Gene and protein expression profile of naive and osteo-chondrogenically differentiated rat bone marrow-derived mesenchymal progenitor cells

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Abstract. Adult mesenchymal progenitor cells (MPCs) are adherent stromal cells of non-haematopoietic origin derived from bone marrow and other tissues. Upon limited *in vitro* expansion, they retain their self-renewal capacity as well as their potential to differentiate into tissues of mesenchymal lineage, such as bone, cartilage, muscle, tendon and connective tissues. Amongst these tissues, cartilage is the only one with insufficient self-renewal capacity, thus MPCs would qualify as an excellent tool for therapeutic regeneration of focal cartilage lesions. However, optimal *in vitro* manipulation of MPCs is a prerequisite; identification and a better understanding of the molecular mechanisms regulating their differentiation pathways are needed. Despite wide usage of rats as a mammalian experimental model for preclinical fracture healing and orthopaedic tissue regeneration studies, basal gene and protein expression profiles of the osteo-chondrogenic differentiation lineages of adult rat MPCs have rarely been investigated. Therefore, this study was carried out for a quantitative RT-PCR based time-course profiling of osteo- and chondrogenesis related gene expression in undifferentiated and differentiated rat adult MPCs. In addition, with an antibody array analysis TIMP-1, MCP-1 and VEGFα-164 were detected in the culture supernatant and CINC-2 and ß-NGF in the cell lysate of MPCs according to their differentiation commitment. Identification of differentially expressed genes and proteins along the osteo-chondrogenic lineage provides a foundation for a more reproducible and reliable quality and differentiation control of rat bone marrow-derived MPCs used for osteo-chondrogenic differentiation studies.

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Abbreviations: MPCs, mesenchymal progenitor cells; TIMP, tissue inhibitor of matrix metalloproteases; MMP, matrix metalloproteases; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor; MCP, monocyte chemoattractant protein; CINC, cytokine-induced neutrophil chemoattractant; NGF, nerve growth factor; Ihh, Indian hedgehog; TGF, transforming growth factor; COMP, cartilage oligomeric matrix protein; CNTF, ciliary neurotrophic factor; IFN, interferon; IL, interleukin; MIP, macrophage inflammatory protein

Key words: mesenchymal progenitor cells, rat, osteogenic, chondrogenic, profiling

Introduction

Adult mesenchymal progenitor cells (MPCs) also known as mesenchymal stem cells (MSCs) are precursors of the tissues of mesenchymal lineage. These osteo-chondroprogenitor MPCs reside as part of the stroma in the medullary cavity of the bone (1) and make up ~0.1% of the bone marrow cellular population which is otherwise dominated by haematopoietic cells. *In vitro* expansion of bone marrow cells leads to separation of the stromal MPCs based on their capacity of adherence to the plastic surface of tissue culture vessels. Upon limited expansion these cells retain not only their self-renewal capacity, but also their differentiation competence (2). This intrinsic competence is invoked under controlled nutritional and mechanical conditions to differentiate MPCs into bone, cartilage, muscle, tendon and connective tissue cells (3). MPCs have already shown promise for *ex vivo* regenerative medicine such as treatment of large bone defects (1,4,5-7) and focal cartilage lesions (8-10).

Lack of precise molecular definition and common standards for initial cell preparations are major obstacles for MPC-based research and application. Nevertheless, genetic profiling of human MPCs for trans-differentiation capacities has shown differential gene expression according to origin and commitment status of MPCs (11). In contrast, very little is known about the genomic and proteomic profile of MPCs from other species which may qualify as osteo-chondroprogenitor cells. Mammalian animal models, like rat, share many aspects of the human genomic, cellular and immunological structures. Several inbred strains are available; parallel to the small size, rapid development of the animals and high experimental reproducibility are advantages of employing rat MPCs for *in vitro* studies. Rats are routinely used for fracture healing studies, which is a specialized post-natal repair process that recapitulates embryological skeletal development (12). Besides

studies using genetically engineered MPCs to analyse their effects on healing critical sized fractures (13,14), many reports exist which analyse factors affecting fracture healing and osteogenic differentiation of precursor cells residing in the callus during endochondral ossification in the rat model (15-18). Importantly, rats are additionally employed for osteogenic and chondrogenic differentiation studies of bone marrow derived or synovial MPCs (19-21). In order to elucidate molecular signalling pathways during fracture healing, a transcriptional profiling of temporal gene expression rates during callus maturation was performed in the rat model. Here, RNA was isolated from total callus tissue which contained a mixture of not well defined cells in various differentiation stages (22). However, genomic and proteomic profiling of rat CD45-depleted MPCs during progression along the osteogenic/chondrogenic differentiation pathway is either incomplete or unavailable.

Therefore, this study undertook mRNA level profiling of selected genes in undifferentiated adult rat MPCs defined as baseline gene expression profile. Changes in gene regulation were then evaluated in an *in vitro* time-course study during osteo-chondrogenic differentiation.

Additionally, a protein profile of selected growth factors and cytokines generated from undifferentiated MPCs was compared with that of cells which were differentiated along osteogenic and chondrogenic lineages.

Establishing a differential, temporal gene and protein expression profile allowed identification of markers suitable for monitoring progression of rat MPCs along the osteogenic and chondrogenic pathway. This knowledge is essential for providing proper and optimized conditions for *in vitro* differentiation studies of MPCs.

