Inhibition of metastasis-associated gene 1 expression affects proliferation and osteogenic differentiation of immortalized human mesenchymal stem cells

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Abstract

Objectives: MTA1 is known to be responsible for independent nucleosome remodelling and deacetylase complexes with ability to regulate divergent cellular pathways. However, additional biological functions have, up to now, remained largely unexplored. The present study was initiated to investigate involvement of MTA1 in osteogenic differentiation of immortalized human mesenchymal stem cells (MSCs).

Materials and methods: MSCs were examined for expression of *MTA1* and stably transfected clones expressing shRNA to *MTA1* were generated. Cells were grown under osteogenic and non-osteogenic conditions. Effects of silencing on cell proliferation, calcium deposition and alkaline phosphatase (ALP) activity were studied. mRNA expression of bone sialoprotein (BSP), osteopontin (OSP), runt-related transcription factor 2 (Runx2), osteocalcin (OC), collagen type I (Col1A) and ALP were analysed.

Results: Transfected cells showed reduction in proliferation and significant increase in calcium deposition and expression of osteogenic marker genes, BSP, OSP, Runx2, OC and Col1A, when they were grown under osteogenic conditions. Under non-osteogenic conditions, expression of BSP and OSP were also markedly upregulated, whereas expression of osteogenic marker genes, *Runx2*, *OC* and *Col1A*, was almost unaffected. Expression of ALP was slightly suppressed under non-osteogenic conditions but significantly increased under osteogenic differ-

entiation conditions, as assessed by enzyme activity and mRNA expression assays.

Conclusions: Our data collectively suggest that endogenously produced MTA1 constrains osteogenic differentiation of MSCs and that targeting of this molecule may provide a novel strategy for enhancing bone regeneration.

Introduction

With aging, bone mass tends to decrease, resulting in onset of many diseases associated with bone mass leakage. For example, osteoporosis, characterized by low bone mass and structural deterioration of bone tissue with increased susceptibility to fractures, is a major public health threat to elderly people (1,2). Maintenance of stable bone mass during adult life, following rapid skeletal growth during childhood, is the result of carefully controlled balance between activities of bone forming (osteoblast) and bone resorbing (osteoclast) cells. Osteoblasts are derived from precursor mesenchymal stem cells (MSCs) that reside in the bone marrow and play key roles in bone homeostasis, because they actively proliferate, migrate and undergo osteogenic, chondrogenic or adipogenic differentiation in response to different stimuli (3–5). For example, bone morphogenic protein-2 and -7, members of the TGF- β superfamily, promote osteoblast differentiation (6,7). Because of their pluripotency, MSCs have shown great potential in cell-based therapies for regeneration of mesenchymal tissues (8–11). Attractiveness of MSCs for therapeutic applications is further strengthened by the fact that these cells can be easily isolated and expanded from a variety of tissues, including adipose tissue (12), skin (13) and bone marrow (14,15), thereby maintaining pluripotency after prolonged *in vitro* culture. MSCs are specially interesting in the context of bone tissue engineering applications because these progenitor cells can be easily differentiated into bone-forming

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osteoblasts and promote osteogenesis upon implantation. However, studies have shown that MSCs undergo senescence-associated growth arrest under current culture conditions, a phenomenon termed replicative senescence (16). Hence, several groups have tried to overcome this hurdle by introducing the gene that codes for human telomerase reverse transcriptase (hTERT) under the control of a constitutive promoter, into MSCs. In human MSCs, ectopic expression of hTERT has abolished senescence-associated phenotype and maintained MSC function including unlimited proliferative capacity and ability to differentiate into multiple cell lineages *in vitro* and *in vivo* (17–21).

