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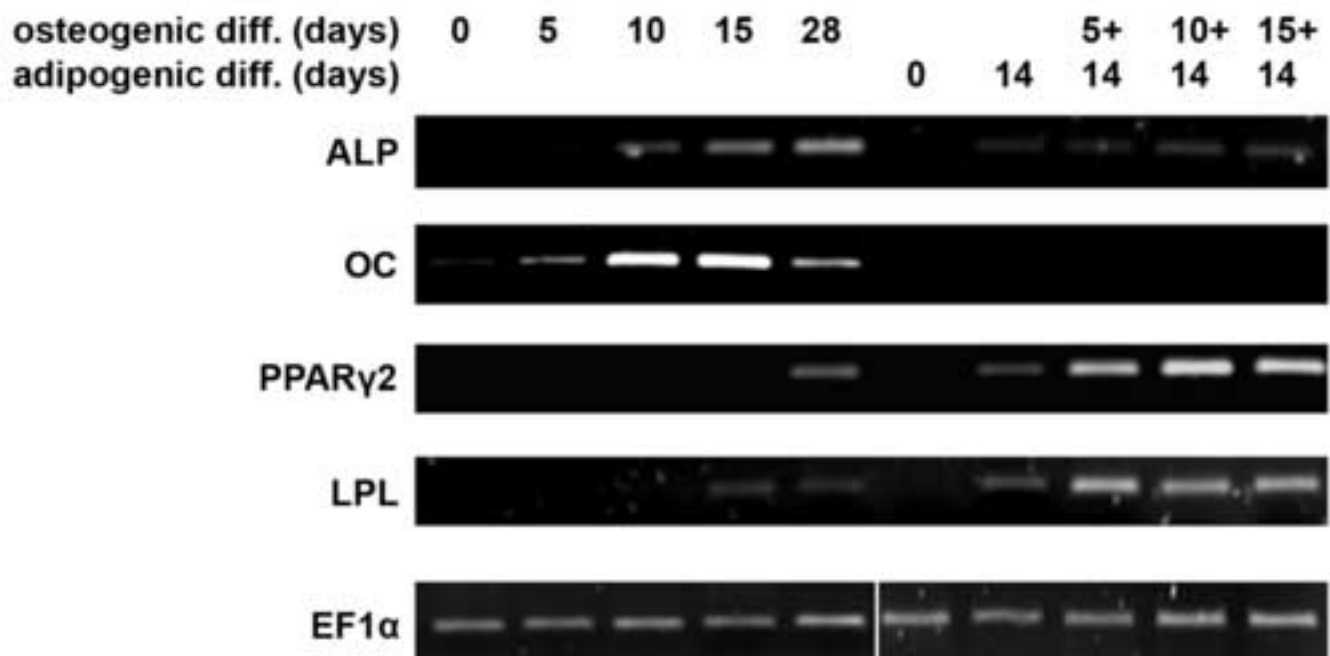
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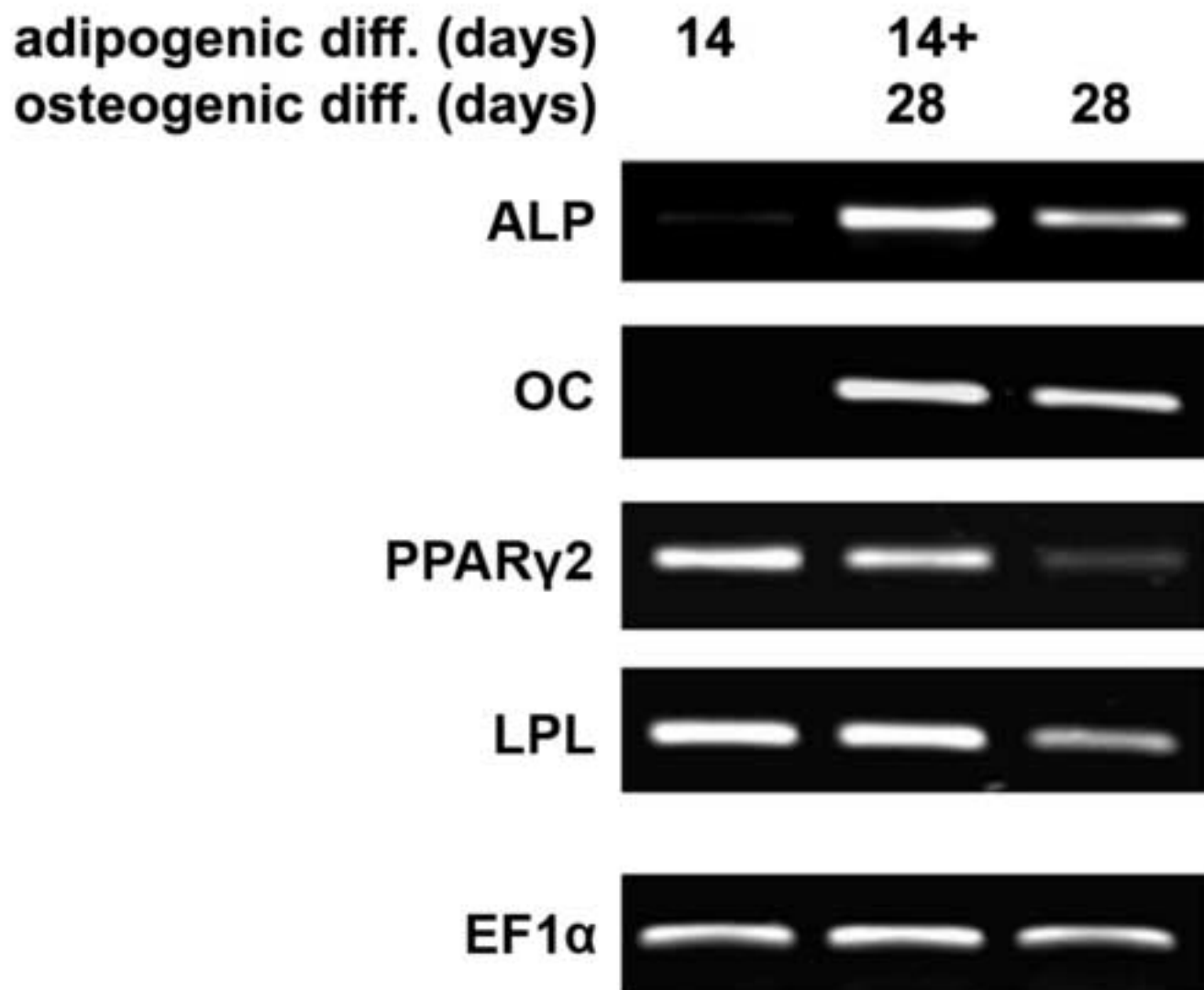
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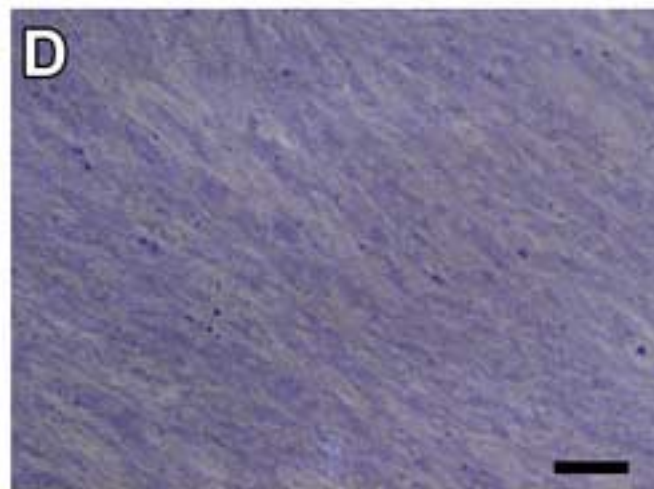
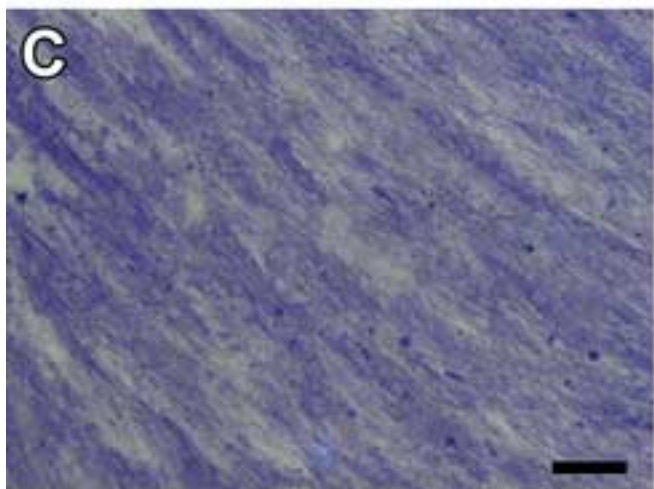
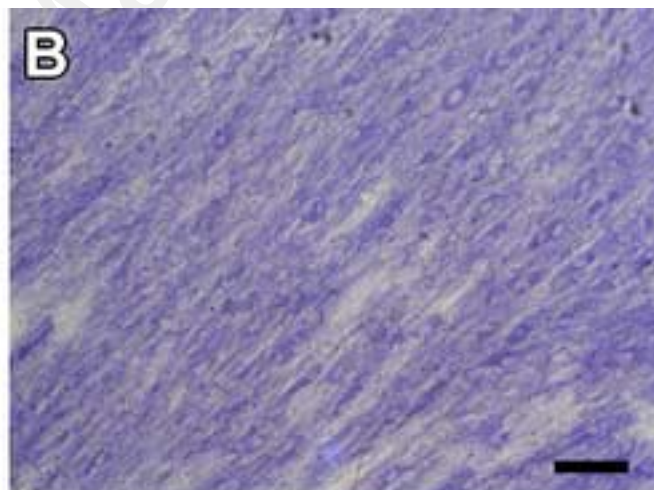
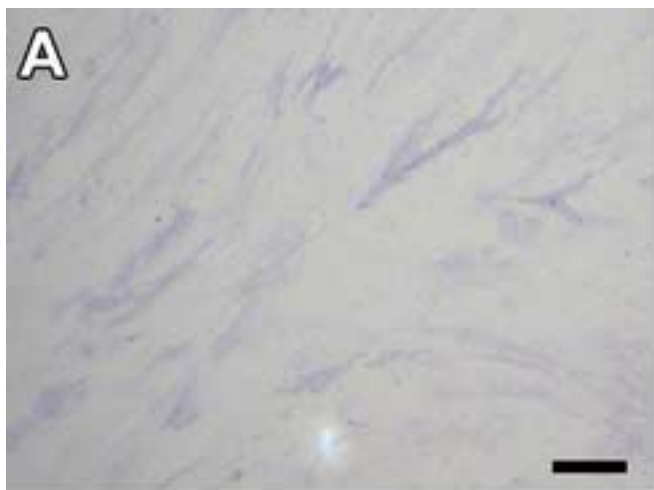
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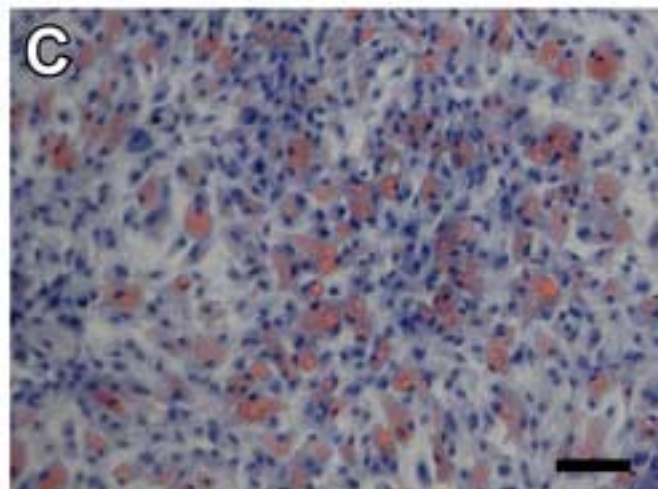
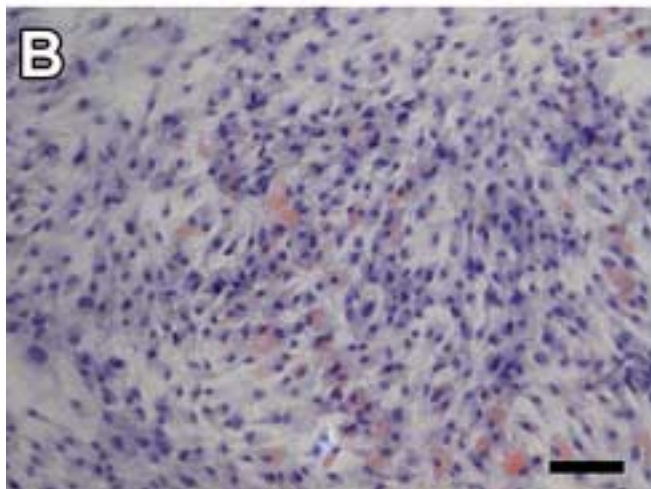
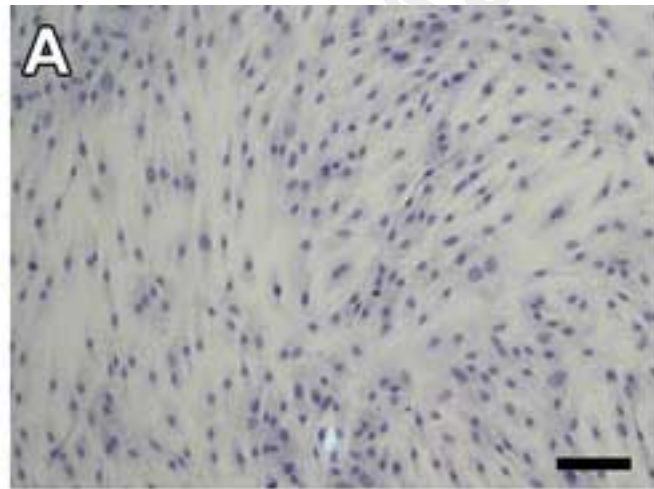
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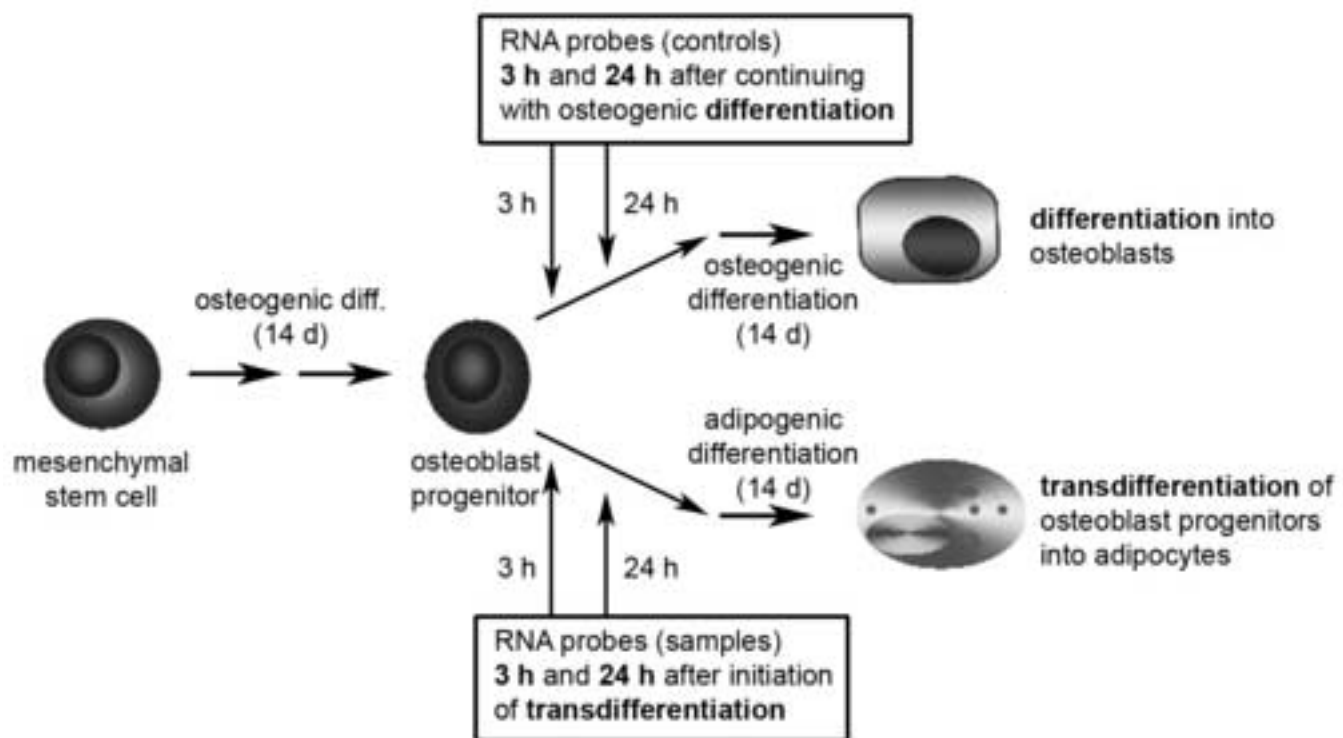




