RESEARCH COMMUNICATION

Chondrocyte expressed protein-68 (CEP-68), a novel human marker gene for cultured chondrocytes

Eric STECK*, Karin BENZ*, Helga LORENZ*, Markus LOEW*, Thomas GRESS† and Wiltrud RICHTER*¹

*Department of Orthopaedic Surgery, University of Heidelberg, Schlierbacher Landstrasse 200a, 69118 Heidelberg, Germany, and †Department of Internal Medicine 1, University of Ulm, Robert Koch Strasse 8, 89081 Ulm, Germany

In the search for new marker genes suitable to distinguish chondrocytes from osteoblasts and mesenchymal stem cells in culture, we have identified and characterized a novel gene called chondrocyte expressed protein-68 (CEP-68), harbouring an N-terminal leader peptide and an epidermal growth factor-like calcium-binding domain. CEP-68 defines a new family of proteins

and complements collagen type II as a new marker for stem-cell-based chondrogenic tissue engineering.

Key words: cartilage, chondrogenic phenotype, chondro-induction, mesenchymal stem cells, representational difference analysis.

INTRODUCTION

The limited regeneration capacity of articular cartilage is ascribed to its lack of vascularization, its low number of cells embedded in matrix and their limited mitotic activity *in vivo*. A newly developed cell-based therapy to enhance cartilage regeneration relies on the transplantation of autologous chondrocytes into the cartilage defect [1,2]. When the cells forming the transplant are harvested from cartilage biopsies, additional defects at less loaded sites of the same joint have to be generated to obtain sufficient cells for transplantation purposes. To avoid such new cartilage defects, mesenchymal stem cells were considered as starting material for cartilage repair protocols [3]. Human mesenchymal stem cells derived from bone marrow have been shown to be able to differentiate into adipocytes, fibroblasts, chondrocytes and osteoblasts *in vitro*, with a preference of most progenitor cells to develop into the osteoblast-like phenotype [4].

To distinguish the chondrocyte-like phenotype from osteoblast-like cells and mesenchymal stem cells, and to identify additional chondrocyte-specific marker genes expressed in cultured monolayers, we compared the gene expression profiles of cultured chondrocytes and osteoblasts using cDNA representational difference analysis (RDA). RDA is a PCR-based subtractive hybridization method useful for identifying differential gene expression between two mRNA populations [5,6]. cDNA RDA eliminates those cDNA fragments constructed from mRNA present in both cell populations, and leaves only the differences with the option to identify previously unknown tissue- [7] or developmental-specific [8] genes.

EXPERIMENTAL

Human samples

Bone and cartilage tissue and mesenchymal stem cells from bone marrow were obtained after written consent from patients undergoing replacement surgery of hip, knee or shoulder.

Cultured osteoblasts and chondrocytes for RDA were obtained from a 69-year-old male donor after humeral head fracture. The study was approved by the local ethics committee.

Cell culture

Cartilage was digested overnight with collagenase (Roche Diagnostics, Mannheim, Germany) and hyaluronidase (Serva, Heidelberg, Germany) in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL), and chondrocytes were cultured in monolayer in DMEM, 10 % fetal calf serum, 1 % penicillin/streptomycin at 37 °C, 5 % CO₂ for up to 6 weeks. Osteoblasts were grown from bone biopsies [9] in monolayer, under the same culture conditions as described above, for up to 9 weeks. Mesenchymal stem cells were isolated from bone marrow by density gradient centrifugation [10] and cultured for 3 weeks as described above. For the induction of the chondrogenic phenotype, mesenchymal stem cells expanded for 2–6 weeks were cultured for 2–3 weeks in DMEM (high glucose) medium with 1 % penicillin/streptomycin containing the following additives: 0.1 µM dexamethasone, 1 mM sodium pyruvate, 0.17 mM ascorbic acid-2-phosphate, 0.35 mM prolin, 6.25 µg/ml bovine insulin, 6.25 µg/ml transferrin, 6.25 µg/ml selenous acid, 1.25 mg/ml BSA and 0.01 µg/ml transforming growth factor (TGF)-β₃ (Sigma).

