*- (I; J) and collagen II- (I; K) positive cells were found to be organized in highly organized structures, so called nodules (I; L). During terminal EB differentiation the chondrocytes became hypertrophic and co-expressed *collagen X* (I; M) and collagen II (I; N) while the nodules started to disaggregate (I; O). Finally, osteogenic proteins, such as osteopontin (I; P) and bone sialoprotein I and II (I; Q), were expressed by cells located in the disaggregating nodules. In parallel, the first nodules staining positive for Alcian blue (II; A) were detected around 12 days after EB plating and during further EB cultivation their number increased. Later, the Alcian blue stained nodules disappeared (II; A) indicating a change in the composition of the extracellular matrix. Furthermore, semiquantitative RT-PCR analysis demonstrated that genes, which are involved in processes of mesenchymal and chondrogenic differentiation, were expressed in a developmentally regulated pattern during EB differentiation *in vitro* (II; B). Bar = 100 μm. Original data: Kramer et al., 2000; Hegert et al., 2002.

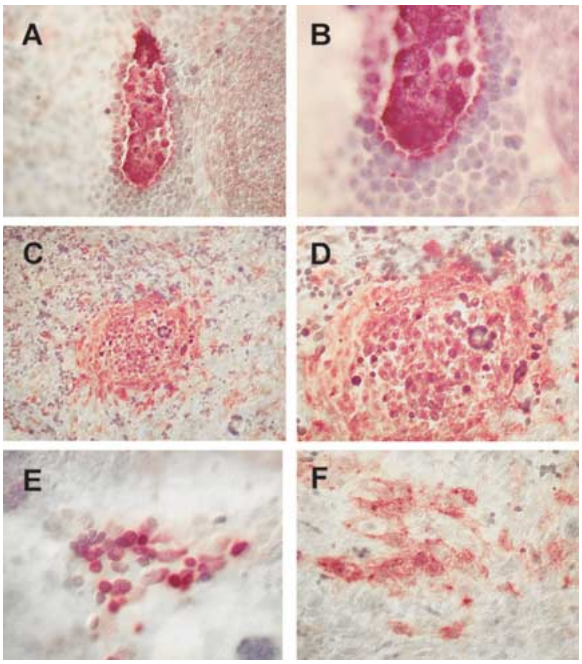

Figure 2. Alkaline phosphatase (AP) expressing osteogenic cells were detected in EB outgrowths. At a terminal differentiation stage AP-positive cell groups were detected inside of disaggregating cartilage nodules (A). Chondrogenic cells adjacent to the AP-positive area started to express AP (B). Areas completely composed of AP-positive cells were detected, which presumably present already disaggregated nodules (C, D). In addition, AP-postive cells organized as single cell clusters were detected outside of nodules (E, F).

Expression of AP was detected in cells located in disaggregating nodules (Figure 2, A–D) or in single cells organized in clusters outside the nodules (Figure 2, E–F).

Generally, the number of chondrogenic nodules spontaneously appearing in the EBs was found to be influenced by many parameters such as the number of ES cells used for preparation of EBs, the time of EB cultivation in suspension, the day of EB-plating, the type of basic cultivation medium, the batch of serum used (reviewed by Kramer et al., in press) and was also varying among different ES cell lines used. The ES cell line BLC6 was particularly suitable for the investigation of terminal chondrogenic differentiation stages (Hegert et al., 2002) because chondrogenic nodules appeared very rapidly during EB cultivation (Rohwedel, unpublished results).

Taken together, murine ES cells are able to differentiate into chondrocytes via mesenchymal, chondrogenic progenitor cells. The mature chondrocytes organized in nodules differentiated further into hypertrophic cells and finally into calcifying osteoblastic cells. A second lineage of osteoblastic cells was found, bypassing the chondrogenic stage.