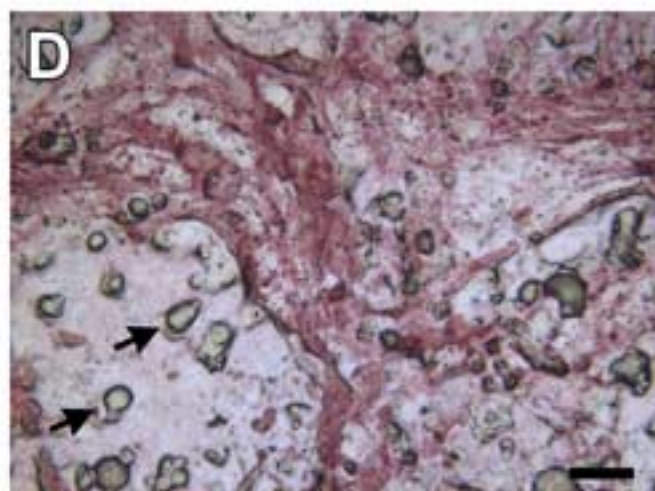
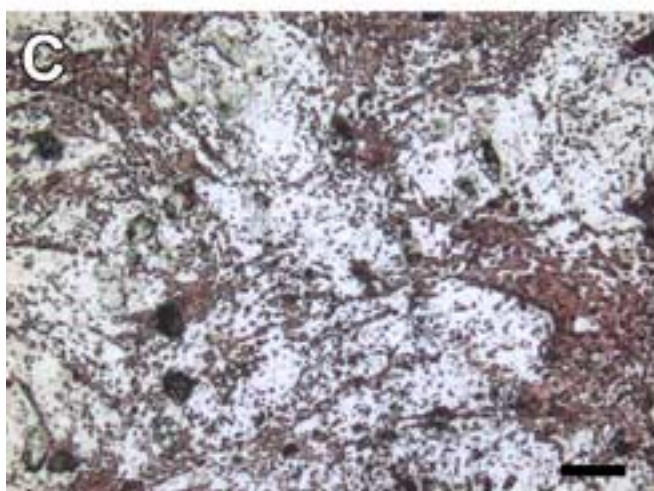
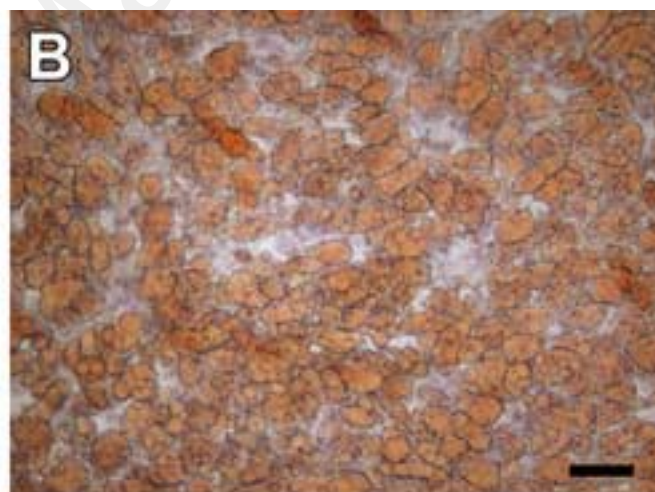
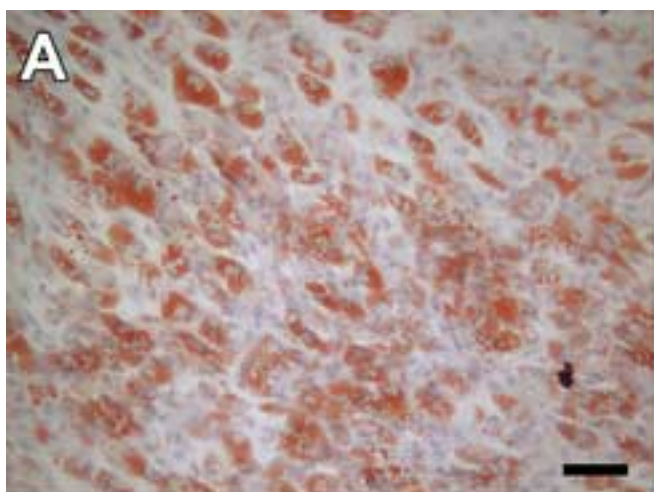