RNA extraction

Total RNA was isolated from cultured cells by using a standard guanidinium thiocyanate/phenol extraction (peqGOLD RNAPure™; Peqlab, Erlangen, Germany). Polyadenylated mRNA was isolated by using oligo d(T) coupled to magnetic beads (Dynabeads™; Dynal, Oslo, Norway) according to the manufacturer's instructions. Cartilage and bone mRNA was directly isolated from the shock-frozen tissue using oligo d(T) coupled to magnetic beads.

Abbreviations used: CEP, chondrocyte expressed protein; DMEM, Dulbecco's modified Eagle's medium; DP1 etc., first difference product, etc.; EGF, epidermal growth factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GP-39, glycoprotein-39; RACE, rapid amplification of cDNA ends; RDA, representational difference analysis; RT, reverse transcriptase; TGF, transforming growth factor.

¹ To whom correspondence should be addressed (e-mail wiltrud.richter@ok.uni-heidelberg.de).

The nucleotide sequence data reported will appear in DDBJ, EMBL, GenBank® and GSDB Nucleotide Sequence Databases under the accession number AJ279016.

RDA

Preparation of representations

cDNA was synthesized by using reverse transcriptase (RT; Superscript II, Gibco BRL) and oligo d(T) primers. A 200 µg portion of the double-stranded cDNA was digested with *DpnII*, phenol-extracted and ethanol-precipitated, followed by ligation with an adapter molecule (R-Bgl-12/24). The DNAs were diluted and PCR-amplified using R-Bam-24 oligonucleotides as primers (20 cycles each of 1 min at 95 °C and 3 min at 72 °C). To obtain enough starting material for the RDA these PCR products were reamplified in an additional PCR step with 5 cycles. The final PCR products were phenol-extracted, ethanol-precipitated and resuspended at 0.5 µg/µl to form the representations. Both tester and driver representations were digested with *DpnII* (New England Biolabs) to remove the R adapters and then phenol-extracted and ethanol-precipitated. The tester representations were gel-purified and ligated to the J-Bgl-12/24 adapter.

Selective amplification

To isolate chondrocyte-specific genes, cDNA representations derived from cultured chondrocytes were used as tester, and representations derived from cultured osteoblasts were used as driver. For the subtractive hybridization the protocol of Hubank and Schatz [5] was followed in detail. Briefly, for the generation of the first difference product (DP1), driver and tester were mixed at a 100:1 ratio, hybridized for 24 h at 67 °C, amplified by PCR with J-Bgl-24 as primer, and treated with mung bean nuclease (New England Biolabs) to digest single-stranded DNA. A final PCR amplification step produced DP1. To generate the further difference products the adapters were substituted as follows: for DP2 J-Bgl against N-Bgl and for DP3 N-Bgl against J-Bgl. The driver to tester ratios were 800:1 for DP2 and 400.000:1 for DP3.

Oligonucleotides

The primer and adaptor oligonucleotides for RDA were: R-Bgl-12, 5'-GATCTGCGGTGA-3'; R-Bgl-24, 5'-AGCACTCTCC-AGCCTCTCACCAGCA-3'; J-Bgl-12, 5'-GATCTGTTTCATG-3'; J-Bgl-24, 5'-ACCGACGTCGACTATCCATGAAC-3'; N-Bgl-12, 5'-GATCTTCCCTCG-3'; N-Bgl-24, 5'-AGGCAACTGT-GCTATCCGAGGGAA-3'. Oligonucleotides used for RT-PCR and sequencing were: glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward, 5'-CCACCCATGGCAAATTCCA-TGGCA-3'; GAPDH reverse, 5'-TCTAGACGGCAGGTCAG-GTCCACC-3'; collagen type II forward, 5'-TGGCCTGAGAC-AGCATGAC-3'; collagen type II reverse, 5'-AGTGTGGG-AGCCAGATTGT-3'; chondrocyte expressed protein-68 (CEP-68) forward 1, 5'-CTCTGGACGCTACTCTATCT-3'; CEP-68 forward 2, 5'-ATGTCCAGGATGTTACCGTTC-3'; CEP-68 reverse 1, 5'-CTCTCCTTAACCACCGACC-3'; CEP-68 reverse 2, 5'-TGTGTCCTAAGATATGGTCATT-3'. Gene-specific oligonucleotides used for 3' rapid amplification of cDNA ends (RACE) were: CEP-68 SP5, 5'-ACATGAACCAGCG-GATGGAG-3'; CEP-68 SP6, 5'-AATTAGGGAGACTCGT-AAGGC-3'. All oligonucleotides were obtained from Interactiva, Ulm, Germany.