Materials and methods

Preparation of MPCs and cell culture. MPCs were isolated from rat bone marrow as described earlier (23). Briefly, bone marrow was removed from the tibiae and femora of 6-week old male Sprague-Dawley rats by centrifugation (800 x g for 3 min in an Eppendorf mini-centrifuge). Homogenized bone marrow was cultured in 175 cm^2 tissue culture flasks in proliferation medium containing 5% glutamate, 1% antibiotics/antimycotics and 10% FBS (Gibco, Invitrogen, UK) in α-MEM (Sigma Aldrich, Germany). Non-adherent cells were removed on the 3rd day and the adherent CFU-cells were proliferated until 70% confluency. Magnetic associated cell sorting procedure (MACS, Miltenyi Biotech, Germany) was carried out according to the manufacturer's instructions. In short, expanded cells from 3-5 different rats were incubated in suspension with 4 μl of selected antibody/10^6 cells for 5 min at 37˚C followed by washing and incubation with goat anti-mouse secondary antibody coupled with magnetic beads for 15 min at 4˚C. This suspension was passed through a magnetic column (Miltenyi Biotec's LS-MACS columns). While labelled cells were retained by the magnetic field, the flow through containing the unlabelled negative fraction was collected by washing the columns with buffer. After removing the column from the magnetic field, the antibody labelled cells (positive fraction) were also flushed out. For both fractions cells were counted and stained. Antibodies used for MACS were directed against rat CD45 (CBL 1502, Chemicon, Germany), rat CD49a (SM 805P, Acris, Germany), rat CD71 (#554890) and rat CD106 (#559165) (both BD Bioscience Pharmingen, USA). The CD45-negative, unlabelled fraction was used for further differentiation studies while the CD45-positive, labelled fraction was discarded.

Chondrogenic and osteogenic differentiation of MPCs. For chondrogenesis, cells were cultured for up to 21 days in high density 3-D alginate bead cultures. To prepare the culture 10^7 cells/ml were suspended in 1.2% alginate. The cell-alginate amalgam was dropped into 102 mM $CaCl_2$ solution via a syringe which resulted in a formation of beads with a diameter of 2-3 mm containing ~10^5 cells/bead. Beads were cultured in 2.3 ml chondrogenic medium in 12-well tissue culture plates. Ten alginate beads per culture set up were used for RNA isolation and gene expression analysis. Cells were released from alginate by incubation at 37˚C for 30 min in 55 mM sodium citrate and 0.15 M sodium chloride buffer followed by cell recovery with a 3 min spin at 750 x g. Chondrogenic medium contained, ITS+ premix 6.25 μg/ml insulin, 6.25 ng/ml selenium acid, 6.25 μg/ml transferrin, 1.25 mg/ml BSA and 5.35 μg/ml linoleic acid (BD Biosciences), 110 μg/ml pyruvate, 40 μg/ml proline, 0.1 μM dexamethasone, 50 μg/ml ascorbic acid and 10 ng/ml TGFß-3 (24) (R&D Systems) in α-MEM high glucose medium (Gibco, Invitrogen). Alginate sodium salts were acquired from Sigma Aldrich. For osteogenesis, $2.5x10^5$ cells/well were cultured for up to 21 days in 6-well culture plates as monolayer in osteogenic medium containing 10% FCS, 1% pen/strep, 10 nM dexamethasone, 50 μg/ml ascorbate 2-PO_4 and 10 mM ß-Na-glycerophosphate (Sigma Aldrich) in α-MEM high glucose medium.

Immunofluorescence. For immunofluorescence analysis, alginate beads were irreversibly polymerized by replacing $CaCl_2$ with 100 mM $BaCl_2$ (25). The beads were fixed with 4% paraformaldehyde and after sequential dehydration embedded in paraffin. Sections (4 μm) were acquired and the deparaffinized and rehydrated sections were used for staining after hyaluronidase digestion for 6 min at RT. Undifferentiated MPCs kept in monolayer culture were fixed with 4% paraformaldehyde. Slides were blocked for 1 h at 37˚C in 5% normal goat serum and 1% BSA in PBS containing Complete Mini 1:5 protease inhibitor solution (Roche, Germany). After washing with PBS, cells were stained overnight at 4˚C with monoclonal antibodies which recognize rat collagen II, diluted 1:100, and rat D7fib, diluted 1:50 (both from Acris) and CD49a, diluted 1:50 (Chemicon). The appropriate Alexa 568 or 488 conjugated secondary antibodies (goat anti-mouse, 5 μg/ml, Molecular Probes, USA) were added for 1 h at RT. After washing, slides were permanently mounted with fluorescent mounting medium (Dako, USA) and covered with cover slips. Slides were evaluated with a scanning laser microscope (C1 confocal microscope from Nikon, Germany) and photos were taken with a Nikon C4 camera and software.

Histological analysis. Osteogenically differentiated cells were washed with PBS and fixed with methanol for 10 min. After rinsing with water, staining was carried out for 2 min with 1%

Table I. Primers used for quantitative RT-PCR.

Gene	NIH accession no.	Amplicon (bp)	Forward primer (5'-3')	Reverse primer (5'-3')
α10	XM_001063132	188	-tttcttcgggaaatcagagc	-tggatggagaagccaatctc
α11	XM_001075650	170	-tggaggtccaacacttcctc	-gggtttcagtccctcctctc
Aggrecan	NM_022190	224	-ggccttccctctggatttag	-ccgcactactgtccaac
ß-Actin	NM_031144	104	-gtagccatccaggctgtgtt	-ccctcatagatgggcagagt
CINC-2	D87926	287	-cactgcttctgctgcttctg	-tgacttctgtctgggtgcag
Col1a1	RGD61817*	59	-tccagggctccaacgaga	-ctgtaggtgaatccactgttgc
Col2a1	NM_012929	60	-cccctgcagtacatgcgg	-ctcgacgtcatgctgtctcaag
Col10a1	AJ131848*	247	-ccctattggaccaccaggta	-tctctgtccgctctttgtga
Col16a1	M92642	97	-gcctggtaccaaaggtgaaa	-catagcctggaggaccttga
COMP	NM_012834	167	-tgacttcgatgctgacaagg	-gaacgatctccattccctga
Ihh	XM_343590	103	-atgaagacggccatcactcag	-cgcgccagtagtccgtacttat
MCP-1	M57441	167	-atgcagttaatgccccactc	-ttccttattggggtcagcac
MMP-2	NM_031054	111	-gaccggtttatttggcgga	-ggcctcatacacagcgtcaat
MMP-13	XM_343345	93	-acctgggatttccaaaagagg	-acacgtccttccctgagaaga
ß-NGF	XM_001067130	182	-ggacgcagctttctatcctg	-ctgtgtcaagggaatgctga
Runx2	XM_34016	86	-gccgggaatgatgagaacta	-agatcgttcaacctggccact
Sox4	XM_344594	58	-ggcccatgaacgcctttat	-ctggatgaacgggatcttgtc
Sox6	XM_215016	51	-gaaatccatgtccaaccaggac	-cgggcctgctcttcatagtaag
Sox9	XM_343981	140	-ctgaagggctacgactggac	-tactggtctgccagcttcct
Tbox2	XM_220810	71	-gcccactctccgtttgtatgag	-aggacgaggcatcggattc
TIMP-1	NM_053819	136	-gattcgacgctgtgggaaat	-tttccgttccttaaacggcc
TIMP-2	NM_021989	140	-ggcaagatgcacattaccctct	-atgtagcatgggatcatagggc
TGFß-3	NM_013174	86	-ttccttcttggccgtatttcc	-tgtgtgggatccagaatcca
VEGFα	NM_031836	71	-tggctttactgctgtacctcca	-tttctgctccccttctgtcgt
VEGFR-2	NM_013062	95	-ttgcctagtcaagcagctcgt	-cgatggtctcaccaatggttg