Nucleosome complexes formed by histone protein and associated DNA are the fundamental units of eukaryotic chromatin (22). Dynamic changes in chromatin architecture include several modifications of histone proteins such as acetylation, phosphorylation, ubiquitination and methylation, which are closely linked to regulation of eukaryotic gene expression (23–25). For instance, histone acetylation contributes to formation of a transcriptionally competent environment by relaxing chromatin structure and allowing general transcription factors to access target DNA sequences (26,27). On the contrary, histone deacetylation makes chromatin structure compact and leads to transcriptional repression. Transcription activators are often associated with histone acetyltransferases (HATs) to increase target gene expression, whereas transcription repressors frequently interact with histone deacetylases (HDACs) to downregulate target gene expression (22,28).

One family of chromatin modifiers that is ubiquitously expressed is that of metastasis-associated proteins (MTA), which are integral parts of nucleosome remodelling and histone deacetylation (NuRD) complexes. *MTA1*, founding member of *MTA* family of genes, was originally identified through differential screening of a cDNA library from rat metastatic breast tumours (29). Subsequent studies indicated that the 80 kDa peptide, part of the NuRD, complex could enhance the aggressive course of several carcinomas by recruiting histone deacetylase and changing status of chromatin remodelling (30,31). Besides strong correlation between *MTA1* upregulation and cancer, growing evidence strongly suggests that *MTA1* could regulate divergent cell pathways by modifying acetylation status of crucial target genes under both pathological and physiological statuses (32). For example, inactivation of *MTA1* homologues *egl-27* and *egr-1* (also known as *lin-40*) in *Caenorhabditis elegans*, leads to abnormal patterning of cells in the embryo, and forced overexpression of *MTA1* in mouse mammary gland epithelium was accompanied by extensive ductal branching and proliferation in virgin glands, suggesting a role for *MTA1* in both embryonic development and mammary gland development

(33,34). However, physiological characteristics of *MTA1* have still been poorly addressed.

In skeletal biology, HDACs have been involved as negative regulators of chondrocyte hypertrophy by binding and inhibiting activity of runt-related transcription factor 2 (*Runx2*) (35). Furthermore, binding of HDAC3 to *Runx2* leads to inhibition of osteogenesis (36). In agreement with this, two groups have recently described that HDAC inhibitors stimulate mineralization phase of mouse MC3T3 and human MSCs (37,38). Recently, functional consequence of HDAC1/2 and *MTA1*/2-containing complex NODE (for Nanog and Oct4 associated deacetylase) on embryonic stem (ES) cell differentiation, has also been identified (39). As HDACs are important modulators of osteogenic differentiation of MSCs, we speculate that *MTA1* has a certain distinct regulatory role in MSCs. Thus, the present study was initiated to investigate potential involvement of *MTA1* in osteogenic differentiation of MSCs. *MTA1* expression level was first explored in immortalized human MSCs and possible participation of *MTA1* in MSC proliferation and osteogenic differentiation was examined. Our combined analysis sheds light on biological relevance of this newly characterized molecule.

Materials and methods

Cell culture

In this study, immortalized hTERT-overexpressing human MSCs were used (18). Cells were cultured in alpha-MEM medium (Gibco-BRL, Eggenstein, Germany) supplemented with 10% foetal calf serum (FCS; Biochrom AG, Berlin, Germany), 50 µg/ml gentamicin (Sigma-Aldrich, Deisenhofen, Germany) under 5% CO₂ at 37 °C (non-osteogenic conditions). For osteogenic differentiation, MSCs were cultured in osteogenic differentiation medium (DMEM supplemented with 10% FCS, 50 µg/ml gentamicin, 10 mM β-glycerolphosphate, 0.1 µM dexamethasone and 50 µM ascorbic acid) for up to 14 days, with media change three times a week.

Stable transfection and clone selection

pSilencer3.1-*MTA1*-short hairpin RNA (shRNA) and pSilencer3.1-Scramble-shRNA plasmids (40) were transfected into MSCs using Amaxa nucleofection kit (Lonza, Basel, Switzerland) and programme U-023. Transfected cells were selected with 200 µg/ml of G418 (Sigma-Aldrich). Stably transfected MSCs were allowed to proliferate under selection pressure. G418-resistant monoclonal cells were pooled together and amplified under non-osteogenic conditions. At 90% confluence, *MTA1* gene expression was verified by quantitative real-time RT-PCR and

western blot analysis. Transfected clones were grown under both non-osteogenic and osteogenic conditions and expression of *MTA1* was studied by quantitative real-time RT-PCR at different time intervals.