Sequence analysis

DP3 fragments were excised from a 3% agarose gel, reamplified by PCR, purified and cloned into the pBluescript KS vector (Stratagene) before transformation into *Escherichia coli* strain

SR101. The plasmid DNA was prepared by using the Qiagen plasmid miniprep kit, and given for insert sequencing to a local company (Genotype, Hirschhorn, Germany). BLAST algorithms were used to screen for DNA and protein sequence similarities in public databases at the National Center for Biotechnology Information (NCBI). Protein motifs were identified by the simple molecular architecture research tool SMART [11,12]

Northern-blot analysis

Per lane 5 µg of total RNA from cultured chondrocytes and cultured osteoblasts were size-fractionated on a 1% agarose, 6% formaldehyde denaturing gel and transferred to Hybond N⁺ membranes (Amersham Pharmacia Biotech). A human multiple tissue Northern was obtained from ClonTech Laboratories. Gene-specific PCR fragments were labelled by random octamer priming (MBI Fermentas, St Leon-Rot, Germany) using α [³²P]dCTP as a radioactive nucleotide. Hybridization was carried out overnight in 0.5 M sodium phosphate buffer, 7% SDS, 1 mM EDTA at 68 °C. Filters were washed in 40 mM sodium phosphate buffer, 1% SDS at 68 °C for 30 min two times.

Semi-quantitative RT-PCR analysis

Reverse transcription was performed with 10 µl of poly-adenylated mRNA (prepared from cartilage and bone tissue), or with 5 µg of total RNA (prepared from cultured cells) by using RT (Superscript II; GibcoBRL) and oligo d(T) primers. The first strand cDNA was subjected to PCR using gene-specific upstream and downstream primers. The CEP-68 specific RT-PCR was performed under long-range PCR conditions using the Expand Long Template PCR System from Roche Diagnostics. After 20, 25, 30 and 35 PCR cycles an aliquot was checked on an ethidium bromide-stained agarose gel. In reference to the intensity of the GAPDH-specific bands, the amount of first strand cDNA from the individual samples was calculated to use equal amounts of DNA template in the gene-specific semi-quantitative RT-PCR experiments.

5'/3'-RACE

To complete the 5'- and 3'-ends of the cDNA the 5'/3'-RACE Kit from Roche was used according to the manufacturer's manual.

In vitro transcription/translation

The CEP-68 PCR-fragment cloned into the pSP64-polyA (Stratagene) expression vector was constructed with the primers: CEP-68 forward 2 and CEP-68 reverse 2. *In vitro* transcription and translation in the presence of [³⁵S]methionine was performed using the 'TNT' Sp6-coupled reticulocyte lysate system (Promega). A 10 µl aliquot of the translation product was loaded onto a 10% SDS/PAGE and analysed by autoradiography.

RESULTS AND DISCUSSION

Identification of genes preferentially expressed in cultured chondrocytes

Chondrocyte-specific gene expression was analysed by RDA of cDNA from cultured chondrocytes (tester) and cultured osteoblasts (driver) obtained from the same donor. A reverse experiment (tester: osteoblasts, driver: chondrocytes), identifying osteoblast-specific expression, was designed as a control. While a smear of cDNA products was evident in the starting material of both cell types, a different band pattern of the final difference

Figure 1 Representational difference products obtained from cultured monolayers of human cartilage and bone

Agarose gel electrophoresis of the starting material for cDNA RDA (left panel) and the final difference products after the third RDA cycle (right panel). The different band pattern obtained with cultured chondrocytes (c) or osteoblasts (b) as tester demonstrates the high specificity during the subtractive hybridization process. M, 100 bp ladder.