*Locus ID.

alizarin red prepared in 25% ammonia. Stained cells were thoroughly washed and photographed with a Nikon C4 camera after complete drying.

RNA isolation and reverse transcription. RNA was isolated by an affinity column chromatography method with Ambion's RNAqueous4-PCR kit according to the manufacturer's protocol. For removal of putative DNA contamination *DNase*I enzyme (*DNA-free*, Ambion, USA) was used. RNA concentration was determined at a 585 nm wavelength with RiboGreen RNA quantification kit (Molecular Probes). RNA (0.5-1 μg) was converted to cDNA with SuperScript II reverse transcriptase kit (Invitrogen) in 20 μl of total reaction volume in the presence of 40 units/μl recombinant ribonuclease inhibitor (RNase OUT®), 500 μg/ml of Oligo-dT primers, 10 mM dNTPs and 200 units of SuperScriptII enzyme in First-Strand Buffer and 0.1 M DTT for 50 min at 42˚C followed by an extension period of 15 min at 70˚C.

Relative quantitative PCR. Relative quantitative PCR was performed using the SYBR Green Dye I on ABI 7000 Prism Sequence detection system (AB Systems, USA) according to the manufacturer's instructions. Briefly, 1 μl of cDNA was amplified in triplicates in 50 μl final volume containing 0.2 μM of each primer suspended in SYBR Green master mix (AB Systems). Amplification parameters were identical for all primer pairs and were repeated for 40 cycles, denaturation occurred at 95˚C for 0.15 min and annealing at 60˚C for 1 min. Mean relative quantification (RQ) values were calculated by the software 'RQ study application v1.1' (ABI Prism 7000 SDS software v1.1) according to the ΔΔCt method using ß-actin as endogenous control and undifferentiated MPCs (day 0) as calibrator. Primers were designed either with freeware Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) or 'Primer Express' software supplied by AB Systems. All primers were manufactured at MWG-Biotech, Germany and are listed in Table I. Prior to gene expression analysis, primer efficiency was determined with $10^{(1-S)}$-1, where S is the slope of the curve of a cycle threshold (Ct) standard curve. Only 90-100% efficient primers were used for analysis (26) and efficiency was included into calculation of gene expression changes according to the method of Pfaffl *et al* (27). For each primer pair cDNA (not pooled) from 4-7 rats was used for PCR.

Antibody array analysis. RayBio™ (Tebu-bio, France) rat cytokine antibody array I was used according to the manufacturer's instructions. Briefly, membranes were blocked for 30 min in 5% BSA in 0.01 M Tris buffer with 0.15 M NaCl (pH 7.6) followed by overnight incubation at 4˚C in 1 ml

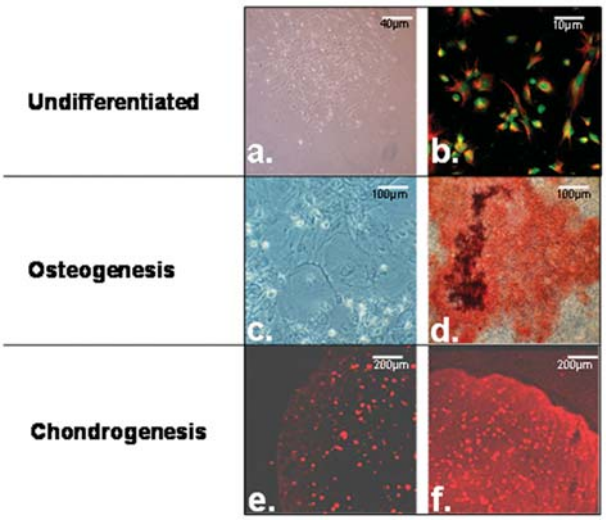

Figure 1. Morphological characterisation of MPCs. CFU-F like marrow derived adherent cells (a) characterized by double-immunofluorescence staining with D7fib (red fluorescence) and CD49a (green fluorescence) (b) exhibited typical stem cell morphology. At day 21 after induction of osteogenic differentiation in monolayer the cells were stained with alizarin red. Positive staining indicates calcified matrix formation (d) while the control culture kept in proliferation medium remained unstained (c). Chondrogenically differentiated cells kept in alginate beads were stained with an antibody against collagen II. After 21 days in 3D-culture the extracellular matrix displayed a positive collagen II signal in the cell surrounding newly synthesized matrix (f) which was undetectable at day 0 of 3D-culture (e).

