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BMP-4 treatment of C3H10T1/2 stem cells blocks expression of MMP-3 and MMP-13

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Abstract

Microarray gene expression profiling was used to identify bone morphogenetic protein-4 (BMP-4) responsive factors involved in late stages of adipocyte commitment in C3H10T1/2 cells. The analysis revealed that the matrix metalloproteinase-3 (MMP-3) gene decreased 100-fold after BMP-4 treatment, and expression of MMP-13 decreased 19.5-fold. Uncommitted C3H10T1/2 cells exhibit dramatic up-regulation of MMP-3 and MMP-13 genes as cells become confluent. Real-time RT-PCR demonstrated that BMP-4 blocks expression of both transcripts. Likewise, a stable committed preadipocyte line derived from C3H10T1/2 cells did not express MMP-3 or MMP-13 at confluence, despite never receiving BMP-4. Active forms of both proteins were detected in media from confluent C3H10T1/2 cells but not in BMP-4 treated cells. Addition of BMP-4 to confluent C3H10T1/2 cells repressed the expression of both genes but did not induce adipocyte differentiation. The findings indicate that BMP-4-induced down-regulation of MMP-3 and MMP-13 is associated with commitment, but is insufficient to induce adipogenesis.

Keywords: Adipogenesis; Bone morphogenetic protein-4; C3H10T1/2; Commitment; Extracellular matrix; Matrix metalloproteinase; Stem cell; Preadipocyte

Adipocytes store energy as triacylglycerol during periods when energy intake exceeds expenditure and utilize this stored lipid as free fatty acids when energy is required. Obesity results when caloric intake persistently exceeds energy expenditure, leading to adipocyte hypertrophy and hyperplasia, due to the recruitment of stem cells and subsequent differentiation of stromal-vascular preadipocytes. The stromal-vascular preadipocyte lineage arises from a multipotent stem cell population of mesodermal origin. These mesenchymal stem cells have the capacity to commit into several distinct cell types, including preadipocytes, myoblasts, osteoblasts, and chondrocytes. Programming to the adipose lineage is a multi-step process comprised of an initial commitment step in which cells become restricted to the adipocyte lineage but do not yet express markers of terminal differentiation, and subsequent activation of a network of transcription factors resulting in the adipocyte phenotype [1]. Factors have been identified that lead to the commitment of mesenchymal stem cells to the adipose lineage *ex vivo*, but the molecular mechanisms by which these pathways are regulated have not been elucidated.

Most of our understanding of adipocyte differentiation has been gained using the 3T3 preadipocyte cell lines [1–4]. However, to understand the processes that occur during adipocyte commitment, a multipotent stem cell line is needed. The C3H10T1/2 (10T1/2) stem cell line was originally isolated from C3H mouse embryos [5] and behaves similarly to mesenchymal stem cells, thus making this cell line ideal for studying factors involved in the adipocyte commitment process.

Bone morphogenetic proteins (BMPs) are members of the transforming growth factor-β superfamily. These proteins bind to serine/threonine kinase receptors and transmit signals to the nucleus through phosphorylation of Smad

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proteins [6]. Studies have demonstrated a role for BMP-2 and BMP-4 in the commitment of mesenchymal stem cells to the adipose, cartilage or bone lineages, depending on culture conditions [7–12]. Notably, BMP-4 treatment of 10T1/2 cells induces nearly complete commitment and subsequent differentiation to the adipocyte lineage [8,11]. This phenomenon should be beneficial in unraveling the processes involved in adipose lineage commitment.

The extracellular matrix (ECM) plays an important role in cell survival, migration, and proliferation, as well as the changes in cell shape that occur during the differentiation of various cell types [13]. Indeed, the ECM undergoes a dramatic transformation throughout adipocyte differentiation [14,15], and studies have demonstrated the importance of both cell shape and ECM remodeling during the course of adipose commitment and development [16–19]. Matrix metalloproteinases (MMPs) represent a family of secreted or transmembrane-associated zinc endopeptidases that degrade ECM components and non-matrix proteins. Most MMPs are secreted from cells as zymogens, requiring cleavage of a propeptide sequence for activation. Plasmin, urokinase-type plasminogen activator, tissue-type plasminogen activator, and furin are known to initiate activation of many MMPs [20,21]. Substrate specificity for each MMP has not been fully characterized and for most of the MMPs, the substrate specificity *in vivo* has not yet been defined [22].

In this study, we utilized microarray gene expression profiling to characterize differences between uncommitted 10T1/2 cells and those that have been committed by BMP-4 with the goal of discovering adipocyte commitment factors. A total of 601 unique genes that changed at least 2-fold with BMP-4 treatment were identified, 19 of which were genes whose products are associated with the ECM. Because ECM remodeling is vital to adipocyte differentiation, we focused our study on two MMPs, MMP-3 and MMP-13, which changed dramatically with BMP-4 exposure. Although the expression patterns of MMP-3 and MMP-13 in adipocyte differentiation have been studied [23–25], the functions of these MMPs during adipocyte commitment have not been examined. This paper describes the characterization of MMP-3 and MMP-13 during BMP-4 commitment of 10T1/2 cells and proposes a role for these two enzymes during the adipocyte commitment process.