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Plasticity in Adipogenesis and Osteogenesis of Human Mesenchymal Stem Cells

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Abstract

We established a cell culture system of human mesenchymal stem cells that allows not only for osteogenic and adipogenic differentiation but also for transdifferentiation between both cell lineages. Committed osteoblasts were transdifferentiated into adipocytes with losing osteogenic but highly expressing adipogenic markers. Adipocytes were transdifferentiated into osteoblasts with most of the resulting cells showing osteogenic but some still displaying adipogenic markers apparently not responding to the reprogramming stimulus. Comparing transdifferentiated adipocytes with committed osteoblasts by microarray analysis revealed 258 regulated transcripts, many of them associated with signal transduction, metabolism, and transcription but mostly distinct from established inducing factors of normal adipogenic and osteogenic differentiation, respectively. The regulation pattern of 20 of 22 selected genes was confirmed by semiquantitative RT-PCR. Our results indicate that the plasticity between osteogenesis and adipogenesis extends into the differentiation pathways of both cell lineages and may contribute to the age-related expansion of adipose tissue in human bone marrow.

Introduction

Human bone marrow-derived stem cells (hMSCs) are multipotential cells that can differentiate into osteoblasts and adipocytes (Prockop, 1997; Pittenger et al., 1999; Muraglia et al., 2000; Noth et al., 2002a; Verfaillie, 2002; Barry and Murphy, 2004). Especially in bone, hMSCs build a reservoir of cells for remodeling and tissue repair to maintain and regenerate bone (Krane, 2005; Martin and Sims, 2005). However, the age-related increase of adipose tissue in bone marrow and the simultaneous decrease of osteoblasts (Beresford et al., 1992; Koo et al., 1998) may contribute to bone-associated diseases like osteoporosis and osteopenia (Meunier et al., 1971; Burkhardt et al., 1987; Nuttall and Gimble, 2000; Pei and Tontonoz, 2004). (Klein et al., 2004) showed that overexpression of 12/15-lipoxygenase that indirectly stimulates adipogenesis in mice resulted in osteoporosis. Besides the possibility of a decreased capability of hMSCs to differentiate into osteoblasts, transdifferentiation of

committed osteoblasts into adipocytes could at least partly account for the age-related shift towards adipogenic degeneration.

Phenotype switches of committed cell types have been described by various groups (Nuttall et al., 1998; Park et al., 1999) also at the single cell level (Song and Tuan, 2004), but the causative molecular events still remain unknown. During transdifferentiation (or reprogramming), a cell type already committed to and developing along a specific cell lineage switches by genetic reprogramming into another cell type of a different lineage. Some of the molecular factors and processes leading to osteogenic or adipogenic differentiation have been identified and partly empirical cell culture systems have been established.

Initiation and continuation of osteogenesis demands runt-related transcription factor 2 (Runx2) (Ducy et al., 1997; Ducy, 2000; Nakashima and de Crombrughe, 2003) and the transcription factor osterix (Osx) (Nakashima et al., 2002). Post-transcriptional regulation of Runx2 by the mitogen-activated protein kinase (MAPK) pathway includes extracellular signal-regulated kinases 1 and 2 (Erk1 and Erk2) that promote Runx2 activity (Jaiswal et al., 2000; Xiao et al., 2002). Further proteins like bone morphogenetic protein 2 (BMP2) and Δ FBJ murine osteosarcoma viral oncogene homolog B (Δ FosB) enhance osteogenesis and inhibit adipogenesis (Gori et al., 1999; Sabatakos et al., 2000). Osteogenic differentiation is achieved *in vitro* by culturing hMSCs in the presence of β -glycerophosphate and a small amount of dexamethasone. Markers of osteogenesis include increasing expression of alkaline phosphatase (alkaline phosphatase, liver/bone/kidney, ALP) and osteocalcin (bone gamma-carboxyglutamate (gla) protein, OC), mature osteoblasts are characterized by deposition of mineralized matrix (Stein et al., 1996; Aubin, 1998).

Adipogenesis requires the sequential action of C/CAAT enhancer binding proteins (C/EBPs) and peroxisome proliferator-activated receptor γ 2 (PPAR γ 2) (Rosen et al., 2000; MacDougald and Lane, 1995; Tanaka et al., 1997; Wu et al., 1996; Darlington et al., 1998) that induce or enhance expression of other adipocyte-specific genes like acetyl CoA carboxylase, solute carrier family 2 (facilitated glucose transporter), member 4 (SLC2A4; formerly glucose transporter type 4, GLUT4), and fatty acid

binding protein 4 (FABP4; formerly adipocyte P2, aP2) (Spiegelman et al., 1993). *In vitro*, adipogenic differentiation is induced by cultivation of hMSCs in medium supplemented with 3-isobutyl-1-methylxanthine, insulin, dexamethasone and indomethacin (Pittenger et al., 1999; Nuttall et al., 1998; Noth et al., 2002a; Song and Tuan, 2004). Early markers of adipogenic differentiation are elevated quantities of mRNAs coding for lipoprotein lipase (LPL) and PPAR γ 2, whereas the formation of lipid vesicles in the cytoplasm of (pre)adipocytes is a late marker of adipogenesis (Gaskins et al., 1989; Fried et al., 1993; Ailhaud et al., 1991; Tontonoz et al., 1995; Barak et al., 1999; Wu et al., 1999; Rosen et al., 2002).

The established markers of osteogenic and adipogenic differentiation allow for tracing of events during transdifferentiation between these lineages and can be followed by analysis of cell type-specific mRNA amounts and histochemical staining. Using these markers, we established a cell culture system of hMSCs wherein the cells are capable of transdifferentiation from osteoblasts into adipocytes and vice versa. Microarray analysis was used, to systematically investigate the possibly disease-related transdifferentiation of committed osteoblasts into adipocytes and showed a high number of differentially regulated gene products.

Materials and Methods

Unless otherwise stated, chemicals were purchased by SIGMA (Munich, Germany), cell culture media by PAA (Cölbe, Germany), and fetal bovine serum (FBS) by Gibco (Karlsruhe, Germany). The microarray analysis was performed using the Affymetrix kits and Gene Chip HG-U 133A (High Wycombe, United Kingdom).

Isolation and culture of hMSCs

After approval by the Local Ethics Committee of the University of Würzburg and informed consent from each patient (8 patients, 6 females, 2 males; aged 10-76 years), trabecular bone was obtained from the femoral head of patients undergoing total hip arthroplasty. hMSCs were isolated as described previously using a modified protocol (Noth et al., 2002b; Schutze et al., 2005a) originally published by (Haynesworth et al., 1992). Briefly, stromal cells were washed out of the cancellous bone with

propagation medium, seeded at a density of $1.33 \times 10^6/\text{cm}^2$ into cell culture flasks and incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2 . Propagation medium consisted of DMEM/Ham's F-12 (1:1) with L-Glutamine supplemented with 10% heat inactivated FBS, 1 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin (PAA, Cölbe, Germany), and 50 $\mu\text{g}/\text{ml}$ L-ascorbic acid 2-phosphate. Adherent hMSCs were separated from non-adherent blood cells after 3 d of cultivation by washing with PBS (Biochrom, Berlin, Germany) and further on cultured in fresh propagation medium that was replaced every 3-4 d. At confluence (after up to 2 weeks), cells were subcultured after exposure to trypsin/EDTA (PAA, Cölbe, Germany).

(Trans)Differentiation of hMSCs

(Trans)Differentiation experiments were started in passage 1 or 2 when hMSCs had reached confluence. Osteogenic differentiation of hMSCs was performed by incubating the cells in osteogenic differentiation medium consisting of propagation medium additionally supplemented with 10 mM β -glycerophosphate and 100 nM dexamethasone for up to 4 weeks (Jaiswal et al., 1997). Adipogenic differentiation of confluent hMSCs was achieved by incubating the cells in adipogenic differentiation medium consisting of DMEM high glucose with L-Glutamine supplemented with 10% heat-inactivated FBS, 1 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, 1 μM dexamethasone, 100 μM indomethacin, 500 μM 3-isobutyl-1-methylxanthine, and 1 $\mu\text{g}/\text{ml}$ insulin for 2 weeks (Pittenger et al., 1999).

Subsequent to 5, 10 and 14/15 d of osteogenic differentiation, committed osteoblasts were transdifferentiated (reprogrammed) into adipocytes by replacing the osteogenic differentiation medium with adipogenic differentiation medium. Incubation in adipogenic differentiation medium was continued for 2 weeks. Analogously, fully differentiated adipocytes obtained after 2 weeks in adipogenic differentiation medium were transdifferentiated (reprogrammed) into osteoblasts by incubation in osteogenic differentiation medium for 4 weeks.

Differentiation media were renewed every 2-3 d, supplements were always added freshly.

Histochemistry

For histochemical analyses of (trans)differentiation, hMSCs were plated onto Lab-Tek Chamber Slides with 2 wells (Nunc, Wiesbaden, Germany) at a density of 1.5×10^5 cells per well. After one day the hMSCs had reached confluence and (trans)differentiation experiments were started.