Figure 2 Cell-type specific expression of GP-39, YKL-49 and CEP-68

Expression of CEP-68, GP-39 and YKL-39 was analysed by RT-PCR on RNA isolated from cultured chondrocytes (c1 and c2), cultured osteoblasts (b1 and b2), cultured mesenchymal stem cells (s1 and s2), primary cartilage (C) and bone (B) tissues using gene-specific primers. Like collagen type II (Col2), CEP-68 was expressed in cultured chondrocytes as well as in primary cartilage, but not in cultured osteoblasts or mesenchymal stem cells. In contrast to collagen type II, however, CEP-68 was expressed in primary bone. GP-39 and YKL-39 showed expression in one of the stem cell samples and YKL-39 was, additionally, positive in one of the cultured osteoblast monolayers. The GAPDH bands indicate equal amounts of cDNA template. For CEP-68, GP-39, YKL-39 and Col2, 35 PCR cycles were performed and for GAPDH, 28 cycles were performed.

products appeared for the chondrocyte- and the osteoblast-specific amplifications after three cycles of RDA with different tester:driver ratios (Figure 1). According to sequence analysis, six of the eight identified chondrocyte-specific fragments belonged to the gene for the cartilage glycoprotein 39 (GP-39, YKL-40) and one to the human YKL-39 precursor protein. Both molecules are known chondrocyte-expressed members of the chitinase family, showing, however, no chitinolytic activity [13,14]. The last fragment was derived from an unknown gene which we designated *CEP-68*. The gene products of the control experiment, being preferentially expressed in cultured osteoblasts, encompassed collagen type I and collagen type IV.

Expression of CEP-68 in cultured chondrocytes but not mesenchymal stem cells and osteoblasts

To verify the chondrocyte-specific RDA results on samples from two additional donors, RT-PCR experiments with gene-specific primers for GP-39, YKL-39 and CEP-68 were carried out on RNA isolated from cultured monolayers of chondrocytes, osteoblasts and mesenchymal stem cells. Figure 2 shows *CEP-68* gene expression only in cultured chondrocytes, but not in cultured osteoblast-like cells or in stromal cells. GP-39 and YKL-39, in contrast, were positive not only in chondrocytes but also in some mesenchymal stem cell samples. Although GP-39 was suitable to distinguish cultured chondrocytes from cultured osteoblasts, GP-39 and YKL-39 may give some background expression in mesenchymal stem cell cultures (Figure 2) used as a source to obtain chondrocyte-like cells by induction of *in vitro* chondrogenesis.

In primary tissue, expression of CEP-68 was evident for both bone and cartilage (Figure 2). This was parallel to YKL-39, whereas GP-39 was positive only in cartilage. Only the collagen type II control amplification was chondrocyte- and cartilage-specific. This suggested that cells expanded from bone lost the expression of CEP-68 when cultured in monolayers, whereas chondrocytes kept the expression of this protein for more than 10 weeks in culture. Indeed, already the earliest osteoblast-like cells growing out from bone biopsies within a few days after culture revealed no detectable CEP-68 expression by RT-PCR. Attempts to re-induce osteoblast differentiation in cultured osteoblast-like cells by a combination of bone morphogenic protein-4, growth and differentiation factor (GDF)-5 and TGF- β_1 [15] did not up-regulate CEP-68 expression, although the activity of alkaline phosphatase, a common marker for osteoblast differentiation, increased 15-fold by this treatment. In summary, RT-PCR confirmed the results obtained by RDA and identified CEP-68 as a stable marker for cultured chondrocytes which is not expressed in mesenchymal stem cells or osteoblasts even after induction of osteoblast differentiation by bone morphogenic protein-4, GDF-5 and TGF- β_1 .

CEP-68 expression in mesenchymal stem cells undergoing chondrogenic differentiation

To evaluate whether CEP-68 expression can be used as a new marker for chondrocyte differentiation, expanded mesenchymal stem cells derived from human bone marrow were shifted to a chondrogenic medium containing TGF- β_3 . RT-PCR after 2 weeks of culture demonstrated that collagen type II and CEP-68 expression were induced in parallel exclusively in cells cultured in chondrogenic medium, whereas the starting culture and cells kept in expansion medium were negative (Figure 3). This suggests *CEP-68* as a new marker gene for chondrocyte differentiation, which will ideally complement the well-established marker collagen type II [16,17] in stem-cell-based chondrogenic tissue-engineering approaches.

Tissue-specific expression of two different CEP-68 transcripts

To determine the transcription length of CEP-68 by Northern blotting, total RNA from cultured chondrocytes and osteoblasts was isolated, size-fractionated on a denaturing formaldehyde agarose gel and blotted on to a nylon membrane. By hybridizing with a CEP-68-specific PCR fragment, we identified a transcript of approx. 3 kb length specifically in cultured chondrocytes (Figure 4A).