Modulation of ES cell-derived chondrogenic differentiation by growth factors

The efficiency of spontaneous chondrogenic differentiation of ES cells is relatively low. We therefore tried to increase the development of chondrogenic nodules by application of growth factors of the TFG-β family at different concentrations and during different cultivation periods and found that they modulated ES cell-derived chondrogenic differentiation (Kramer et al., 2000). Treatment of EBs with TGF-β_1 at 2 ng ml^{-1} during the entire cultivation period resulted in slightly reduced or unaltered ES cell-derived chondrogenic differentiation. However, BMP-2 at 2 ng ml^{-1} and BMP-4 at 10 ng ml^{-1} enhanced the number of chondrogenic nodules in EBs if applied during the whole cultivation procedure. In addition, a time-dependent effect of BMP-2 to increase chondrogenic differentiation was observed. BMP-2 caused activation of chondrogenesis when added from two up to five day of EB development (2–5 d). This time window previously was described as a crucial period of early mesodermal development (Rohwedel et al., 1998). Hence, other external signaling molecules were added during this stage and tested for their effect on differentiation. But neither 10^{-8} M all-trans RA nor TGF-β_1 (2 ng ml^{-1}) in combination with bFGF (2 ng ml^{-1}) modulated chondrogenic differentiation when supplemented from two to five day of EB differentiation (reviewed by Kramer et al., in press).

Differentiation plasticity of isolated chondrocytes from EBs

To investigate whether chondrocytes isolated from ES cell-derived chondrogenic nodules are stable in culture, nodules were cut from the EBs using a microscalpel, dissociated into single cells by collagenase treatment and replated for differentiation. Cryosections of the undissociated nodules showed expression of cartilage-matrix proteins collagen II and X and osteogenic markers such as osteopontin and bone sialoprotein I and II as analyzed by immunostaining (Hegert et al., 2002). Single cells released from nodules initially dedifferentiated into fibroblastoid cells in culture, which showed decreasing expression of collagen II and X and increasing expression of collagen I. During further cultivation the cells redifferentiated into chondrocytes reexpressing collagen II and X. These cells again formed chondrogenic nodules (Hegert et al., 2002). However, the isolated chondrogenic cells exhibited a certain differentiation plasticity. Differentiation of other mesenchymal cell types besides chondrocytes was observed after prolonged cultivation of the isolated cells (Hegert et al., 2002).

Isolation of chondrocyte-associated factors by differential display analysis

To search for as yet unkown genes which might play an important role during chondrogenesis, RNA from ES cell-derived chondrocytes and from undifferentiated ES cells was analyzed for differential gene expression. A T_{11}GC-primer was used for reverse transcription of RNA and combined with random primer to amplify transcribed fragments by PCR according to Lohmann et al. (1995). Fragments expressed in chondrocytes but not in undifferentiated ES cells were cloned and sequenced (Table 1). Specific primers were designed for three fragments, which carried a putative open reading frame and used to analyze their expression during *in vitro* differentiation of ES cells by semiquantitative RT-PCR analysis. The three fragments were expressed during the entire cultivation period, but only for the fragment GC2-4 a developmentally regulated expression pattern could be detected (Figure 3). GC2-4 was initially upregulated and continously expressed from four up to 17 days after EB plating (5+4 d up to 5+17 d). Finally, a significant downregulation of GC2-4 expression was observed (5+16 d up to 5+24 d), followed by an again increasing expression at the end of cultivation (5+30 d). A search in the sequence library of the European Bioinformatics Institute (EBI) for homologous sequences revealed that this fragment did not show significant homologies to any sequences contained in the library (Table 1). Taken together, we were able to isolate a new cDNA fragment, which may have a developmental function during ES cell-derived chondrogenic differentiation.