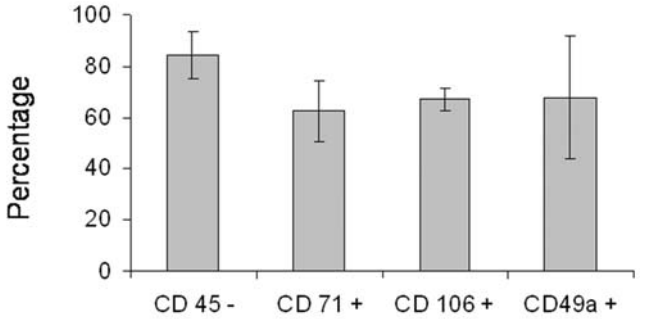

Figure 2. Antigen profiling of MPCs. Separation of bone marrow-derived adherent cells by magnetic antibody labelling resulted in a mixture of 60-75% CD71-, CD49a- and CD106-positive cells. About 85% of the cells were CD45-negative stromal cells indicating a slightly heterogeneous population with ~15% of CD45-positive haematopoietic cells. n=3-5 independent experiments.

of cell culture supernatant or cell lysates containing 100 μg of total protein. Both, cell culture supernatant and cell lysates were harvested at the endpoint (21 days) of culture duration. Cell lysates were prepared by using cell lysis buffer provided by the manufacturer. After washing and incubation with biotin coupled anti-cytokine antibodies for 2 h a second series of washing was performed and then the membranes were incubated with horseradish peroxidase-conjugated streptavidin for 2 h. Signals were detected with the provided detection solution using a CURIX 60 film developer (Agfa, Germany).

Statistics. Changes in gene expression <2-fold were not considered physiologically relevant and therefore described as unchanged (27,28) even if statistically significant. Expression rates of genes were compared between the two groups (undifferentiated and differentiated) using one-repeated measure ANOVA with the assumption of homogeneity of variance used as appropriate. Only where a statistical significance was found, pair-wise comparisons of the groups were performed using the t-test. A p-value <0.05 was considered statistically significant.

Results

Cellular morphology and surface antigen marker profile of MPCs. Formation of CFU-F is one of the basic classifications of bone marrow derived stem cells along with rapid adhesion and extended proliferation capacity (29). After *in vitro* expansion of bone marrow isolated cells, colony forming units of fibroblast like cells (CFU-F) designated as MPCs readily adhered to the culture flasks and showed typical fibroblast-like morphology (Fig. 1a). Immunofluorescence analysis of the adherent cells exhibited positive staining for fibroblast marker D7fib which is described as a specific marker for MPCs as suitable as STRO-1 and CD105 (30). Positive staining was also detected for α1 integrin marker CD49a (Fig. 1b). To confirm that isolated cells are indeed MPCs, cells were tested for their retained differentiation potential after expansion *in vitro*. MPCs were induced for up to 21 days in monolayer culture to undergo osteogenesis and, in 3-D high density alginate cultures for chondrogenesis. Osteogenic cultures stained positive for alizarin red indicating formation of a calcified matrix while uninduced control cultures remained unstained (Fig. 1c and d). Chondrogenically induced cells exhibited extensive collagen II staining in the surrounding matrix on day 21 of the culture which was undetectable at day 0 (Fig. 1e and f).

Since a unique MPC marker is not yet identified, culture purity is usually determined by employing a consortium of positive and negative markers. In this study adherent cells were screened by magnetic cells sorting (MACS™) using the three MPC-positive markers CD71 (transferrin receptor) (31), CD106 (VCAM) (32) and CD49a (integrin subunit α1) (33,34) (Fig. 2). These markers reveal 60-65% of the cells as CD71 positive and 75% positive for CD106 and CD49a. In addition, CD45 (leukocyte common antigen) (35,36), a haematopoietic surface marker used for negative selection, revealed strong enrichment of MPCs with a remaining 15% of CD45-positive cells.

Gene expression profiling of undifferentiated and differentiated MPCs. With the help of quantitative RT-PCR and specific primers (Table I) mRNA expression of selected genes was determined in un-induced, undifferentiated MPCs (day 0) and defined as baseline or calibrator gene expression (data not shown).

After application of osteogenic or chondrogenic favourable conditions to the cell cultures for up to 21 days, relative changes in mRNA level were determined on days 7, 14 and 21 (Fig. 3A-H). During induction of chondrogenic differentiation, tissue inhibitor of matrix metalloproteases (TIMP-1) became downregulated at day 21 while TIMP-2, MMP-2 and -13 remained upregulated until day 21 (Fig. 3A). Integrin subunit α11 was upregulated non-physiologically (2x) according to