Cytoplasmic ALP of differentiating osteoblasts was stained using the Alkaline Phosphatase, Leukocyte Kit 86-C (Sigma, Munich, Germany) according to the manufacturer's instructions. Mineralized extracellular matrix of osteoblasts was visualized by staining for calcium hydrogen phosphate using Alizarin Red S (Chroma-Gesellschaft Schmid & Co., Stuttgart, Germany) as described by (Bodine et al., 1996). Intracellular lipid vesicles of adipogenic monolayer cultures were stained with Oil Red O (Merck, Darmstadt, Germany) as described by (Pittenger et al., 1999).

RNA isolation and semiquantitative reverse transcriptase polymerase chain reaction (RT-PCR)

Total cellular RNA was isolated from cells undergoing transdifferentiation as well as controls. Control RNA probes were obtained from confluent undifferentiated hMSCs at day 0 prior to initiation of differentiation, additional control RNA probes were received from (pre-)differentiated cells (5, 10, 14/15, and 28 d) at the time point of initiation of transdifferentiation and from fully differentiated cells, respectively. Further samples were isolated 14 d and 28 d after initiation of adipogenic and osteogenic transdifferentiation, respectively, and used for semiquantitative RT-PCR.

3 h and 24 h after initiation of transdifferentiation from adipocytes into osteoblasts, RNA samples from transdifferentiated cells and cells continued with normal differentiation (controls) were collected in parallel and applied for semiquantitative RT-PCR as well as for Affymetrix Gene Chip analysis (see next section). Thereby for control samples, osteogenic differentiation medium was freshly added at the same time as transdifferentiation was induced by addition of adipogenic supplements.

RNA isolation was performed using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. cDNA was synthesized from identical amounts of total RNA (1 μ g) using the M-MLV RT, Rnase H(-) Point Mutant with the provided buffer system (Promega, Mannheim,

Germany) according to the manufacturer's instructions. PCR was run using a PTC-200 Peltier thermal cycler (Biozym, Hessisch Oldendorf, Germany) in a volume of 30 μ l containing 1 μ l cDNA for the house keeping gene eukaryotic translation elongation factor 1 alpha 1 (EF1 α), for osteogenic (ALP, OC) and adipogenic (LPL, PPAR γ 2) marker mRNAs and selected gene products (see Tab. 1 for primer sequences). Primer sequences were obtained by using the online software at http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi developed by the Whitehead Institute for Biomedical Research (Rozen and Skaletsky, 2000). Primer oligo-nucleotides were purchased by Operon (Köln, Germany). The PCR reaction mix for each sample consisted of 1.5 units Taq-polymerase and 1 x reaction buffer (Amersham Biosciences, Freiburg, Germany). Final standard assay concentrations were 10 mM Tris-HCl, pH 9.0, 1.5 mM MgCl₂ and 50 mM KCl, 0.3 mM dNTPs plus 5 pmol forward and 5 pmol reverse primer. Additional modified assays contained dimethylsulfoxide (DMSO) and/or higher amounts of MgCl₂ as stated in Tab. 1. The PCR reaction steps were as follows: 3 min at 94°C, 23 - 49 cycles of 94°C for 45 s, 51 - 60°C for 45 s and 72°C for 1 min, with a final 72°C step of 3 min.

To verify the specificity of PCR products, sequence analyses were performed using the Big Dye Terminator v1.1 Cycle Sequencing Kit and ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Darmstadt, Germany) according to the manufacturer's instructions.

Gel electrophoresis and densitometry of differential PCR product intensities was performed as described previously (Schutze et al., 2005b) using the LTF Bio ID software (LTF, Wasserburg, Germany) and normalizing on house keeping mRNA amounts.

Microarray analysis

As described above, 3 h and 24 h after initiation of adipogenic transdifferentiation, total RNA was extracted from transdifferentiated cells and committed osteoblasts (controls) in parallel. As additional step, Trizol Reagent (Invitrogen, Karlsruhe, Germany) was used according to the manufacturer's instruction prior to RNA purification by the RNeasy Mini Kit. The microarray assay was performed according to the Affymetrix GeneChip Expression Analysis Technical Manual (www.affymetrix.com).

For all samples, 10 μ g of biotinylated cRNA was hybridized onto an Affymetrix Gene Chip HG-U 133A containing more than 22,000 25-mer oligonucleotides standing for 18,400 transcripts and 14,500 genes, respectively. Arrays were scanned with the Affymetrix GeneArray 2500 scanner. Gene expression data were obtained using the Affymetrix software Microarray Suite 5.0, GeneChip Operating Software 1.2, and Data Mining Tool 3.0. The gene expression of transdifferentiated cells was compared to the gene expression of committed osteoblasts 3 h and 24 h after initiation of transdifferentiation, respectively. Differentially expressed genes were obtained as follows: only genes with an increase or decrease call in at least one of the two comparisons were selected. Further analysis was only performed for genes that fulfilled following criteria: signal \log_2 ratio < -1.32 or > 1.32 (representing a fold change of more than 2.5), change p-value < 0.001 or > 0.999 , and present call in at least one of the two compared samples.

Further analysis of differentially expressed genes concerning their grouping into gene ontology classes was performed by using the online tools of the Affymetrix NetAffx Analysis Center at <http://www.affymetrix.com/analysis/index.affx> and the Gostat program at <http://gostat.wehi.edu.au> (Beissbarth and Speed, 2004). For Gostat analysis, the Benjamini and Hochberg correction was employed (Benjamini and Hochberg, 1995) and all transcripts on the applied Gene Chip showing at least one P detection call in the two compared samples served as reference. By semiquantitative RT-PCR, the microarray results of 22 gene products selected due to strong regulation and/or their potential functional relevance in transdifferentiation were re-evaluated (see Tab. 1 for primer sequences and RT-PCR conditions).

Results

Differentiation of hMSCs into osteoblasts and adipocytes

As described previously, monolayer cultures of hMSCs incubated with differentiation-specific supplements underwent the osteogenic and adipogenic differentiation processes, respectively (Noth et al., 2002a). Undifferentiated cells lacked expression of adipogenic (LPL, PPAR γ 2) and osteogenic (OC, ALP) markers on the mRNA level (Fig. 1). Semiquantitative RT-PCR showed the appearance of

an ALP mRNA band after 10 d of incubation with osteogenic supplements whose intensity augmented with proceeding differentiation. According to these results on the mRNA level, only few undifferentiated hMSCs stained positive for ALP on the protein level whereas homogenous staining was obtained by progressing osteogenic differentiation (Fig. 3). After 4 weeks, fully differentiated osteoblasts expressed the osteoblast-specific marker genes ALP and OC (Fig. 1) and had formed mineralized extracellular matrix as we previously showed (Schutze et al., 2005b). Whereas ALP expression increased during the osteogenic differentiation process OC expression was highest after 15 d under osteogenic incubation conditions. After 4 weeks of osteogenic incubation, also weak expression of adipogenic markers LPL and PPAR γ 2 was visible. After 14 d of incubation in adipogenic differentiation medium, the adipocyte-specific marker genes LPL and PPAR γ 2 were expressed whereas osteogenic marker mRNAs were not detectable (Fig. 2). Cytoplasmic lipid vesicles characteristic of fully differentiated adipocytes had formed during adipogenic differentiation (Fig. 4).

Adipogenic and osteogenic transdifferentiation

Since fully differentiated osteoblasts obtained after 28 d in osteogenic differentiation medium showed weak expression of adipogenic marker mRNAs, we took committed osteoblasts that were already expressing OC or OC in combination with ALP as osteogenic markers and tested their capability to transdifferentiate. Accordingly, we have arbitrarily chosen three time points during differentiation, namely 5, 10 and 14/15 d of osteogenic pre-differentiation, and incubated these pre-osteoblasts in adipogenic differentiation medium for 2 weeks, i. e. for the same period that is necessary for hMSCs to produce fully differentiated adipocytes. The described treatment resulted in cells with adipogenic phenotype and therefore in adipogenic transdifferentiation. Transdifferentiated cells expressed only small amounts of ALP mRNA and no OC mRNA but showed strong expression of LPL and PPAR γ 2 (Fig. 1). Oil Red O staining of the transdifferentiated monolayer showed homogeneous accumulation of lipids in cytoplasmic vesicles as in normally differentiated, hMSC-derived adipocytes (Fig. 5A and B). Independent of the period of pre-differentiation examined, i. e. 5, 10 and 14/15 d of incubation in osteogenic differentiation medium prior to initiation of adipogenic transdifferentiation, the committed osteoblasts developed an adipogenic phenotype.

To achieve osteogenic transdifferentiation, fully differentiated adipocytes that lacked any osteogenic markers were incubated in osteogenic differentiation medium for a period of 4 weeks as required for normal in vitro osteogenic differentiation of hMSCs. After this 4 week period, the cells showed strong expression of osteoblast markers, i. e. mRNAs for ALP and OC (Fig. 2). Nevertheless, adipogenic marker expression was not abolished, the cells still expressed mRNA for LPL and PPAR γ 2. Histochemical staining showed mineralized matrix of the monolayer typical for fully differentiated osteoblasts, but in single cells lipid vesicles were still visible (Fig. 5C and D).

Microarray analysis during transdifferentiation

Because transdifferentiation was especially efficient taking pre-differentiated osteoblasts, the adipogenic transdifferentiation was assessed by microarray analysis. Committed osteoblasts undergoing transdifferentiation into adipocytes were compared with committed osteoblasts continuing with osteogenic differentiation (Fig. 6). Samples were arbitrarily taken at two time points, 3 h and 24 h after initiation of transdifferentiation to detect genes regulated early during reprogramming of the cells. Microarray analysis revealed 258 regulated transcripts coding for 202 gene products with at least 2.5-fold regulation (see Materials and Methods section) at one or both time points examined (supplementary Tab. 1 and 2). 3 h after initiation of transdifferentiation, 114 transcripts showed differential expression. In particular, 61 transcripts were down regulated and 53 transcripts were up regulated. 24 h after initiation of transdifferentiation, 155 transcripts were detected for differential expression, 88 transcripts showed down regulation, 67 transcripts showed up regulation. The adipogenic markers LPL (3.1- and 3.6-fold in two different transcripts), C/EBP α (10.5-fold), and FABP4 (14.5-fold) showed up regulation 24 h after initiation of transdifferentiation.