Summary and future prospects

Our studies demonstrate that ES cell-derived chondrogenic differentiation *in vitro* recapitulates early as well as terminal cellular processes of chondrogenic and osteogenic differentiation *in vivo* (Kramer et al., 2000; Hegert et al., 2002). We were able to characterize a sequence of differentiation stages from pluripotent ES cells via mesenchymal and prechondrogenic cells, followed by mature chondrocytes organized in nodules. The chondrocytes expressed the major cartilage matrix protein collagen II and stained positive

Table 1. Characteristic features of cDNA fragments cloned from ES cell-derived chondrocytes after differential display analysis

Primer	Clone	Length in nucleotides	Search for homologies in the EBI-database	Putative open reading frame
GC1	GC1-19-1	322	Homology of 100% to EST of different murine tissues	95 AA
	GC1-17-2	76	Not significant	–
	GC1-10	74	Homologous of 92% to a EGF repeat region	–
GC2	GC2-10	540	Not significant	79 AA
	GC2-4	339	Not significant	58 AA (rev)
GC4	GC4-12-4	426	Homologous to the murine repetitive DNA family L1	–
	GC4-1	435	Homologous to the murine repetitive DNA family B2	–

Using the GC1, GC2 or GC4 random primer for differential display analysis (see the Materials and methods Section) differentially expressed fragments were amplified, cloned and sequenced. The length of the cloned fragments is given. A search for homologies in the database of the European Bioinformatics institute (EBI) showed that three fragments did not show any significant homologies. Two of these fragments contained a putative open reading frame. AA = amino acids; rev = reverse; EGF = epidermal growth factor; EST = expressed sequence tag.

for Alcian blue indicating the presence of cartilage proteoglycans. Later, the chondrocytes expressed *collagen X*, a marker for hypertrophic chondrocytes, the nodules lost their stainability for Alcian blue and expression of collagen II decreased. Finally, the nodules disaggregated and the cells increasingly expressed osteoblastic marker proteins like bone sialoprotein and osteopontin. During this terminal stage of differentiation positive AP-staining of the nodules confirmed transdifferentiation of chondrocytes into osteoblasts. In parallel, the expression pattern of collagen marker genes recapitulated nicely the pattern observed *in vivo* (Perälä et al., 1997). The juvenile splice variant A of *collagen II* was expressed throughout EB cultivation, whereas the adult splice variant B, *in vivo* specifically expressed by mature chondrocytes (Sandell et al., 1991, 1994; Ng et al., 1993), was predominantly expressed during late differentiation stages in EBs. Finally, *collagen X* expression was detected in EBs during terminal differentiation stages when hypertrophic chondrocytes appeared. Moreover, RT-PCR analysis revealed that the cartilage associated genes *scleraxis*, *Pax-1*, *Sox9* and *aggrecan* were found to be expressed in EBs of different cultivation stages (Kramer et al., 2000). Taken together, these results indicate a regulated process of chondrogenesis during EB cultivation *in vitro*, reflecting the cellular events during cartilage and bone development *in vivo*. It has been described for many cell types that differentiation of ES cells *in vitro* closely recapitulates differentiation processes *in vivo*. For example, the genes coding for the signaling molecules BMP-4 and Wnt-1 and the transcription factors Brachyury and Pax-6, *in vivo* activated during early processes of neuroectodermal and mesodermal determination, are expressed in a developmentally regulated pattern during ES cell differentiation *in vitro* (Rohwedel et al., 1998). Furthermore, differentiation of ES cells into cardiomyocytes, skeletal muscle and neurogenic cells resembled celluar differentiation events *in vivo* (Maltsev et al., 1993, 1994; Strübing et al., 1995; Rose et al., 1994; Rohwedel et al., 1994, 1998).