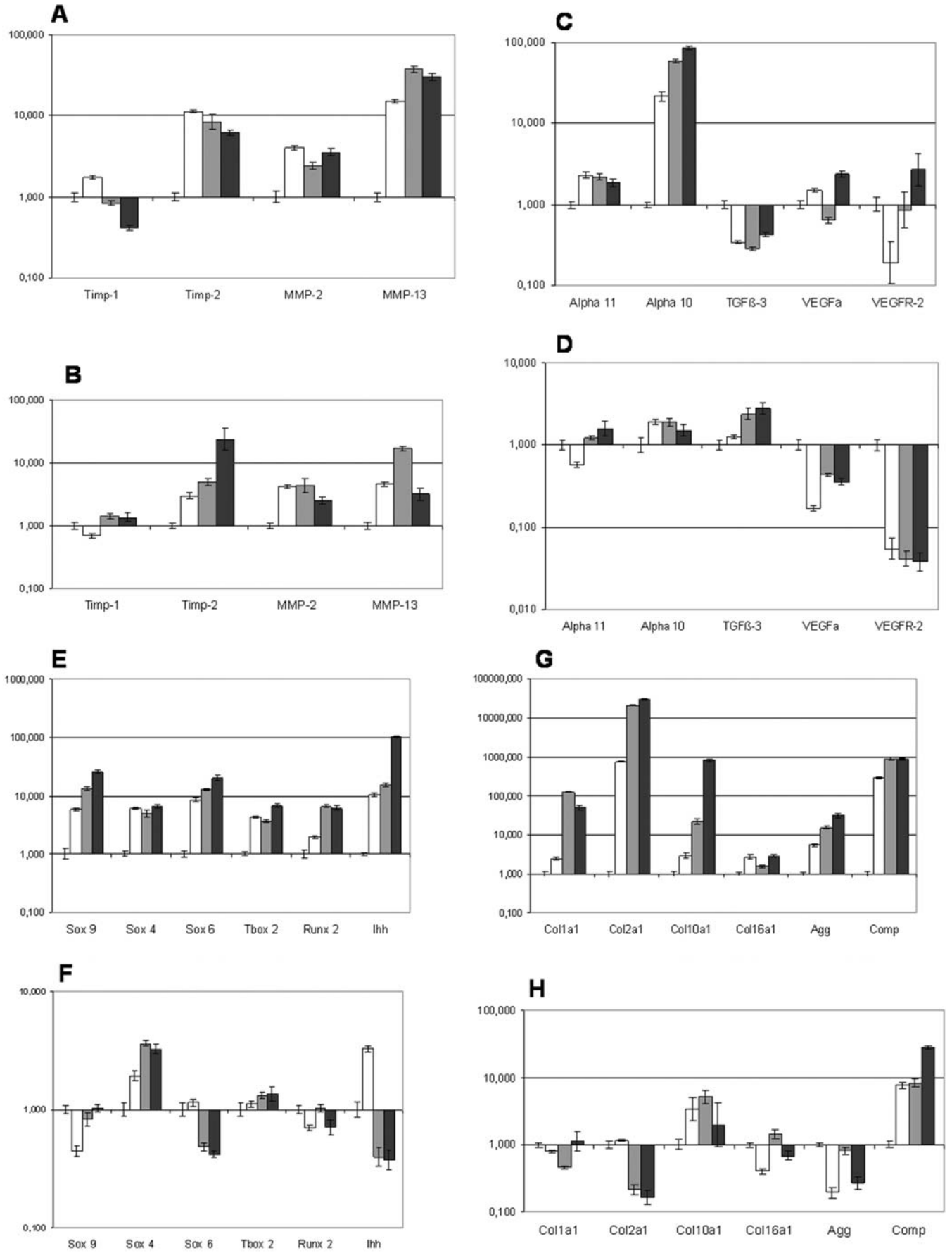

Figure 3. Differential gene expression profile of MPCs during osteo-chondrogenic differentiation. Relative quantitative mRNA expression level (RQ) of selected TIMPs, MMPs, Integrin subunits, collagens, transcription factors and growth factors was determined by the ΔΔCT method using ß-actin gene expression as endogenous control and gene expression of undifferentiated MPCs as calibrator which was set at 1. Expression level of each gene was determined at day 7 (white bars), day 14 (grey bars) and day 21 (black bars) during chondrogenic (A, C, E, G) and osteogenic (B, D, F, H) differentiation of MPCs. n=4-7 independent experiments.

Table II. Regulation of gene expression during osteo-chondrogenic differentiation of MPCs at day 21 (end point of culture regimen) expressed as relative quantification values (RQ).

Gene	RQ chondrogenesis	RQ osteogenesis	Ratio RQ of chondrogenesis/osteogenesis
Col2a1	30818.00***	0.20***	181276.00
Col10a1	808.00***	2.00 n.s.	404.00
Ihh	103.00***	0.40***	274.00
Aggrecan	32.00***	0.30***	107.00
VEGFR-2	2.70**	0.04***	67.50
Integrin α10	85.00***	1.50***	57.00
Sox6	20.50***	0.40***	51.00
Col1a1	50.00***	1.00 n.s.	50.00
COMP	897.00***	28.00***	32.00
Sox9	26.00***	1.00 n.s.	26.00
CINC-2	1.50***	0.15***	10.00
MMP-13	30.00***	3.00***	10.00
Runx2	6.00***	0.70*	8.60
VEGF α	2.40***	0.35***	6.90
Tbox2	6.70***	1.35***	5.00
Col16a1	2.80***	0.70***	4.00
Sox4	6.50***	3.25***	2.00
MMP-2	3.60***	2.50***	1.40
Integrin α11	2.00***	1.60***	1.25
TIMP-2	6.20***	23.60***	0.30
TIMP-1	0.40***	1.40*	0.30
ß-NGF	0.20***	0.60***	0.30
TGFß-3	0.40***	2.80***	0.14
MCP-1	0.05***	1.00 n.s.	0.05

Values >1 indicate upregulation, values <1 indicate downregulation of the gene relative to the calibrator (undifferentiated MPCs set as 1). *p<0.05; **p<0.01; ***p<0.001; n.s., non-significant.

Pfaffl (27,28) while integrin subunit α10 became strongly induced. Gene expression of TGFß-3 was downregulated from day 7 while vascular endothelial growth factor (VEGF) α and VEGFR-2 mRNA level were moderately increased at the end of culture time after an earlier suppression (Fig. 3C). Gene expression of transcription factors Sox9, Sox6, Sox4, Tbox2, Runx2 and Ihh was clearly upregulated with an increase towards the culture end point (Fig. 3E). Notably, Ihh mRNA level increased up to 100x at day 21. All collagens were upregulated during culture time with a profound increase in gene expression for Col2a1 (>30,000x) and the lowest change for Col16a1 (2.8x). Gene expression of aggrecan and COMP was strongly induced over culture time (Fig. 3G).

Induction of osteogenic differentiation resulted in a mostly altered expression profile of these genes. TIMP-1 was not regulated in physiologically relevant terms (<2x) while gene expression of TIMP-2 was more profoundly upregulated at day 21 than in chondrogenic differentiation (23x versus 6x). MMP-2 gene expression was similarly regulated as under chondrogenic differentiation while MMP-13 became downregulated at the end of culture compared to chondrogenic conditions (3x versus 30x) (Fig. 3B). During osteogenic differentiation changes in expression for integrin subunits α10 and α11 remained below physiological relevance. Contrary to chondrogenesis TGFß-3 mRNA level was moderately upregulated during osteogenic culture time (2.8x) while both VEGFα and VEGFR-2 mRNAs were downregulated, gene expression of VEGFR-2 decreased 25x compared to its expression in undifferentiated MPCs (Fig. 3D). Gene expression profiles of all transcription factors (except for Sox4) was different under osteogenic compared to chondrogenic culture conditions. Sox9 was downregulated at day 7 while at the end of culture time gene expression was similar to undifferentiated MPCs. Sox6 and Ihh mRNA level were strongly suppressed towards later time points while Tbox2 remained unaltered and Runx2 was slightly downregulated (Fig. 3F). Also, collagen gene expression profiles were different from chondrogenic induced MPCs. During osteogenesis Col1a1 mRNA level did not change compared to undifferentiated MPCs while Col2a1 was profoundly downregulated during progression of osteogenesis. Col10a1 was upregulated until day 14 and almost reduced to the level of uninduced MPCs at day 21. Gene expression of Col61a1 and aggrecan decreased over culture time while COMP became upregulated, however, less profoundly as under chondrogenic differentiation conditions (28x versus 900x) (Fig. 3H). Regulation data from the end