Inserting the differentially regulated transcripts into the Affymetrix NetAffx Analysis Center allowed to search for gene ontology (GO) classes. In the GO category of molecular function, regulated genes mostly belonged to the GO classes of binding and catalytic activity, independent of the time point and of the direction of regulation (Fig. 7). Child categories included protein binding, nucleic acid binding or nucleotide binding, and ion binding. A higher number of down regulated genes were also grouped into the class of signal transducer activity 3 h as well as 24 h after initiation of transdifferentiation

(Fig. 7C and D). Up regulated transcripts additionally showed a higher number in the category of transporter activity 24 h after initiation of transdifferentiation (Fig. 7B). Searching for regulated transcripts in the GO class of biological process revealed the classes of cellular process, physiological process, and regulation of biological process for both time points and regulation directions (Fig. 8). Thereby, many transcripts in the class cellular process belonged to the subclass of cell communication. Represented child categories of physiological process were cellular physiological process and metabolism. Down and up regulated genes could also be found in the GO class of development. For further branching into subclasses of GO see Fig. 7 and 8. Many up or down regulated members of the subclasses of cell differentiation, lipid metabolism, and morphogenesis encode transcription or growth factors and signaling molecules (supplementary Tab. 3). While almost all cell differentiation-associated transcripts showed increasing mRNA amounts 3 h after initiation of transdifferentiation, the majority of transcripts of the other subclasses was expressed differentially 24 h after initiation of transdifferentiation.

GOstat analysis of regulated transcripts 3 h after initiation of transdifferentiation revealed significant overrepresentation of genes associated with transcription (Tab. 2). Additionally, a few GO classes of development, metabolism, and signal transduction are enriched in the list of regulated genes at this time point. 24 h after initiation of transdifferentiation a number of cellular processes is overrepresented including communication, cell adhesion, differentiation, morphogenesis and development (Tab. 3). GO classes on molecular function revealed overrepresented transcripts important for signal transduction. Interestingly, the subgroup cellular component revealed overrepresented GO classes containing transcripts for gene products acting extracellularly.

Re-evaluation of microarray data

To verify the data of the microarray concerning differential expression of genes during transdifferentiation, we performed semiquantitative RT-PCR analyses for selected gene products listed in Tab. 1. Gene expression in cells progressing along adipogenic transdifferentiation was compared with gene expression in cells maintained in osteogenic differentiation. RT-PCR reactions were performed for 22 different mRNA species that showed significant regulation according to the

microarray data, i. e. fulfilling the criteria stated in the Materials and Methods section, and could be considered having a potential functional role in the transdifferentiation process. Using the same RNA samples that were taken for microarray analysis, 10 of 11 gene products that should be up or down regulated 3 h after initiation of transdifferentiation according to the microarray data could be confirmed by densitometric evaluation of RT-PCR products (Tab. 4). 24 h hours after initiation of transdifferentiation, 11 of 12 gene products showed the same regulation pattern as predicted by microarray analysis (Tab. 5). Only the regulation of two hypothetical proteins (DKFZp434F0318 and MGC4655) were not in accordance with the microarray data using identical RNA for re-evaluation. Taken together, regulation could be confirmed for 20 of 22 gene products, i. e. accordance of microarray data and semiquantitative RT-PCR was 91%.

Assessing those gene products whose regulation patterns were confirmed in identical RNA samples, the comparison of the microarray data with RNA samples from two additional transdifferentiation experiments showed accordance with the microarray data in 16 of the 20 confirmed genes with accordance of at least one of the two additional RNAs (Tab. 4 and 5). In 9 of the 20 confirmed gene regulations, even RNA samples of both additional transdifferentiation experiments matched the microarray results.

Discussion

Adipocytes and osteoblasts as well as various other mesenchymal lineages originate from multipotential hMSCs (Caplan, 1991; Pittenger et al., 1999; Prockop, 1997; Noth et al., 2002a). In this study, we established a cell culture system of hMSCs that was not only capable of adipogenesis and osteogenesis with the corresponding differentiation markers as described previously (Schutze et al., 2005b) but also showed plasticity between osteoblasts and adipocytes. In this case, plasticity means the phenotype switch of committed osteoblasts and fully differentiated adipocytes into the other cell lineage, respectively, which was achieved by changing the respective differentiation media.

Osteogenic differentiation was accompanied by the increase of known osteogenic markers, i. e. increasing amounts of ALP and OC mRNA as well as calcium hydrogen phosphate deposition in the

extracellular matrix of differentiated osteoblasts (Stein et al., 1996; Aubin, 1998). The weak expression of adipogenic markers in the late stage of osteogenic differentiation could on the one hand result from some pre-osteoblasts spontaneously transdifferentiating into adipocytes. This phenomenon could also contribute to the increase of adipose tissue in human bone marrow observed during aging (Beresford et al., 1992; Koo et al., 1998). On the other hand, even in undifferentiated hMSCs very low expression levels of PPAR γ were detected in further microarray analysis experiments of our group (data not shown) indicating that detection of PPAR γ alone is apparently not sufficient to state an adipogenic phenotype but its adipogenic function is accelerated by inductive factors in the adipogenic medium. We chose a period of 14/15 d for osteogenic pre-differentiation prior to initiation of adipogenic transdifferentiation, because in our cell culture system at this time point high amounts of OC mRNA were observed, ALP mRNA was detected, and staining of ALP protein stretched homogeneously over the monolayer. Thus, these markers indicated a homogenous differentiation into pre-osteoblasts that were devoid of any adipogenic marker expression unlike weakly detected in fully differentiated osteoblasts. Transdifferentiation of committed pre-osteoblasts into adipocytes was characterized by strong expression of adipogenic (PPAR γ 2, LPL) and loss of osteogenic mRNA markers (ALP, OC), thereby excluding significant contribution of undifferentiated hMSCs to the adipogenic phenotype. The marker gene expression in transdifferentiated adipocytes was the same as for normally differentiated hMSC-derived adipocytes, transdifferentiated adipocytes even showed higher amounts of lipid vesicle accumulation than normally differentiated ones. As mentioned before, this phenomenon could also result from some pre-osteoblasts capable of spontaneous adipogenic reprogramming that is accelerated by adipogenic differentiation medium. Strong appearance of adipogenic markers (PPAR γ 2, LPL, cytoplasmic lipid vesicles) (Pittenger et al., 1999; Muraglia et al., 2000; Rosen et al., 2000) was detected after 14 d of adipogenic incubation in our cell culture system (Noth et al., 2002a; Schutze et al., 2005b). Thus, we initiated osteogenic transdifferentiation in these differentiated adipocytes. Transdifferentiated osteoblasts were obtained after 4 weeks of subsequent osteogenic incubation expressing osteoblast markers on the mRNA level (ALP, OC) and staining positive for mineralized extracellular matrix. In contrast to the marker expression after transdifferentiation of pre-osteoblasts into adipocytes, monolayers subjected to osteogenic

transdifferentiation still showed adipocyte markers mRNAs indicating that the transdifferentiation into adipocytes was more efficient than into the other direction.

Since our system does not show events at the single cell level and originates from a heterogeneous population of hMSCs, the lower efficiency of osteogenic transdifferentiation could be caused by cells in the adipocyte monolayer that do not respond to the change of differentiation medium. In this case, only a part of the cells in the monolayer would transdifferentiate into osteoblasts whereas the non-responders would maintain their adipogenic phenotype. Due to strong expression of adipocyte-specific markers and homogeneous distribution of lipid vesicles in the adipogenic monolayer prior to the initiation of transdifferentiation, it seems unlikely that the osteogenic phenotype of the reprogrammed monolayer could be generated solely by possibly undifferentiated hMSCs. Furthermore, the empirical composition of the differentiation media serves well for direct differentiation of hMSCs (Jaiswal et al., 1997; Pittenger et al., 1999; Nuttall et al., 1998; Noth et al., 2002a) but might not be strong and specific enough for osteogenic transdifferentiation of all adipocytes in the monolayer, in particular due to the little difference in the composition of normal culture medium and osteogenic differentiation medium. The fact that for osteogenic differentiation, the inductive components of the osteogenic medium must turn on the complex Wnt-signaling pathway supports this view (Gregory et al., 2005; Rawadi et al., 2003).

In spite of literature questioning the existence of transdifferentiation in vivo and rather tracing back changes in lineage phenotype on cell fusion (Terada et al., 2002; Ying et al., 2002) or heterogeneity (Verfaillie, 2002; Orkin and Zon, 2002), in vitro experiments of other groups reported plasticity of differentiated bone marrow-derived adipocytes (Park et al., 1999) and human trabecular bone-derived osteoblasts (Nuttall et al., 1998). Furthermore, (Song and Tuan, 2004) proved transdifferentiation at the single cell level for hMSC-derived osteoblasts, adipocytes and chondrocytes. As possible mechanisms of transdifferentiation, de-differentiation followed by re-differentiation and direct change of the phenotype have been assumed (Song and Tuan, 2004; Verfaillie, 2002).

The adipogenic switch of osteoblast progenitors might also occur in vivo and could contribute to age-related diseases like osteoporosis and osteopenia that are accompanied by increased adipose tissue and a decreased number of osteoblasts in the bone marrow (Beresford et al., 1992; Koo et al., 1998; Nuttall and Gimble, 2000; Abdallah et al., 2006). The association of the adipogenic conversion with aging indicates a systemic quality of plasticity, but fatty degeneration also occurs locally, e. g. in the first decade of life in the diaphysis of human long bones (Moore and Dawson, 1990; Zawin and Jaramillo, 1993) or independent of age in muscle tissue after trauma in the rotator cuff of the shoulder (Goutallier et al., 1994; Nakagaki et al., 1996).

To elucidate underlying molecular mechanisms of fatty degeneration in bone – especially regarding the initiation of transdifferentiation – we applied Affymetrix Gene Chip analysis to early steps in adipogenic transdifferentiation. The comparison of transdifferentiating pre-osteoblasts developing towards adipocytes with pre-osteoblasts continuing with osteogenic differentiation yielded a high number of regulated genes.