According to our results, it has been shown recently that ES cells are able to differentiate into bone nodules *in vitro* (Buttery et al., 2001). In addition, we present evidence that two separate lineages of osteoblast differentiation exist in EBs. We found osteoblast-like cells expressing osteogenic proteins in EB outgrowths, which formed single cell clusters located outside of the nodules (Hegert et al., 2002). It is conceiving that these cells are osteocytes differentiating from non-chondrocytic precursor cells, bypassing the chondrogenic stage. Similarly, *in vivo* bone is formed either by replacing a cartilaginous template as most bones of the skeleton or directly from mesenchyme such as the flat bones or the skull (Erlebacher et al., 1995). In summary, embryonic cellular differentiation events are nicely recapitulated during ES cell

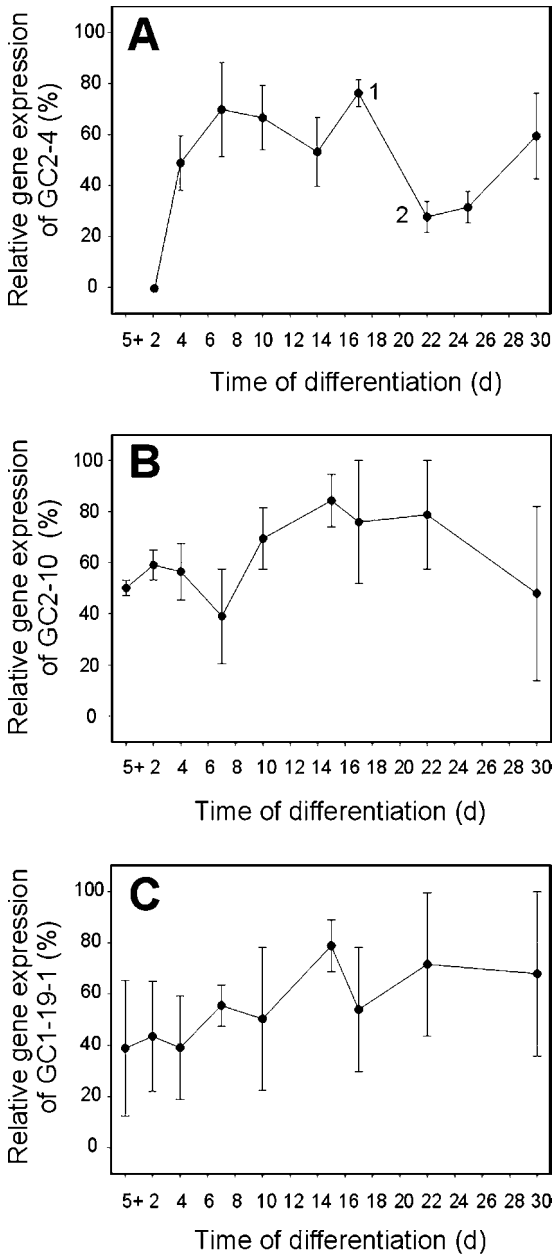

Figure 3. Expression of cDNA fragments isolated by differential display analysis from ES cell-derived chondrocytes. The expression of the fragments GC2-4 (A), GC2-10 (B) and GC1-19-1 (C), which were generated via differential display analysis, was investigated by RT-PCR analysis during *in vitro* differentiation of EBs. Only the fragment GC2-4 showed a developmentally regulated expression pattern. Expression initially increased and was significantly (1–2, $p = 0.0004$) downregulated later. The expression levels of the fragments GC2-10 and GC1-19-1 were relatively constant during EB differentiation. The ratio of gene expression of the analysed fragments in relation to the 'house keeping' gene *hypoxanthine guanine phosphoribosyl transferase (HPRT)*, which was used as an internal standard is shown. Data were derived from independent experiments (n = 3).

differentiation *in vitro* from pluripotent up to terminally differentiated cells, making this a unique *in vitro* model system for developmental biology.

We asked the question, whether ES cell differentiation *in vitro* could also be helpful to isolate as yet unknown genes which have important functions during chondrogenic development. Because EBs are heterogenous differentiation systems, consisting of many cell types, a differential display approach using whole EBs would result in the isolation of too many differentially expressed genes. Using a simple method for isolation of chondrocytes from EBs by micro manipulation we were able to demonstrate that it is possible to obtain as yet unknown cDNA fragments which are expressed during ES cell-derived chondrogenic differentiation. Combined with the microchip technology it might be possible to perform high-throughput screenings for detection of genes differentially expressed during chondrogenesis.