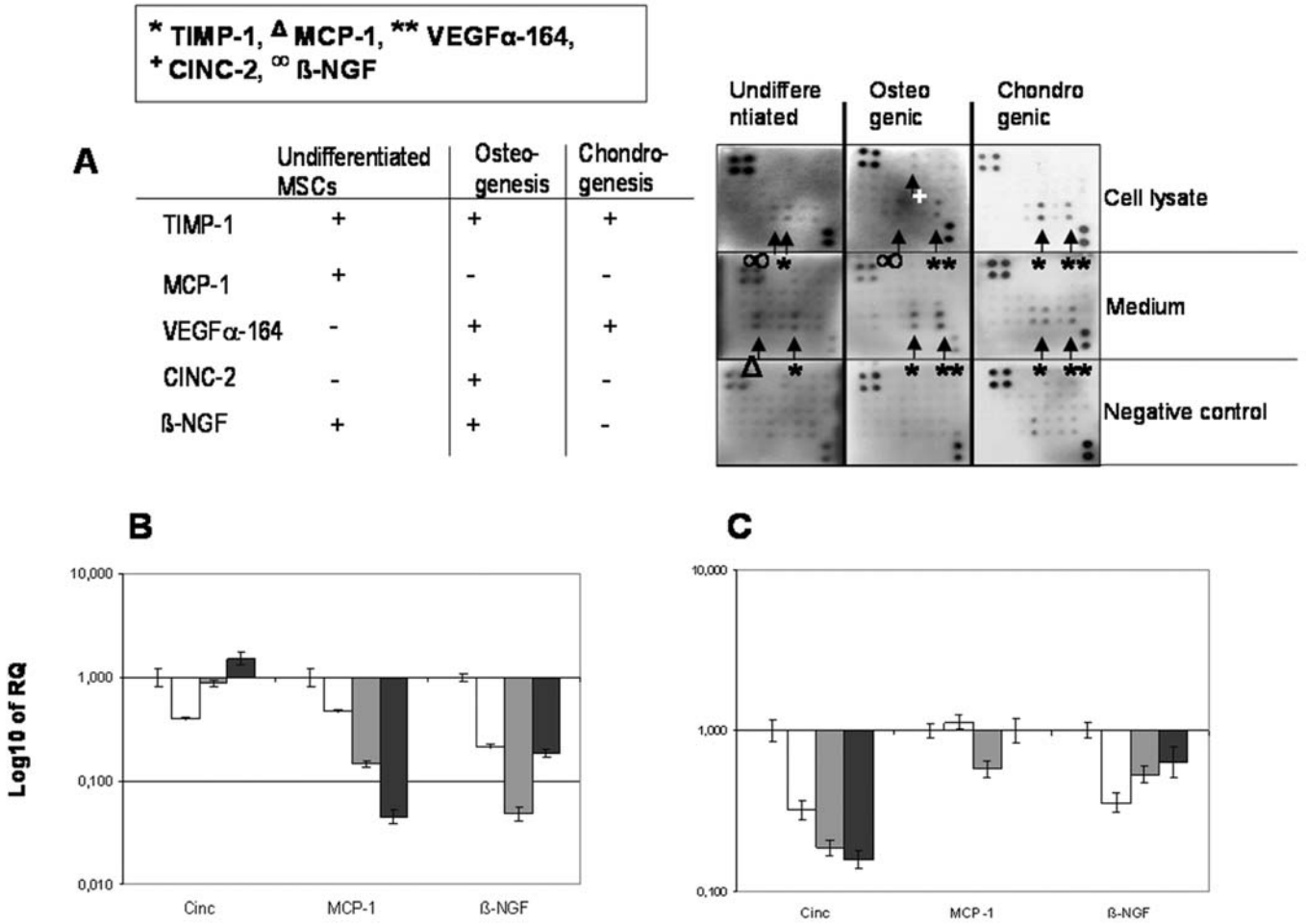

Figure 4. Comparison of cytokine and growth factor profiles of undifferentiated and differentiated MPCs. An antibody array containing 14 different cytokines plus TIMP-1, ß-NGF, VEGFα-165, leptin and CNTF was used to compare protein profiles of proliferated undifferentiated, uninduced MPCs and MPCs after osteogenic and chondrogenic induction (A). *TIMP-1, **VEGFα-164, ΔMCP-1, ∞ß-NGF, +CINC-2. Representative blots of a total of 5 independent experiments are shown; n=3 for culture supernatants and n=2 for cell lysates. Temporal gene expression profiles of MCP-1, CINC-2 and ß-NGF was determined by qRT-PCR as described above (B and C).

point of the different culture regimen (day 21) are displayed in Table II.

Cytokine and growth factor protein profile of undifferentiated and differentiated MPCs. Cell culture supernatants and lysates from undifferentiated and differentiated MPCs at day 21 were analyzed for secretory molecules using an antibody array covering 14 cytokines plus ß-NGF, TIMP-1, CNTF, leptin and VEGFα-164 (Fig. 4). TIMP-1 was the only molecule secreted from undifferentiated as well as differentiated MPCs, while the cytokine monocyte chemoattractant protein (MCP-1) was exclusively secreted from undifferentiated MPCs. Secretion of VEGF (variant VEGFα-164) was observed only after induction of osteogenic and chondrogenic differentiation. When the same antibody array was examined with the cell lysate two additional molecules were identified. Nerve growth factor (ß-NGF) was detected in undifferentiated and osteogenically induced cells only and cytokine induced neutrophil chemoattractant (CINC-2) was detected as a weak spot after osteogenesis. Both factors were undetectable after chondrogenic induction (Fig. 4A). The remaining 14 proteins of the array were undetectable in all culture conditions analysed, these include, CINC-3, ciliary neurotrophic factor (CNTF), fractalkine, GM-CSF, IFN-γ, IL-1α, -1ß, -4, -6, -10, lipopolysaccharide induced c-x-c chemokine (LIX), leptin, macrophage inflammatory protein (MIP-3α) and TNF-α.