Materials and methods

Cell culture. A33 and 3T3-L1 cells were propagated and differentiated as described [8,26]. The 10T1/2 cell line (American Type Culture Collection, Manassas, VA) was maintained in Dulbecco's modified Eagle's media (DMEM) supplemented with 10% calf serum, and antibiotics. To commit the cells to adipocytes, 10T1/2 cells were plated at a density of 8×10^4 cells/6-cm dish, and 50 ng/ml BMP-4 (R&D Systems, Minneapolis, MN) was added two days after plating and replenished two days later. Once the cells reached postconfluence, the media was switched to DMEM, 10% fetal calf serum and adipocyte differentiation inducers were added [26].

Microarray gene expression profiling. Total RNA from uncommitted and BMP-4-committed 10T1/2 cells was harvested at postconfluence with the RNeasy Mini Kit (Qiagen, Valencia, CA) and was processed according to Affymetrix specifications. RNA from both sets was analyzed in triplicate. Briefly, 5 μg total RNA was used to synthesize first strand cDNA using oligonucleotide probes with 24 oligo-dT plus T7 promoter as primer and the SuperScript Choice System (Invitrogen, Carlsbad, CA). Following the double-stranded cDNA synthesis, the product was purified by phenol–chloroform extraction, and biotinylated anti-sense cRNA was generated through *in vitro* transcription using the BioArray RNA High Yield Transcript Labeling Kit (ENZO Life Sciences Inc., Farmingdale, NY). Fifteen micrograms of biotinylated cRNA was fragmented at 94 °C for 35 min and 10 μg total fragmented cRNA was hybridized to the Affymetrix murine genome GeneChip array (MOE430 2.0; Santa Clara, CA) for 16 h at 45 °C with constant rotation. Affymetrix Fluidics Station 450 was used to wash and stain the chips, removing the non-hybridized target and incubating with a streptavidin–phycoerythrin conjugate to stain the biotinylated cRNA. The staining was amplified using goat IgG as blocking reagent and biotinylated anti-streptavidin antibody, followed by a second staining step with a streptavidin–phycoerythrin conjugate. Fluorescence was detected with the Affymetrix GS3000 GeneArray Scanner, and image analysis of each GeneChip was done through the GeneChip Operating System software from Affymetrix (GCOS1.3) using default settings. For comparison between different chips, global scaling was used, scaling all probe sets to a user defined target intensity of 150.

Method of microarray analysis. To estimate the gene expression signals, data analysis was conducted on the chips' CEL file probe signal values at the Affymetrix probe pair (perfect match probe and mismatch probe) level, using the statistical technique GCRMA [27] with the bioconductor package gcrma. This probe level data processing includes a normalization procedure utilizing the quantile normalization method [28] to reduce the obscuring variation between microarrays, which might be introduced during the processes of sample preparation, manufacture, fluorescence labeling, hybridization, and/or scanning. With the signal estimates, principle component analysis was performed with *R* function *prcomp* to assess sample variability. Exploratory data analysis was performed on the signal estimates to narrow down the genes of interest in terms of evidence for differential expression. A volcano plot was made with the *p*-values from *t*-tests between the control and treated samples versus their fold changes on the base 2 logarithm. All computations were performed under *R* environment. The bioconductor package is available at www.bioconductor.org.

Real-time RT-PCR. All real-time RT-PCR experiments were performed with the Mx3000P Quantitative PCR System (Stratagene, La Jolla, CA), as described [29]. The sequences of the primers were: 18S forward primer, 5′-tggttgatcctgccagtag-3′; 18S reverse primer, 3′-tcaataccaaggaaaccagc-5′; MMP-3 forward primer, 5′-acatggagactttgtccctttg-3′; MMP-3 reverse primer, 3′-ccctgagatggtgagtcggtt-5′; MMP-13 forward primer, 5′-ttagaggtgactggcaaact-3′; MMP-13 reverse primer, 3′-ggtgacttaaagtggtctta-5′. Data analysis was performed with Mx3000P software. Standard curves were used to determine RNA levels, and values were normalized to 18S rRNA amounts. RNA samples were analyzed in triplicate.

Western blot analysis.To follow MMP-3 and MMP-13 protein levels after BMP-4 treatment, 10T1/2 cells were plated and treated or not with BMP-4 as described above. Once the cells reached postconfluence, monolayers were rinsed and incubated for 20 h in serum-free media. Conditioned media were collected and concentrated by centrifugation through Centriprep YM-10 membranes (Millipore, Bedford, MA). Aliquots were subjected to SDS–PAGE using 10% polyacrylamide gels and immunoblotted with antibodies against MMP-3 (R&D Systems) and MMP-13 (Calbiochem, San Diego, CA).

Substrate zymography. Conditioned media from postconfluent 10T1/2 cells treated or not with BMP-4 was prepared as described above. Aliquots were electrophoresed under non-reducing conditions on 10% SDS–PAGE gels containing 0.1% casein or gelatin. Gels were washed in 2.5% Triton X-100 to facilitate protein renaturation and then were incubated for 18 h in a development buffer (50 mM Tris–HCl, pH 7.4, 5 mM $CaCl_2$). They were stained with 0.5% Coomassie Brilliant Blue R-250. The presence of MMP activity was visualized as unstained areas on a blue background. Migration of proteins was compared with that of pre-stained molecular weight markers.