Dedifferentiation is a well known process during cultivation of primary chondrocytes (Von der Mark et al., 1977). We therefore tested the differentiation behaviour of ES cell-derived chondrogenic cells, which we isolated from EBs, and found that after initial dedifferentiation the cells rapidly redifferentiated into mature chondrocytes. This indicates that these ES cell-derived chondrocytes posses a high potential for regeneration (Hegert et al., 2002). However, additional mesenchymal cell types, such as adipocytes, skeletal muscle and epithelial cells, appeared in the cultures. A possible explanation for these additional cell types may be transdifferentiation. By clonal analysis we were able to demonstrate that at least adipocytes transdifferentiated from the chondrocytes isolated from EBs (Hegert et al., 2002). These results underline the finding that it is difficult to generate homogenous cell populations from ES cells for therapeutic applications. Selection strategies to obtain homogenous populations of transplantable differentiated cell types from mouse ES cells such as transfection with selection constructs (Klug et al., 1996; Li et al., 1998) and reporter constructs (Kolossov et al., 1998) have been established. But even then it can not be excluded that the selected cells are able to transdifferentiate into inappropriate cell types. Nevertheless, differentiated cells derived from murine ES cells have been enriched, used for transplantation and formed morphological and functional stable cellular grafts (Klug et al., 1996; Dinsmore et al., 1996; Brüstle et al., 1999; Soria et al., 2000). Because murine and human ES cells show a similar differentiation capacity *in vitro* (Thomson et al., 1998; Itskovitz-

Eldor et al., 2000), protocols to obtain a specifc cell type from mouse ES cells may also be used for human ES cells (Odorico et al., 2001). However, one has to consider other sources of stem cells, which may be more suitable for transplantation approaches such as adult stem (AS) cells. There is increasing evidence that AS cells have a greater differentiation potency than expected. For example, it has been reported that bone marrow stem cells are capable to differentiate into hepatocytes in humans (Alison et al., 2000) and into cardiomyocytes in rats (Orlic et al., 2001). But it has been shown recently that bone marrow stem cells are able to fuse spontaneously with other cells and subsequently acquire the phenotype of the recipient cell (Terada et al., 2002; Ying et al., 2002). Therefore, it is still questionable whether AS cells can suffice as a cell source for tissue repair (Vogel, 2001).

We investigated the influence of TGF-β growth factors on chondrogenic and osteogenic differentiation of ES cells and found that they modulated differentiation time- and concentration-dependently. Our data showed that BMP-2 and BMP-4 were able to increase ES cell-derived chondrogenic differentiation. The enhancing effect of BMP-2 was limited to a time window between two and five days of EB development (2–5 d) corresponding to the cultivation of EBs in suspension. This early differentiation stage of EBs was characterised as a period of early mesodermal development (Yamada et al., 1994; Rohwedel et al., 1998). Previous studies demonstrated that this time window is also sensitive for the influence of other differentiation factors. For example, treatment of EBs during cultivation in suspension (2–5 d) with RA induced ES cell-derived differentiation of skeletal muscle cells and adipocytes, but inhibited cardiogenic differentiation (reviewed by Rohwedel et al., 1999). Until now only BMP-2 and BMP-4 have been characterised as stimulating factors for ES cell-derived chondrogenic differentiation (reviewed by Kramer et al., in press). Although they showed a stimulating effect on chondrogenic differentiation, BMPs were not at all powerful enough to induce an amount of chondrocytes that could be sufficient for therapeutic applications. Thus, creating a homogenous chondrogenic population using growth factor treatment may not be practicable. Nevertheless, a directed and efficient differentiation for mouse ES cells into neurons (Lee et al., 2000) or pancreatic islet-like cells (Lumelsky et al., 2001) by a sophisticated, multistep culture procedure using several different signaling molecules has been established.

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