Western blot analysis

Total protein was isolated from scramble shRNA and *MTA1*-shRNA clones using lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.25% Na-deoxycholate, 1% NP-40, 1 mM phenylmethane sulphonylfluoride, 1 mM sodium orthovanadate, 1 µg/ml leupeptin, 1 µg/ml aprotinin and 1 µg/ml pepstatin). Equivalent amounts (20 µg) of protein were separated on 7.5% sodium dodecyl sulphate-polyacrylamide gel electrophoresis gels and subsequently transferred to Hybond enhanced chemiluminescence (ECL) nitrocellulose membranes. Membranes were probed with anti-*MTA1* antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA). To confirm equal loading of gels, membranes were stripped and re-probed with a monoclonal antibody against beta-actin (Sigma). Visualization of protein bands was accomplished using ECL (GE Healthcare, Munich, Germany) and captured on Hyperfilm ECL (GE Healthcare).

Cell proliferation assay

Cells were seeded in six-well plates (1.5×10^4 cells/well) in alpha-MEM containing 10% FCS and 50 µg/ml gentamicin and incubated at 37 °C in humidified atmosphere of 5% CO₂ for 21 days. Medium was changed three times a week. Cell counting was performed by CASY1 (Innovatis, Reutlingen, Germany) at indicated time points.

MTS assay

Effect of gene silencing of *MTA1* on cell proliferation was examined by CellTiter 96 Aqueous one solution (Promega, Heidelberg, Germany) according to manufacturer's instructions. Briefly, 1×10^4 cells were seeded in triplicate in 96-well plates and incubated at 37 °C in 5% CO₂ atmosphere for 24, 48 and 72 h. Twenty microlitres of MTS solution was added per well and plates were incubated for 2 h at 37 °C. Formazan synthesis in each well was measured by absorbance at 490 nm (SpectraFluor-Plus; Tecan, Crailsheim, Germany).

Cytochemical staining

MSCs were seeded in 24-well plates (6×10^4 cells/well) in osteogenic as well as in non-osteogenic differentiation media and cultured for 14 days. Medium was changed three times per week. Mineral deposition was assessed by

von Kossa staining after 14 days. Monolayers in 24-well plates were washed in PBS and fixed in 4% formalin, then treated with 5% silver nitrate. After that, cells were exposed to UV light for 1 h, 5% thiosulphate was added and cells were placed at room temperature after washing step in distilled water. Mineral deposition was also assessed by staining in alizarin-red after 14 days. Cells were washed once in PBS and fixed in phosphate-buffered formalin for 20 min. Fixed cells were washed in PBS and subsequently stained in 1% alizarin-red S (Sigma) dissolved in distilled water, for 15 min. Remaining dye was washed out with distilled water, and cells were washed once more and air-dried. Images of stained cells were captured using a light microscope and appropriate image analysing software (Leica, Solms, Germany). Stained plates were photographed using a digital camera.

Calcium content assay

MSCs were seeded in 24-well plates (6×10^4 cells/well) in osteogenic as well as in non-osteogenic differentiation media and cultured for 14 days. Medium was changed three times per week and mineralized matrix was quantified by measuring the Ca²⁺-levels. Calcium content assay was performed by dissolving crystals in 0.4 ml of 0.5 N acetic acid overnight, and quantifying them using a calcium assessment kit (BioAssay System, Hayward, CA, USA) according to manufacturer's instructions. Calcium levels were normalized to total protein content using BCA protein assay kit (Thermo Scientific, Rockford, IL, USA).