Expression of CEP-68 outside the skeleton was further evaluated by a human multiple-tissue Northern. A CEP-68 transcript

Figure 3 CEP-68 expression in mesenchymal stem cells undergoing chondrogenic differentiation

Expression of CEP-68 was analysed by RT-PCR on RNA isolated from mesenchymal stem cells after different culture conditions: cells of the starting culture (passage 0) after 4 weeks (lane 1); cells kept in expansion medium (passage 1) after an additional 2 weeks in culture (lane 2); and cells shifted into chondrogenic medium for 2 weeks (lane 3). Like collagen type II (Col2), CEP-68 was only expressed in cultures kept in chondrogenic medium. The GAPDH bands indicated equal amounts of cDNA templates. For CEP-68 and Col2, 35 PCR cycles were performed and for GAPDH, 25 cycles were performed.

of 2.7–3 kb length was detected in lung, while heart, placenta, liver, skeletal muscle, pancreas (Figure 4B) and blood (results not shown) were negative. A smaller transcript (2.4 kb) of CEP-68 was found in brain. Thus outside the skeleton, at least two different CEP-68 transcripts were observed in lung and brain. In contrast, GP-39 mRNA had previously been detected in liver and YKL-39 mRNA in heart and lung [13,14].

Determination of the CEP-68 sequence and its genomic organization

The fragment of CEP-68 identified by RDA was highly similar to a human cDNA sequence (FLJ10320, accession number AK001182) with an open reading frame coding for 408 amino acids with unknown function and to a much shorter rat sequence (W307, accession number U78304). Whereas the rat sequence harboured an epidermal growth factor (EGF)-like motif, the human sequence FLJ10320 contained a 41 bp deletion, leading to a reading frame shift missing the EGF-like domain. Three human expressed sequence tags (accession numbers T77333, R60637 and Z43948) extended the FLJ10320 sequence at the 5'-end and three related human genomic clones (accession numbers AC015630, AL139239 and AL358938) from the human high throughput genomic sequences database at the NCBI allowed to predict the genomic structure of CEP-68 (Figure 5). The created consensus cDNA sequence was composed of 14 exons with a genomic distance of at least 74 kb. All introns possessed the consensus 5'- and 3'-splice sites and a polyadenylation signal was found 463 nucleotides after the stop codon within the last exon. Since two of the three genomic clones harbouring at least one exon of this gene were derived from chromosomal 10 genomic libraries (for the third no information was given), and three similar sequences in sequence tagged sites databases (accession numbers G27517, G25373 and AL159715) also identified this human chromosome, we suggest chromosome 10 as the genomic localization of CEP-68.

After selection of primers, the complete open reading frame of CEP-68 was amplified by PCR, cloned, and the fragment was sequenced. To complete the entire mRNA sequence including 5'- and 3'-untranslated regions 5'- and 3'-RACE were performed. The sequence obtained by 3'-RACE was included in Figure 5,

Figure 4 Transcript length and expression pattern of CEP-68

(A) Northern-blot analysis of CEP-68 was performed on total RNA isolated from cultured chondrocytes (c) and osteoblasts (b). The ethidium bromide-stained denaturing gel on the left indicates equal amounts of loaded RNA samples. On the right, the hybridization with a CEP-68-specific probe detected a transcript of approx. 3 kb length exclusively expressed in cultured chondrocytes. The autoradiograph was exposed for 24 h at -80°C . (B) Northern-blot analysis was performed on a human multiple-tissue Northern blot (ClonTech Laboratories) with a CEP-68 specific probe. An approx. 2.7–3.0 kb transcript was present in lung while in brain an additional smaller transcript of 2.4 kb was detectable. The autoradiograph was exposed for 5 days at -80°C . Hybridization with β -actin is shown in the lower panel, indicating equal amounts of mRNA in each lane.

while 5'-RACE was unsuccessful. Our sequence confirmed the predicted open reading frame of 1959 nucleotides encoding a 653 amino acid protein with a calculated molecular mass of approx. 71 kDa. CEP-68 harboured a 19 amino acid leader peptide at the N-terminus arguing in favour of a correctly predicted start codon. The molecular mass without the leading peptide was calculated as 68 kDa. Therefore we named this new protein chondrocyte-expressed protein (CEP-68).