Gene expression level of MCP-1 was profoundly down-regulated during chondrogenic differentiation while unchanged along osteogenic differentiation pathway expression. CINC-2 was downregulated upon osteogenic differentiation and remained unaltered during chondrogenic differentiation (<2x at day 21) while gene expression of ß-NGF was decreased upon induction of MPC differentiation (Fig. 4B and C).

RQ values and ratios. Table II summarizes regulation of gene expression at the end point of the osteo-chondrogenic differentiation culture regimens (day 21). Opposite regulated genes include Col2a1, Ihh, aggrecan, VEGFR-2, Sox6, Runx 2, VEGFα, Col16a1 and TGFß-3. Except for TGFß-3, the other genes listed above were downregulated upon induction of osteogenesis and upregulated upon chondrogenesis. Genes that are not regulated upon osteogenesis but upregulated upon chondrogenesis were Col10a1, integrin subunit α10, Col1a1, Sox9, Tbox2, integrin subunit α11 while TIMP-1 and MCP-1 were downregulated upon chondrogenesis. Only CINC-2 mRNA level was suppressed in osteogenesis and

remained unchanged upon chondrogenesis. Genes upregulated under both osteogenic and chondrogenic differentiation conditions include COMP, MMP-13, Sox4, MMP-2 and TIMP-2. The only gene downregulated under both differentiation pathways was ß-NGF.

The ratio of RQ reflects whether the gene is up- or down-regulated in chondrogenesis versus osteogenesis. Values >1 indicate upregulation of mRNA levels during chondrogenesis while values <1 indicate downregulation during chondrogenesis compared to osteogenesis.

Discussion

Rat is an appropriate animal model for MPC based pre-clinical studies due to high reproducibility and at the same time provides a sufficient number of bone marrow derived cells. However, a representative gene expression profile of rat naïve and differentiated MPCs is not available and as the expression of known genes is not identical to adult human stem cells, a comprehensive profiling of rat gene and protein expression is necessary for accurate interpretation of data generated with rat MPCs.

Here, we present a time course profiling of a selected panel of genes mostly differentially regulated in undifferentiated and osteo-chondrogenically differentiated rat MPCs. These genes include growth factors, transcription factors, metalloproteinases (MMPs) and their inhibitors (TIMPs), structural components of the matrix and cell adhesion molecules. Several of these genes routinely serve as markers to determine osteo-chondrogenic differentiation status of human chondrocytes, osteocytes and MPCs (col1a1, col2a1, col10a1, Runx2, Sox9, aggrecan) others are not yet implicated in osteo-chondrogenic differentiation of MPCs (Col16a1, Sox4, TIMP1, -2, MCP-1, ß-NGF and CINC-2). Not much is known about the temporal involvement of all of these markers in osteo-chondrogenic differentiation processes of rat MPCs and whether their regulation is comparable to the human system.

Identification of genomic marker for monitoring osteogenesis and chondrogenesis. Integrin subunit α11, expressed in undifferentiated rat MPCs, is present in various mesenchymal tissues around the cartilage anlage in the developing skeleton. In comparison, integrin subunit α10, known as a highly chondrocyte specific, collagen II and IX-binding integrin (37,38), was not detected in naïve MPCs. Chondrogenic differentiation of MPCs leads to ~85-fold increase in the mRNA level of α10, whereas the α11 mRNA level remains mostly unchanged. Gene expression profile of both integrin subunits remained unaltered during the osteogenic differentiation pathway. Thus, the ratio of α11/α10 gene expression is highly appropriate for monitoring the progression of chondrogenic versus osteogenic differentiation of MPCs.

Gene expression profiles of growth factors TGFß-3 and VEGFα, including its receptor VEGFR-2, are differentially regulated upon induction of osteo-chondrogenically differentiation.

During endochondral bone formation angiogenic factor VEGF modulates matrix mineralization in hypertrophic cartilage in an autocrine manner by upregulating the expression of its receptor (39). Here, VEGFα and VEGFR-2 gene expression levels become induced in MPCs after entering the chondrogenic pathway while commitment to osteogenic differentiation regimen results in strong downregulation of VEGFα and VEGFR-2 mRNA. Hence, comparison of relative VEGFα and VEGFR-2 mRNA levels are useful indicators of MPC differentiation stage and lineage commitment.

Transcription factors which regulate osteo- and chondrogenic lineage commitment as, Tbox2 (brachyury), Sox9, Sox6 and Runx2 are clearly upregulated during chondrogenic differentiation. Both, Sox9 and Tbox2 are essential components for the BMP-dependent onset of chondrogenesis (40). Except for Sox4, the other four transcription factors were differentially regulated under osteogenic culture conditions. Differential expression levels of these transcription factors might serve as markers for harvesting osteo-chondroprogenitor cells at different stages of lineage commitment thus allowing reproducible separation and identification of these early committed cells. Similarly, Sox4 was not implicated in being involved in the osteogenic differentiation pathway and not much is known regarding its involvement in chondrogenic differentiation except that Sox4 and BMP-2, together with collagens I and IV are upregulated in early chondrogenic differentiating human MPCs kept in alginate beads (41). During chondrogenic differentiation, gene expression of both Sox9 and Ihh is strongly induced, indicating cellular arrest in the early hypertrophic stage. Sox9 is expressed in all essential stages of chondrogenic development, but its expression is abrogated in hypertrophic chondrocytes (42,43) which may be regulated by a negative feedback mechanism of the PTHrP and Ihh signal cascade in pre-hypertrophic chondrocytes (44,45). However, this expression profile is accompanied by strong upregulation of collagen X gene expression which is restricted to terminally differentiated hypertrophic chondrocytes (46). Possibly, upregulation of collagen X is connected to increased expression of Runx2 since multiple Runx2 consensus binding elements are located in the collagen X promoter region (47). However, collagen X expression *in vitro* is not in line with the expression of other differentiation markers. This limitation in using collagen X as a marker for chondrogenesis of human MPCs is described in previous literature (48,49). Our data suggest the same for rat MPCs during chondrogenic differentiation.