Alkaline phosphatase enzyme activity assay

Experiments were performed in 12-well (5×10^4 cells/well) plates as described previously (41). MSCs were seeded in osteogenic as well as in non-osteogenic differentiation media and cultured for 3, 6, 9 and 14 days. Cell lysates were prepared with 300 µl assay buffer containing 25 mM Tris-HCl (pH 8.5) and 0.5% Triton X-100. Twenty microlitres of each sample was mixed with 100 µl of CSPD substrate (Applied Biosystems, Foster City, CA, USA) and incubated at 37 °C for 30 min. Light output was measured as relative luminescence units (RLU) using a plate luminometer (SpectraFluorPlus; Tecan). Cell alkaline phosphatase (ALP) activities were normalized to total protein content using BCA protein assay kit (Thermo Scientific).

Quantitative real-time RT-PCR

TaqMan RT-PCR was carried out as described previously (42). MSCs were seeded in six-well plates (2×10^5 cells/well) in osteogenic and non-osteogenic differentiation

media, and total RNA was prepared at indicated time points, using RNeasy Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. Total RNA (3 µg) was treated with 3 U of deoxyribonuclease I (DNase I; Invitrogen, Karlsruhe, Germany) to digest genomic DNA contamination. Random-primed cDNA synthesis was performed using 3 µg of DNase I-treated total RNA and 1 µl of AffinityScript reverse transcriptase according to the manufacturer's instructions (Stratagene, La Jolla, CA, USA). TaqMan PCR assays were performed in 384-well optical plates on LightCycler (Roche, Mannheim, Germany) using Absolute QPCR ROX Mix (Abgene, Hamburg, Germany) according to manufacturer's instructions. Oligonucleotide primers and probes for human GAPDH (GAPDH forward: 5'-TGGGCTACACTGAGCACCAG-3'; GAPDH reverse: 5'-CAGCGTCAAAGGTGGAGGAG-3', GAPDH probe: 5'-FAM-TCTCCTCTGACTTCAACAGCGACACCC-TAMRA-3') and human osteocalcin (OC forward:

5'-TGAGCTCAATCCGGACTGTG-3', OC reverse: 5'-GCCGTAGAAGCGCCGATAG-3', OC probe: 5'-FAM-CGAGTTGGCTGACCACATCGGCTT-TAMRA-3') were designed using Primer Express software (Applied Biosystems) according to company guidelines. Oligonucleotide primers and TaqMan probes for human MTA1 (cat.-no.: Hs00183042_m1), ALP (cat.-no.: Hs01029144_m1), Runx2 (cat.-no.: Hs00231692_m1), osteopontin (OSP; cat.-no.: Hs00959006_g1), collagen type I (Col1A; cat.-no.: Hs01076765_g1) and bone sialoprotein (BSP; cat.-no.: Hs00913377_m1) were purchased from Applied Biosystems. Thermal cycling conditions were 95 °C for 15 min followed by 50 cycles at 95 °C for 15 s and at 60 °C for 1 min. Data were analysed using the relative standard curve method, with each sample being normalized to GAPDH to correct for differences in RNA quality and quantity. Results from three independent experiments, performed in triplicates, are expressed as mean arbitrary units $\pm$ SD.

Figure 1. Silencing MTA1 by shRNA in immortalized MSCs. (a) Quantitative real-time RT-PCR of MSCs stably transfected with the scrambled (SCR)-shRNA or MTA1-shRNA under non-osteogenic conditions. mRNA expression levels were normalized to GAPDH. Results are expressed as mean arbitrary units $\pm$ SD ($n = 3$). Asterisks represent statistically significant differences between the groups (** $P < 0.005$, *** $P < 0.0005$). (b) Western blot analysis of wild-type (wt) MSCs and MSCs stably transfected with SCR-shRNA or MTA1-shRNA under non-osteogenic conditions. Cell proteins (20 µg/lane) were separated by 7.5% SDS-PAGE, blotted on nitrocellulose membranes and probed with an anti-MTA1 antibody. Equal loading of the gel was confirmed by re-probing the membrane with antibody to beta-actin. (c) MTA1 expression levels were studied in transfected cells under non-osteogenic conditions for indicated time periods by quantitative real-time RT-PCR. (d) MTA1 expression levels were studied in transfected cells under osteogenic conditions for indicated time periods by quantitative real-time RT-PCR. Results are expressed as mean arbitrary units $\pm$ SD ($n = 3$). Asterisks represent statistically significant differences between the groups (** $P < 0.005$, *** $P < 0.0005$).