CEP-68 defines a new family of proteins and harbours an EGF-like domain

To confirm that the CEP-68 sequence encodes a 68 kDa protein, the full length PCR-fragment was cloned into an expression vector and expressed by *in vitro* translation. As expected, SDS/PAGE revealed a protein with a molecular mass of approx.

SDS-PAGE gel showing a single band at approximately 66 kDa for the purified protein. Molecular weight markers are indicated on the left: 220, 97.4, 66, 46, 30, and 21.5 kDa.

Autoradiography of *in vitro* translated [³⁵S]Met-labelled CEP-68 revealed a protein band with a molecular mass of approx. 70 kDa resolved by 10% SDS/PAGE, confirming the predicted mass of 68 kDa. The cloud at the bottom of the gel indicates unincorporated [³⁵S]Met at the dye front.

EGF-like domains were named after the EGF polypeptide, the first protein characterized harbouring this motive. They are shared by a large number of proteins of divergent families. The domain itself consists of approx. 40 amino acids with a characteristic distribution pattern of six conserved cysteine residues which are also present in CEP-68 (Figure 5). These cysteines build up three disulphide bonds between cysteine-1 and cysteine-3, cysteine-2 and cysteine-4, and cysteine-5 and cysteine-6. The EGF-like domain in CEP-68 is located close to the C-terminus (Figure 5), and the four conserved amino acids aspartic acid-551, asparagine-553, glutamic acid-554 and asparagine-571 indicate a calcium-binding property associated with this EGF-like domain. Ca^{2+} -binding EGF domains are found in a spectrum of proteins with diverse functions like extracellular matrix molecules (e.g. fibrillin, fibulin, etc.) and coagulation factors (e.g. factor IX) [18,19]. Since the strongest sequence similarity within the EGF-like Ca^{2+} -binding domain of CEP-68 was detected to the EGF-like domain of the extracellular matrix protein fibulin-1 [20], and a leader peptide at the N-terminus indicates secretion of CEP-68, we suggest that CEP-68 may be an extracellular matrix protein expressed in bone, cartilage and cultured chondrocytes.

In summary, we identified CEP-68 as a member of a new protein family harbouring an EGF-like calcium-binding domain, which may presumably function as an extracellular matrix protein. CEP-68 allows better discrimination of cultured chondrocytes from osteoblast-like cells and mesenchymal stem cells compared with GP-39 and YKL-39, and will ideally complement collagen type II as a new marker for stem-cell-based chondrogenic tissue-engineering approaches.

We thank H. G. Simank and M. Lukoschek for providing human mesenchymal stem cells. This work was supported by a grant of the research fund of the Stiftung Orthopädische Universitätsklinik Heidelberg.

- 1 Grande, D. A., Piñhán, M. I., Peterson, L., Menche, D. and Klein, M. (1989) The repair of experimentally produced defects in rabbit articular cartilage by autologous chondrocyte transplantation. *J. Orthop. Res.* **7**, 208–218
- 2 Brittberg, M., Lindahl, A., Nilsson, A., Ohlsson, C., Isaksson, O. and Peterson, L. (1994) Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation. *N. Engl. J. Med.* **331**, 889–895

Nucleotides and amino acids are numbered on the left and right respectively. The leader peptide (underlined), the EGF-like Ca^{2+} -binding domain (double underlined), with the conserved cysteine residues (**bold**) and the residues responsible for the calcium binding motive (bold and italic), the stop codon (asterisk) and the polyadenylation site (italic underlined) are indicated. The vertical lines indicate the separate exons identified by sequence comparison with the genomic clones AC015630, AL139239 and AL358938. The protein motifs were identified by the simple molecular architecture research tool (SMART) at EMBL, and by database homology searches at the NCBI via the Internet.