MMPs alter the activity or function of numerous proteins by proteolytic processing and they are also involved in various cellular functions and matrix formation during development. Expression of MMPs and TIMPs in MPC cultures was reported on in earlier studies on human MPCs of different origins (50,51). MMP-13 together with its inhibitors, TIMP-1 and -2 is expressed in MPCs indicating a role in stem cell migration and proliferation as it is assigned to MMP-2 (52). While TIMP-1 gene expression remains mainly unaltered or is downregulated, MMP-13 and TIMP-2 were upregulated during both, chondrogenic and osteogenic culture regimen. A relatively higher MMP-13 mRNA level during chondrogenesis might obtain relevance in progressing mineralization as MMP-13 is involved in endochondral and intramembranous ossification (53).

We report, for the first time, on gene expression of collagen XVI in undifferentiated MPCs which is upregulated during chondrogenesis while downregulated during osteo-

genesis. This observation indicates an unknown role of this fibril-associated collagen with interrupted triple helices (FACIT) in proliferation of un-induced MPCs and/or chondrogenic differentiation. As known for human MPCs, collagen II and aggrecan gene expression are upregulated during chondrogenesis and reduced in osteogenesis, therefore their differential expression profiles obtained in this study are also usable for rat MPCs as marker of chondrogenic commitment.

Identification of proteomic marker for monitoring osteogenesis and chondrogenesis. Secretory cytokines, proteases and growth factors are key players in cell proliferation and differentiation. Therefore, we investigated MPC differentiation state-specific protein profile. TIMP-1, the only identified molecule secreted from undifferentiated as well as differentiated MPCs is a multi-functional molecule. In addition to its physiological activity as inhibitor of MMPs, it is also thought to be involved in regulation of cell growth and differentiation of a variety of cell types including mesenchymal and epithelial cells (54). Hence, TIMP-1 secreted differentiation independently of MPCs act either as a growth factor or as an inducer/repressor of specific factors necessary for commitment to the osteo-chondrogenic lineage of MPCs.

MCP-1, which recruits monocytes into ischemic tissue and stimulates chemotaxis, is secreted exclusively from un-induced MPCs while its gene expression remains unchanged or is repressed during osteo-chondrogenic differentiation, respectively. It is speculated to be involved in homing of circulating stem and progenitor cells. MPCs secrete this chemokine to the region of injuries which contribute to healing processes (55,56). MCP-1 secretion appears suitable as a marker for un-induced rat MPCs not having entered the chondrogenic and osteogenic differentiation cascade.

Secretion of the rat-specific splice variant of VEGF, VEGFα-164, was induced only after cells were subjected to osteogenic and chondrogenic differentiation. VEGF as a multi-faceted protein has a role in induction of metalloproteinases, such as MMP-13 during the late stages of chondrogenesis marking the onset of hypertrophy (57). Lack of VEGF secretion in undifferentiated MPCs are taken as an indicator for a relatively pure MPC population, mostly devoid of VEGF secreting haematopoietic and endothelial progenitor cells (58,59).

Analysis of the cellular lysates leads to the identification of two additional molecules. ß-NGF, detected in undifferentiated as well as in cells after osteogenic induction and cytokine-induced neutrophil chemoattractant (CINC-2), which was detected after osteogenesis only. CINC-2 attracts neutrophils and was suggested to inhibit acute inflammation indicating an anti-inflammatory role in osteogenesis (60). So far, this chemokine has not been determined in rat MPCs undergoing osteogenic differentiation, however, our data suggest that CINC-2 is suitable as one of several reliable protein indicators for successfully induced osteogenesis in MPCs.

Undifferentiated, non-committed bone marrow derived cells are known to express ß-NGF and its receptor (61) together with other neurotrophic factors (62). This falls in line with the observed production of ß-NGF in undifferentiated MPCs. The naïve MPCs loose ß-NGF biosynthesis upon chondrogenic differentiation but keep it under osteogenic favourable conditions which suggests a role of ß-NGF in osteogenesis as a trophic factor known to stimulate collagen synthesis and expression of specific osteogenic marker, such as alkaline phosphatase (63).

The discrepancy in reduced gene expression and protein synthesis/secretion profile of VEGFα, ß-NGF and CINC-2 after differentiation is likely due to post-translational regulation. VEGF production is known to be regulated at the translational level as over-expression of transcription factor c-Myc related with rapid induction of tumors, also leads to 10-fold higher VEGF production while the gene expression level remains unaltered. The mechanisms of action are most likely higher ribosome biogenesis rates which increase translation efficiency of selected mRNAs (64). Also, in rat smooth muscle cells regulation of NGF production does not solely occur at the level of transcription. Post-transcriptional mechanisms, such as increased NGF mRNA stability and elevated synthesis of NGF protein per unit NGF mRNA, can operate (65,66).

In conclusion, we demonstrated a specific gene and protein expression/secretion profile of rat $CD45^{-low}$ bone marrow-derived progenitor cells. The defined single genes and gene combinations are considered suitable as markers allowing reliable and reproducible discrimination between the un-induced MPCs and the osteogenically and chondrogenically differentiating MPCs (Table II). Discrimination is carried out also at protein level with the help of the two cytokines MCP-1 and CINC-2 indicative for un-induced and osteogenically differentiated MPCs, respectively. The knowledge of differentially expressed genes and proteins provides a foundation for optimizing cell culture conditions necessary to properly induce and maintain desired differentiation pathways. This is a prerequisite for not only manipulating MPCs in animal models to regenerate complex tissues such as cartilage and bone but also for future tissue engineering studies with human adult MPCs.

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