GO analyses provided insight into groups of regulated gene products associated with the same cellular component, molecular function or biological process. Both GO graphs and Gostat tables revealed an enrichment of regulated genes associated with transcription and several members of signal transduction 3 h after initiation of transdifferentiation, of which the majority displayed up regulation. 24 h after initiation of reprogramming, the regulation of extracellular region-associated genes suggested differential regulation of secreted proteins. Besides complex regulation in the GO class of biological process, numerous genes related to signal transduction were found in the GO class of molecular function at this time point, whereas the GO class of transcription was no longer represented. Furthermore, up regulated catalytic and transporter activity-associated transcripts as well as lipid metabolism-correlated gene products appeared 24 h after initiation of transdifferentiation. The reverse regulation pattern of down regulated non-adipogenic gene products (e.g. jagged 1, JAG1 correlated with osteogenesis (Nobta et al., 2005) and angiogenesis (Uyttendaele et al., 2000), CYR61 involved in angiogenesis (Lau and Lam, 1999; Schutze et al., 2005a), muscle-associated tropomyosin 1 (alpha), cysteine and glycine-rich protein 2, CSR2, myosin heavy polypeptide 11 smooth muscle, and villin 2

(ezrin), VIL2 (MacLeod and Gooding, 1988; Louis et al., 1997; Babu et al., 2000; Moyen et al., 2004)) and up regulated adipogenesis-related gene products (C/EBP α , LPL and acetyl-CoA carboxylase β (Rosen et al., 2002; Wu et al., 1999; Fried et al., 1993; Spiegelman et al., 1993), and two glucose transporter molecules SLC2A3 and SLC2A14) could contribute to the switch of pre-osteoblasts into adipocytes during transdifferentiation. Furthermore, the initiation of transdifferentiation could involve other factors and signaling pathways that have not been described in the context of direct differentiation or whose functions have not been revealed so far. Thus, the molecular events of transdifferentiation seem more complex than the regulation of differentiation of hMSCs into either osteoblasts or adipocytes, where the expression of factors of one cell lineage partly inhibits the differentiation into the other lineage (Akune et al., 2004; Nuttall and Gimble, 2004). Re-evaluation of interesting candidates for transdifferentiation by RT-PCR including identical and additional RNA specimens resulted in 91% accordance in identical RNA and 45% accordance in both of two additional RNA isolations of independent experiments. This indicated the reliability and significance of Affymetrix microarray data, but also showed some variance between individual transdifferentiation experiments. Whereas the re-evaluation of up regulated, adipocyte-specific gene products (APM1 and FABP4) seemed reasonable in the process of adipogenic transdifferentiation, the confirmation of the down regulation of DUSP6 that inhibits osteogenesis did not meet our expectations but further supported the reliability of the microarray data.

In conclusion, we established a cell culture system that enables monitoring of the transdifferentiation process. Thereby, reprogramming of committed pre-osteoblasts into adipocytes was as efficient and followed the same kinetics as the direct differentiation of adipocytes from hMSCs. Osteogenic transdifferentiation of fully differentiated adipocytes was less efficient but also yielded expression of osteoblast markers. In addition, reproducible molecular changes associated with the transdifferentiation of mesenchymal stem cell-derived osteoblasts into adipocytes were shown by comparison of microarray and semiquantitative RT-PCR data. Our results suggest that many more molecules apart from established adipogenesis-related factors are involved in the induction propagation and maintenance of transdifferentiation. Functional examination of a selection of highly

regulated candidate gene products should provide further knowledge of the molecular events during transdifferentiation and help elucidate the underlying pathways that initiate the switch between both cell lineages.

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Figure 1. Transdifferentiation of committed osteoblasts into adipocytes

hMSCs were incubated in osteogenic differentiation medium for up to 4 weeks as indicated. Day 0 probes represent confluent, undifferentiated hMSCs. After 5, 10, and 15 d of osteogenic differentiation, cells were reprogrammed by incubation in adipogenic differentiation medium for another 14 d as indicated. RNA samples were isolated at the indicated time points, thereby also controls of undifferentiated hMSCs, fully differentiated osteoblasts (28 d under osteogenic conditions) and adipocytes (14 d of adipogenic incubation) were investigated. RT-PCR was performed for osteogenic marker genes (ALP, OC) as well as adipogenic marker genes (PPAR γ 2, LPL), EF1 α reported the quality of the cDNA as house keeping gene. diff. differentiation

Figure 2. Transdifferentiation of adipocytes into osteoblasts

hMSCs were incubated in adipogenic differentiation medium for 14 d as indicated. Osteogenic incubation was performed for 28 d as indicated. After 14 d of adipogenic differentiation, fully differentiated adipocytes were reprogrammed by incubation in osteogenic differentiation medium for another 28 d. RNA samples were isolated at the indicated time points, thereby also controls of fully differentiated adipocytes (14 d in adipogenic differentiation medium) and osteoblasts (28 d in osteogenic conditions) were investigated. RT-PCR was performed for osteogenic marker genes (ALP, OC) as well as adipogenic marker genes (PPAR γ 2, LPL), EF1 α reported the quality of the cDNA as house keeping gene.

Figure 3. ALP staining during osteogenic differentiation

Staining for cytoplasmic ALP was performed for undifferentiated hMSCs (A) as well as after 7 d (B), 14 d (C) and 28 d (D) of incubation in osteogenic differentiation medium. Scale bar = 100 μ m.

Figure 4. Oil Red O staining during adipogenic differentiation

Oil Red O staining for intracellular lipid vesicles was performed for undifferentiated hMSCs (A) as well as after 7 d (B) and 14 d (C) of incubation in adipogenic differentiation medium. Cell nuclei were counterstained with hemalaun. Scale bar = 100 μ m.

Figure 5. Histochemical staining of (trans)differentiated hMSCs

HMSCs were differentiated or transdifferentiated and stained for lipid vesicles by Oil Red O (A, B) and for mineralized extracellular matrix by Alizarin Red S (C, D), respectively. Control hMSCs were differentiated into adipocytes by incubation in adipogenic differentiation medium for 14 d and stained for lipid vesicles (A). Another group of control hMSCs was differentiated into osteoblasts by incubation in osteogenic differentiation medium for 28 d and stained for mineralized extracellular matrix (C). HMSCs that were pre-differentiated into pre-osteoblasts in osteogenic differentiation medium for 14 d were afterwards subjected to transdifferentiation by incubation in adipogenic differentiation medium for 14 d and stained for lipid vesicles (B). HMSCs that were differentiated into adipocytes after 14 d of incubation in adipogenic differentiation medium were subsequently transdifferentiated by incubation in osteogenic differentiation medium for 28 d and stained for mineralized extracellular matrix (D). Arrows in D indicate residual lipid vesicles, scale bar = 100 μ m.

Figure 6. RNA isolation during transdifferentiation of osteoblast progenitors into adipocytes

Osteoblast progenitors were transdifferentiated by incubation in adipogenic differentiation medium. RNA was extracted 3 h and 24 h after initiation of transdifferentiation. In parallel, osteoblast progenitors continued in osteogenic differentiation provided control RNA probes. The RNA isolations of transdifferentiated cells were compared to the control of the corresponding time point using microarray analysis and RT-PCR.

Figure 7. GO graphs of regulated genes grouped into the GO classes of molecular function

Introduction of the regulated transcripts obtained by microarray analysis into the Affymetrix NetAffx Analysis Center provides classification of these transcripts into different and hierarchical structured GO classes. The GO classes of molecular function are displayed for those categories that contain at least 10 members. The analysis is displayed for up regulated genes 3 h (A) and 24 h (B) after initiation of transdifferentiation as well as for down regulated genes 3 h (C) and 24 h (D) after initiation of transdifferentiation. Each rectangular node contains the name of the GO class and the number of regulated transcripts grouped into this class in parentheses. The color of each node correlates with the number of included transcripts, changing from high numbers in red boxes to low numbers in blue boxes. Inferior GO classes, also called child categories are linked to the parent category by curves.

Figure 8. GO graphs of regulated genes belonging to the GO classes of biological process

Regulated transcripts were grouped into different and hierarchical structured GO classes by introducing them into the Affymetrix NetAffx Analysis Center. GO classes of biological process with at least 10 members are displayed. For A and C, an extract of the results is shown. A and B show up regulated transcripts 3 h and 24 h after initiation of transdifferentiation, respectively. C and D show down regulated gene products 3 h and 24 h after initiation of transdifferentiation, respectively. Each rectangular node displays the name of the GO class and the number of regulated genes grouped into this class in parentheses. The color of each node correlates with the number of included transcripts, changing from high numbers in red boxes to low numbers in blue boxes. Inferior GO classes also called child categories are linked to the parent category by curves.