Statistical analysis

Statistically significant differences between groups were determined using an unpaired Student's *t*-test. Statistical significance was defined when $P < 0.05$.

Results

Silencing *MTA1* gene in MSCs by shRNA transfection

To determine functional significance of *MTA1* on osteogenic differentiation, endogenous expression was suppressed in MSCs using shRNA interference. *MTA1*-shRNA and scrambled (SCR) shRNA containing plasmid pSilencer 3.1 was stably transfected into human immortalized MSCs. Transfected MSCs showed clear reduction in expression level of *MTA1* compared to MSCs transfected with SCR, at RNA level in quantitative real-time PCR under non-osteogenic conditions (Fig. 1a). *MTA1* protein level in MSCs expressing *MTA1*-shRNA was also reduced in comparison to SCR-shRNA-transfected and wild-type MSCs under non-osteogenic conditions (Fig. 1b). To confirm effective gene silencing of *MTA1*, quantitative real-time RT-PCR under non-osteogenic (Fig. 1c) and osteogenic conditions (Fig. 1d) was performed for indicated time periods. Results clearly show that silencing *MTA1* gene expression was effectively maintained.

Effect of *MTA1* gene silencing on MSC proliferation

To investigate effects of *MTA1* gene knockout on proliferation, MSCs were plated in non-osteogenic growth medium containing 10% FCS. We observed drastic changes in cell proliferation of *MTA1*-shRNA-transfected MSCs, as shown in Fig. 2a. There was significant decrease in cell number of *MTA1*-shRNA-transfected MSCs ($6.5 \times 10^4 \pm 0.37$) in comparison to SCR-shRNA-transfected ($75 \times 10^4 \pm 2.16$) and wild-type ($91.3 \times 10^4 \pm 2.49$) MSCs on day 21. In the context of the MTS cell proliferation assay, *MTA1*-shRNA-transfected MSCs (0.92 ± 0.02) also showed reduced proliferation rate in comparison to SCR-shRNA-transfected (1.6 ± 0.181) and wild-type (1.8 ± 0.15) MSCs at 72 h (Fig. 2b).

Effect of *MTA1* gene silencing on osteogenic differentiation of MSCs

To evaluate effects of *MTA1* gene silencing on osteogenic differentiation, MSCs were grown either under osteogenic growth conditions or under non-osteogenic differentiation medium, for 14 days. For assessment of mineralized-matrix production, MSCs were stained with von Kossa's stain (Fig. 3a) or alizarin-red (Fig. 3b). In addition, extra-

Figure 2. Effect of *MTA1* gene silencing on proliferation of MSCs. (a) Wild-type (wt) MSCs and MSCs transfected with scrambled (SCR)-shRNA or *MTA1*-shRNA were plated in six-well plates and incubated for indicated time periods in growth medium containing 10% FCS. Results shown are mean $\pm$ SD ($n = 3$). (b) MSCs (wild type and transfected) were seeded in 96-well plates in growth medium for indicated time points. Proliferative activities were quantified by MTS assay. Result shown are mean $\pm$ SD ($n = 3$). Asterisks represent statistically significant differences between the groups (* $P < 0.05$; ** $P < 0.005$, *** $P < 0.0005$).

cellular Ca^{2+} was quantitatively measured by a Ca^{2+} assay (Fig. 4a,b). As expected, in all the three mineralization assays, mineralized-matrix production was strongly increased on incubation of immortalized MSCs under osteogenic differentiation conditions. Results of staining indicated a significant increase in mineralized-matrix production in *MTA1*-shRNA-transfected cells in comparison to wild-type and SCR-shRNA-transfected MSCs, when grown under osteogenic differentiation conditions (Fig. 3a,b).