70 kDa (Figure 6). Neither by similarity searches in protein or nucleotide databases, nor by computer-based protein motif prediction programmes, was any significant similarity or known

- 3 Pittenger, M. F., Mackay, A. M., Beck, S. C., Jaiswal, R. K., Douglas, R., Mosca, J. D., Moorman, M. A., Simonetti, D. W., Craig, S. and Marshak, D. R. (1999) Multilineage potential of adult human mesenchymal stem cells. *Science* (Washington, DC) **284**, 143–147
- 4 Muraglia, A., Cancedda, R. and Quarto, R. (2000) Clonal mesenchymal progenitors from human bone marrow differentiate in vitro according to a hierarchical model. *J. Cell Sci.* **113**, 1161–1166
- 5 Hubank, M. and Schatz, D. G. (1994) Identifying differences in mRNA expression by representational difference analysis of cDNA. *Nucleic Acids Res.* **22**, 5640–5648
- 6 Geng, M., Wallrapp, C., Muller-Pillasch, F., Frohme, M., Hoheisel, J. D. and Gress, T. M. (1998) Isolation of differentially expressed genes by combining representational difference analysis (RDA) and cDNA library arrays. *Biotechniques* **25**, 434–438
- 7 De Smet, C., Martelange, V., Lucas, S., Brasseur, F., Lurquin, C. and Boon, T. (1997) Identification of human testis-specific transcripts and analysis of their expression in tumor cells. *Biochem. Biophys. Res. Commun.* **241**, 653–657
- 8 Cooper, P., Mueck, B., Yousefi, S., Potter, S. and Jarai, G. (2000) cDNA-RDA of genes expressed in fetal and adult lungs identifies factors important in development and function. *Am. J. Physiol. Lung Cell Mol. Physiol.* **278**, L284–L293
- 9 Beresford, J. N., Gallagher, J. A., Poser, J. W. and Russell, R. G. (1984) Production of osteocalcin by human bone cells in vitro. Effects of 1,25(OH)2D3, 24,25(OH)2D3, parathyroid hormone, and glucocorticoids. *Metab. Bone Dis. Relat. Res.* **5**, 229–234
- 10 Jaiswal, N., Haynesworth, S. E., Caplan, A. I. and Bruder, S. P. (1997) Osteogenic differentiation of purified, culture-expanded human mesenchymal stem cells in vitro. *J. Cell. Biochem.* **64**, 295–312
- 11 Schultz, J., Copley, R. R., Doerks, T., Ponting, C. P. and Bork, P. (2000) SMART: a web-based tool for the study of genetically mobile domains. *Nucleic Acids Res.* **28**, 231–234
- 12 Schultz, J., Milpetz, F., Bork, P. and Ponting, C. P. (1998) SMART, a simple modular architecture research tool: identification of signaling domains. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 5857–5864
- 13 Hakala, B. E., White, C. and Recklies, A. D. (1993) Human cartilage gp-39, a major secretory product of articular chondrocytes and synovial cells, is a mammalian member of a chitinase protein family. *J. Biol. Chem.* **268**, 25803–25810
- 14 Hu, B., Trinh, K., Figueira, W. F. and Price, P. A. (1996) Isolation and sequence of a novel human chondrocyte protein related to mammalian members of the chitinase protein family. *J. Biol. Chem.* **271**, 19415–19420
- 15 Benz, K., Lorenz, H., Ewerbeck, V., Gerner, H. J. and Richter, W. (2000) Proliferation and differentiation of osteoblasts by combinations of growth factors. *Osteologie* **9**, 97
- 16 Benay, P. D. and Shaffer, J. D. (1982) Dedifferentiated chondrocytes reexpress the differentiated collagen phenotype when cultured in agarose gels. *Cell* (Cambridge, Mass.) **30**, 215–224
- 17 Johnstone, B., Hering, T. M., Caplan, A. I., Goldberg, V. M. and Yoo, J. U. (1998) In vitro chondrogenesis of bone marrow-derived mesenchymal progenitor cells. *Exp. Cell Res.* **238**, 265–272
- 18 Rao, Z., Handford, P., Mayhew, M., Knott, V., Brownlee, G. G. and Stuart, D. (1995) The structure of a Ca(2+) -binding epidermal growth factor-like domain: its role in protein-protein interactions. *Cell* (Cambridge, Mass.) **82**, 131–141
- 19 Van Zoelen, E. J., Stortelers, C., Lenterink, A. E. and Van de Poll, M. L. (2000) The EGF domain: requirements for binding to receptors of the ErbB family. *Vitam. Horm.* **59**, 99–131
- 20 Argraves, W. S., Dickerson, K., Burgess, W. H. and Ruoslahti, E. (1989) Fibulin, a novel protein that interacts with the fibronectin receptor beta subunit cytoplasmic domain. *Cell* (Cambridge, Mass.) **58**, 623–629

Received 25 September 2000/10 November 2000; accepted 20 November 2000