Table 1. Primer sequences and conditions for RT-PCR

Gene Name	Forward Primer 5'-3' Sequence	Reverse Primer 5'-3' Sequence	Annealing Temperature [°C]	Length of PCR Product [bp]	Annotation ID
house keeping gene:					
eukaryotic translation elongation factor 1 alpha 1 (E1F1 ^a)	AGGT GATTATCCT GAACCATCC	AAAGGTGGATAGTCTGA GAAGC	54	235	NM_001402
marker genes of differentiation:					
alkaline phosphatase, liver/bone/kidney (ALP) ^a	TGGAGCCTCAGAAAGCTCAACACCA	ATCTCGTGTCTGA GTACCA GTCC	51	454	NM_000478
lipoprotein lipase (LPL) ^a	GAGATTTCTCTGTATGGCACC	CTGCAAATGAGACACTTCTC	51	275	NM_000237
osteocalcin = bone gamma-carboxyglutamate (gla) protein (OC) ^a	ATGAGAGCCCTCACACTCCTC	GCCGTAGAAGCCGCCGATAGGC	60	294	NM_199173
peroxisome proliferator-activated receptor gamma 2 (PPARgamma2) ^a	GCTGTTATGGGTGAAACTCTG	ATAAGGTGGAGATGCAAGCTC	51	350	NM_005037, NM_015869, NM_138711, NM_138712
selected gene products for microarray re-evaluation:					
transcriptional regulators					
core promoter element binding protein (COPEB) ^a	CTCATGGGAAGGGTGTGA GT	CAGGATCCACCTCTCTGCTC	55	179	NM_001300
D site of albumin promoter (albumin D-box) binding protein (DBP) ^f	CACCTGGA GTGTGCTGGT GAC	CTTCCGCTCCTTTCTCTC	59	244	NM_001352
Kruppel-like factor 4 (gut) (KLF4) ^a	GCCACCCACACTTGATTAA	ATGTGTAAAGCGGAGGTGGTC	57	245	NM_004235
nuclear receptor subfamily 4, group A, member 2 (NR4A2) ^a	TTTCTGCCTCTCTCTGCATT	TGTGTGCAAAAGGGTACGAA G	55	205	NM_006186, NM_173171, NM_173172, NM_173173
zinc finger protein 331 (ZNF331) ^a	GGCCTGTCTGA ACTCTGCTC	ACGGCCAAAGGGATTTACTTC	55	184	NM_018555
signaling molecules					
cysteine-rich, angiogenic inducer, 61 (CYR61) ^a	CAACCCCTTACAAGGCCAGA	TGGTCTTGCTGCATTTCTTG	55	206	NM_001554
interleukin 8 (IL8) ^a	AAGGAAAAGCTGGGTGCAAG	CCCTACAAAGACCCACACA	57	163	NM_000584
protein kinase C-like 2 (PRKCL2) ^a	ATGATGTCTGTGCTGTTTGAAG	GCCAAATCAGCCAAATAACT	55	150	NM_006256
prostaglandin E receptor 4 (subtype EP4) (PTGER4) ^c	TCACTTACTCATTGCCACTC	TACAGAAAGCAATTCGGATG	58	150	NM_000958
regulator of G-protein signalling 2, 24kDa (RGS2) ^a	AGCTGTCTCAAAAGCAAGG	CCCTTTCTGGCGA GTTGTGA	55	150	NM_002923
regulator of G-protein signalling 4 (RGS4) ^a	AGTCCCAAGCCAAAAGAT	ACGGGTGACCAATCAAGA	55	220	NM_005613
tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin) (TNFRSF11B) ^a	TAAAACGGCAACACAGCTCA	GCCTCAAGTGCCTGAGAAAC	55	568	NM_002546
inhibitor of osteogenesis					
dual specificity phosphatase 6 (DUSP6) ^a	ACA GTGGTCTCTACGACGA	CAGTGA CTGACGGCTAATG	55	190/627 ^g	NM_001946, NM_022652
adipocyte-associated genes					
adipose most abundant gene transcript 1 (APM1) ^a	GCTGGGAGCTGTTCTACTGC	CGATGTCTCCCTTAGGACCA	59	233	NM_004797
fatty acid binding protein 4, adipocyte (FABP4) ^a	AACCTTAGATGGGGTGTCC	ATGCGA ACTCA GTCCAGGT	57	179	NM_001442
solute carrier family 2 (facilitated glucose transporter), member 3 (SLC2A3) ^b	TCGCACTCATTGCACTCTAGC	AAATGGGACCTGCCTTACT	55	167	NM_006931
cell cycle-, cell differentiation- and cell growth-associated genes					
dual specificity tyrosine-(Y)-phosphorylation regulated kinase 2 (DYRK2) ^b	GCCATGTAAACAAGAAACC	TGCCGTCTATGAATGCTGTC	55	255	NM_003583, NM_006482
insulin-like growth factor binding protein 5 (IGFBP5) ^a	GACCGCAAGAAAGAA GCTGAC	GAATCCTTTGCGGTCA CAAT	55	210	NM_000599
putative lymphocyte G0/G1 switch gene (G0S2) ^a	CGTGCACCTAAGGTCATTCC	TGCA CA CA GTCTCCATCA GG	57	186	NM_015714
cytoskeleton-associated genes					
serine/threonine kinase 38 like (STK38L) ^a	GAAAGGCCAGCA GCAATC	GGGATAGAGCCACGTGAGT	55	191	NM_015000
genes for hypothetical proteins					
hypothetical protein DKFZp434F0318 (DKFZp434F0318) ^c	CCAGGGTACTCGGAAAG	AGCAGCA GTCCCTGGAA G	55	136	NM_030817
hypothetical protein MGC4655 (MGC4655) ^d	GTCCCCCTTCTGCCAAC	AGCCCTTGCCTGTTCT	55	175	NM_033309

^a standard PCR mix; ^b 2.25 mM MgCl₂; ^c 3.75 mM MgCl₂; ^d 4% DMSO; ^e 6% DMSO; ^f 3.75 mM MgCl₂ and 8% DMSO; ^g two alternative transcripts of the same gene, the shorter transcript lacks exon

2 of the longer one, in identical RNA samples both transcripts were expressed, in additional RNA samples only the smaller one was detected;

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Table 2. Gostat analysis 3 h after initiation of adipogenic transdifferentiation

GO ID	GO Term	Gene Symbol	Regulated Genes	Genes on Array	Gostat p-Value
<i>biological process</i>					
GO:0050794	regulation of cellular process	STK38L, HOXA1, DUSP6, KLF5, RGS16, TRAF1, NR4A2, PIM1, CX3CL1, KLF7, SOD2, CREM, CBX4, ID4, CRSP9, EGR2, GATA6, RGS2, NEDD9, HMOX1, LIF, ZNF331, HIVEP1, VGLL3, CITED2, HMGA2, SPRY2, CYR61, ZNF217, EGR3, TNFAIP8, TCF8	32	1362	0.000483
GO:0050789	regulation of biological process	STK38L, HOXA1, DUSP6, KLF5, RGS16, TRAF1, NR4A2, PIM1, CX3CL1, KLF7, SOD2, CREM, CBX4, ID4, CRSP9, EGR2, GATA6, RGS2, NEDD9, HMOX1, LIF, ZNF331, HIVEP1, VGLL3, CITED2, HMGA2, SPRY2, CYR61, ZNF217, EGR3, TNFAIP8, TCF8	32	1451	0.000178
GO:0050791	regulation of physiological process	STK38L, HOXA1, DUSP6, KLF5, TRAF1, NR4A2, KLF7, PIM1, CX3CL1, CBX4, CREM, SOD2, EGR2, CRSP9, ID4, GATA6, NEDD9, LIF, HIVEP1, ZNF331, HMGA2, CITED2, VGLL3, CYR61, EGR3, ZNF217, TNFAIP8, TCF8	28	1293	0.00155
GO:0051244	regulation of cellular physiological process	STK38L, HOXA1, DUSP6, KLF5, TRAF1, NR4A2, PIM1, KLF7, CBX4, CREM, SOD2, EGR2, CRSP9, ID4, GATA6, NEDD9, LIF, HIVEP1, ZNF331, HMGA2, CITED2, VGLL3, CYR61, EGR3, ZNF217, TNFAIP8, TCF8	27	1262	0.00253
GO:0045449	regulation of transcription	HOXA1, KLF5, NR4A2, KLF7, SOD2, CBX4, CREM, EGR2, ID4, CRSP9, GATA6, ZNF331, HIVEP1, VGLL3, HMGA2, CITED2, ZNF217, EGR3, TCF8	19	755	0.00339
GO:0006355	regulation of transcription, DNA-dependent	HOXA1, KLF5, NR4A2, KLF7, SOD2, CBX4, CREM, EGR2, ID4, CRSP9, GATA6, ZNF331, HIVEP1, HMGA2, CITED2, ZNF217, EGR3, TCF8	18	711	0.00384
GO:0019219	regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism	HOXA1, KLF5, NR4A2, KLF7, SOD2, CBX4, CREM, EGR2, ID4, CRSP9, GATA6, ZNF331, HIVEP1, VGLL3, HMGA2, CITED2, ZNF217, EGR3, TCF8	19	773	0.00384
GO:0006350	transcription	HOXA1, KLF5, NR4A2, KLF7, SOD2, CBX4, CREM, EGR2, ID4, CRSP9, GATA6, ZNF331, HIVEP1, VGLL3, HMGA2, CITED2, ZNF217, EGR3, TCF8	19	797	0.006
GO:0006351	transcription, DNA-dependent	HOXA1, KLF5, NR4A2, KLF7, SOD2, CBX4, CREM, EGR2, ID4, CRSP9, GATA6, ZNF331, HIVEP1, HMGA2, CITED2, ZNF217, EGR3, TCF8	18	741	0.00635
GO:0007275	development	HOXA1, APOLD1, SLC2A14, PDLIM5, PIM1, EGR2, GATA6, NEDD9, LIF, DACT1, HMGA2, CITED2, SPRY2, CYR61, EGR3, GREM1	16	642	0.0107
GO:0031323	regulation of cellular metabolism	HOXA1, KLF5, NR4A2, KLF7, SOD2, CBX4, CREM, EGR2, ID4, CRSP9, GATA6, ZNF331, HIVEP1, VGLL3, HMGA2, CITED2, ZNF217, EGR3, TCF8	19	844	0.0123
GO:0019222	regulation of metabolism	HOXA1, KLF5, NR4A2, KLF7, SOD2, CBX4, CREM, EGR2, ID4, CRSP9, GATA6, ZNF331, HIVEP1, VGLL3, HMGA2, CITED2, ZNF217, EGR3, TCF8	19	880	0.0229
GO:0007165	signal transduction	PDE4D, STK38L, DUSP6, RGS16, TRAF1, NR4A2, ARL4C, TOP2A, CX3CL1, CREM, RAB20, RGS2, NEDD9, PTGER4, HMOX1, LIF, DACT1, GNAI1, SPRY2, ADM	20	1001	0.0509
<i>molecular function</i>					
GO:0030528	transcription regulator activity	GATA6, CITED2, HOXA1, VGLL3, KLF5, ZNF217, NR4A2, EGR3, TCF8, KLF7, CREM, CBX4, EGR2, CRSP9, ID4	15	485	0.00231
GO:0003700	transcription factor activity	GATA6, CITED2, HOXA1, ZNF217, NR4A2, EGR3, TCF8, KLF7, CREM, EGR2	10	288	0.061
GO:0003677	DNA binding	GATA6, HIVEP1, ZNF331, HMGA2, CITED2, HOXA1, KLF5, EGR3, NR4A2, ZNF217, TCF8, KLF7, TOP2A, CREM, EGR2	15	685	0.1
GO:0043565	sequence-specific DNA-binding	NR4A2, GATA6, TCF8, HMGA2, HOXA1, CREM	6	143	0.1

Gostat analysis was performed for target transcripts that were reliably measured (at least one P detection call in the compared samples) and displayed differential regulation 3 h after initiation of adipogenic transdifferentiation. Thereby, 156 target transcripts were analyzed to all 11548 reliably measured probe sets on the gene chip serving as reference. Only those overrepresented GO classes are listed that comprise at least 5 genes and show a Gostat p-value ≤ 0.1 . For the GO class of cellular component, no relevant overrepresentation was found under the defined criteria. Abbreviation of gene names follows the HUGO gene nomenclature.

Table 3. Gostat analysis 24 h after initiation of adipogenic transdifferentiation

GO ID	GO Term	Gene Symbol	Regulated Genes	Genes on Array	Gostat p-Value
<i>cellular component</i>					
GO:0005576	extracellular region	MMP13, CHL1, IGFBP1, CSPG4, IL32, LPL, IGF1, STC1, TNFRSF11B, MMP1, CXCL12, CYR61, LEP, IGFBP5, CXCL5, IL6, SERPINE1, IL8, ADIPOQ	19	318	0.000000322
GO:0005615	extracellular space	MMP13, IGFBP1, IL32, LPL, CXCL12, LEP, CXCL5, IL6, IL8, MMP1, ADIPOQ	11	128	0.000231
GO:0044421	extracellular region part	MMP13, CHL1, IGFBP1, CSPG4, IL32, LPL, CXCL12, LEP, CXCL5, IL6, IL8, MMP1, ADIPOQ			
<i>biological process</i>					
GO:0050874	organismal physiological process	TPM1, CD97, IL32, EYA1, LPL, IGF1, PDE7B, CYP1B1, AOC2, PLN, CALD1, ADA, KIAA1199, KLF6, OXTR, SLC22A4, CXCL12, SORT1, CXCL5, IL6, SERPINE1, CNN1, IL8	23	518	0.0000296
GO:0007275	development	CHL1, IGFBP1, CSPG4, EYA1, IGF1, CYP1B1, CSRP2, TNFRSF11B, CHGN, CACNB2, MAFB, NEDD9, KLF6, DACT1, TNFRSF12A, CYR61, SORT1, IGFBP5, DSP, CEBPA, IL8, MID1	22	639	0.0126
GO:0048513	organ development	KLF6, CSPG4, IGF1, TNFRSF12A, SORT1, CYP1B1, CSRP2, TNFRSF11B, CEBPA, IL8, CACNB2, MAFB	12	210	0.0126
GO:0007165	signal transduction	CHL1, IGFBP1, CD97, STK38L, CSPG4, IGF1, PDE7B, STC1, ARHGAP29, CLIC3, TNFRSF11B, ARL4C, SLC20A1, RGS4, NEDD9, RAB3B, PLK2, OXTR, DACT1, CXCL12, ANKRD1, LEP, IGFBP5, CXCL5, IL6, CEBPA, IL8, RASL11B, DIRAS3	29	982	0.0126
GO:0006936	muscle contraction	TPM1, CNN1, OXTR, PLN, CALD1	5	39	0.0378
GO:0007154	cell communication	CHL1, IGFBP1, CD97, STK38L, CSPG4, IGF1, PDE7B, STC1, ARHGAP29, CLIC3, TNFRSF11B, ARL4C, SLC20A1, RGS4, NEDD9, RAB3B, PLK2, OXTR, DACT1, CXCL12, ANKRD1, LEP, IGFBP5, CXCL5, IL6, CEBPA, IL8, RASL11B, DIRAS3	29	1051	0.0378
GO:0007155	cell adhesion	NEDD9, CHL1, CD97, NRXN3, IL32, CXCL12, TNFRSF12A, SIRPA, CYR61, IL8	10	207	0.0598
GO:0030154	cell differentiation	CHL1, KLF6, CSPG4, TNFRSF12A, SORT1, CSRP2, DSP, CEBPA, MAFB	9	177	0.0598
GO:0009653	morphogenesis	NEDD9, IGFBP1, CSPG4, EYA1, TNFRSF12A, CYR61, IGFBP5, CSRP2, CHGN, MID1, IL8	11	254	0.0598
GO:0006928	cell motility	TPM1, CD97, IL8, S100P, IGF1, TNFRSF12A, CALD1	7	111	0.0598
GO:0040011	locomotion	TPM1, CD97, IL8, S100P, IGF1, TNFRSF12A, CALD1	7	111	0.0598
GO:0051674	localization of cell	TPM1, CD97, IL8, S100P, IGF1, TNFRSF12A, CALD1	7	111	0.0598
GO:0007267	cell-cell signaling	CD97, CXCL12, PDE7B, STC1, LEP, CXCL5, IL6, IL8	8	149	0.069
<i>molecular function</i>					
GO:0005125	cytokine activity	CXCL5, IL6, TNFRSF11B, IL32, IL8, CXCL12, ADIPOQ	7	65	0.0196
GO:0005102	receptor binding	IL32, CXCL12, IGF1, STC1, LEP, CXCL5, IL6, TNFRSF11B, IL8, ADIPOQ	10	229	0.0976
GO:0004871	signal transducer activity	CD97, EVI2A, CSPG4, MMD, IL32, IGF1, STC1, TNFRSF11B, SLC20A1, RGS4, PLK2, OXTR, CXCL12, TNFRSF12A, SORT1, LEP, CXCL5, IL6, IL8, ADIPOQ	20	695	0.0976

Results of the Gostat analysis for genes showing differential regulation 24 h after initiation of adipogenic transdifferentiation are depicted under the same prerequisites as described for Tab. 2. Thereby, 114 target transcripts were analyzed to all 11617 reliably measured probe sets on the gene chip serving as reference.

Table 4. Gene regulation 3 h after initiation of adipogenic transdifferentiation

Gene Name	Identical RNA probes			Additional RNA probes		Number of Accordances with Microarray Data
	Microarray Analysis	RT-PCR Analysis	Accordance with Microarray Data	1 st Additional RT-PCR Analysis	2 nd Additional RT-PCR Analysis	
cysteine-rich, angiogenic inducer, 61 (CYR61)	-5.2 -4.1	-5.2	++	1.6	-1.8	1
dual specificity phosphatase 6 (DUSP6)	-4.0 -4.8	-7.4	++	-22.1	-8.7	2
hypothetical protein DKFZp434F0318 (DKFZp434F0318)	4.5	-5.5	-	n. r.	n. r.	n. r.
Kruppel-like factor 4 (gut) (KLF4)	2.8	2.8	++	1.5	1.7	2
nuclear receptor subfamily 4, group A, member 2 (NR4A2)	9.6 7.4 9.5	2.9	++	2.2	1.5	2
prostaglandin E receptor 4 (subtype EP4) (PTGER4)	-4.0	-4.2	++	1.1	1.1	0
protein kinase C-like 2 (PRKCL2)	-2.6	-1.5	+	21.5	1.3	0
regulator of G-protein signalling 2, 24kDa (RGS2)	6.1	1.2	+	-1.2	1.4	1
serine/threonine kinase 38 like (STK38L)	-3.8 -3.5	-1.5	+	1.4	-1.4	1
solute carrier family 2 (facilitated glucose transporter), member 3 (SLC2A3)	2.9 3.5 4.7	1.3	+	1.2	1.6	2
zinc finger protein 331 (ZNF331)	7.6	3.0	++	1.2	-3.9	1

Numbers in columns for microarray analysis and RT-PCR analysis indicate the fold change of gene product regulation, positive values correspond to up regulation, negative values correspond to down regulation. Indication of more than 1 value per gene product in the column microarray analysis represents fold changes of different transcripts for the same gene.

Criteria for accordance evaluation between microarray analysis and RT-PCR analysis in identical RNA probes:

+ having the same algebraic sign and a fold change of ≤ -1.2 or ≥ 1.2 in RT-PCR

++ having the same algebraic sign and a fold change of ≤ -2.0 or ≥ 2.0 in RT-PCR

- not meeting the above described criteria

n. r. not re-evaluated

Table 5. Gene regulation 24 h after initiation of adipogenic transdifferentiation

Gene Name	Identical RNA probes			Additional RNA probes		Number of Accordances with Microarray Data
	Microarray Analysis	RT-PCR Analysis	Accordance with Microarray Data	1 st Additional RT-PCR Analysis	2 nd Additional RT-PCR Analysis	
adipose most abundant gene transcript 1 (APM1)	5.7	4.0	++	-4.7	1.3	1
core promoter element binding protein (COPEB)	-3.1 -2.6	-3.8	++	-1.5	-1.2	2
D site of albumin promoter (albumin D-box) binding protein (DBP)	8.3	4.9	++	n. a.	n. a.	n. a.
dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 2 (DYRK2)	-2.7	-1.2	+	1.1	-1.0	0
fatty acid binding protein 4, adipocyte (FABP4)	14.5	1.8	+	9.5	9.4	2
hypothetical protein MGC 4655 (MGC 4655)	-2.8	3.7	-	n. r.	n. r.	n. r.
insulin-like growth factor binding protein 5 (IGFBP5)	3.5 3.5	6.5	++	n. d/c.	1.2	2
interleukin 8 (IL8)	-3.1 -3.6	n. d/s.	++	-2.1	-1.1	1
putative lymphocyte G0/G1 switch gene (G0S2)	9.6	2.1	++	11.8	2.6	2
regulator of G-protein signalling 4 (RGS4)	-7.7 -7.0 -4.1	-3.4	++	-4.0	-2.0	2
serine/threonine kinase 38 like (STK38L)	-3.3 -3.2	-2.7	++	-1.1	-1.7	1
tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin) (TNFRSF11B)	-2.9 -29.4	-7.2	++	8.3	-2.4	1

Numbers in columns indicate the fold change of gene product regulation as indicated for Tab. 4, for criteria of accordance evaluation see description of Tab. 4.

n. a. not available, mRNA could not be detected in examined probes,

n. d./s. not determined, quantitation was not possible due to absence in transdifferentiated sample

n. d./c. not determined, quantitation was not possible due to absence in control