Quantification of extracellular Ca^{2+} revealed moderate, but statistically significant, increase in Ca^{2+} deposition in

Figure 3. Effect of *MTA1* gene silencing on mineralized-matrix production of MSCs. MSCs (wild type and transfected) were seeded in 24-well plates and grown either under osteogenic growth conditions or non-osteogenic differentiation medium for 14 days. Medium was changed three times per week. Cells were stained with (a) von Kossa and (b) alizarin-red reagents. Images of stained cells by different staining procedures were obtained by light microscopy at 100× magnification. Macroscopic images from stained plates were also photographed using a digital camera.

Figure 4. Effect of *MTA1* gene silencing on extracellular Ca^{2+} formation. Wild-type (wt) MSCs and MSCs stably transfected with scrambled (SCR)-shRNA or *MTA1*-shRNA were seeded in 24-well plates and grown either under (a) non-osteogenic growth conditions or in (b) osteogenic differentiation medium for 14 days. At day 14, extracellular Ca^{2+} was measured using a Ca^{2+} assay kit. Measurement of Ca^{2+} was carried out in triplicate. Shown are mean $\pm$ SD ($n = 3$). Asterisks represent statistically significant differences between the groups (** $P < 0.005$, *** $P < 0.0005$).

MTA1-shRNA-transfected cells in comparison to mock-transfected or wild-type MSCs (Fig. 4a). However, this difference was much more pronounced when cells were grown under osteogenic differentiation conditions (Fig. 4b).

Results of ALP activity measurements of cell extracts showed that ALP activity was slightly suppressed by silencing *MTA1* gene, when MSCs were grown under non-osteogenic growth conditions (Fig. 5a). However, *MTA1* silencing dramatically increased ALP activity in comparison to mock-transfected or wild-type MSCs, when MSCs were grown under osteogenic differentiation conditions (Fig. 5b).

Effect of *MTA1* gene silencing on osteogenic marker gene expression

We next investigated effects of *MTA1* gene silencing on expression of osteogenic marker genes, ALP, OC, Runx2,

Figure 5. Effect of *MTA1* gene silencing on ALP activity. MSCs were seeded in 12-well plates and grown either under (a) non-osteogenic growth conditions or in (b) osteogenic differentiation medium for the indicated time periods. Medium was changed three times per week. ALP activity was then measured in cell lysates. Shown are mean $\pm$ SD ($n = 3$). Asterisks represent statistically significant differences between the groups (* $P < 0.05$; ** $P < 0.005$; *** $P < 0.0005$).

OSP, Col1A and BSP, by quantitative real-time RT-PCR. MSCs were grown under non-osteogenic (Fig. 6) and osteogenic growth conditions (Fig. 7) for indicated time periods. Results show that BSP mRNA expression was dramatically enhanced on *MTA1* silencing day 1 (3.5-fold), day 7 (67.7-fold) and day 14 (13.6-fold) in MSCs grown under non-osteogenic growth conditions (Fig. 6a) and at day 1 (3.2-fold), day 7 (24-fold) and day 14 (10.5-fold) under osteogenic differentiation conditions (Fig. 7a). Similarly, OSP mRNA expression was upregulated on *MTA1* silencing in cells grown under non-osteogenic conditions (Fig. 6b) at day 1 (6.6-fold), day 7 (12.3-fold) and day 14 (16.21-fold) as well as in osteogenic differentiation medium (Fig. 7b) on day 1 (9.35-fold), day 7 (21.37-fold) and day 14 (16.5-fold). mRNA expression of Runx2, OC and Col1A also increased 1.8-, 1.74- and 1.6-fold, respectively, on day 14 on *MTA1* gene silencing in MSCs grown under osteogenic differentiation conditions (Fig. 7c–e), but expression of these genes remained almost unaffected

Figure 6. Effect of *MTA1* gene silencing on osteogenic marker gene expression under non-osteogenic conditions assessed by quantitative real-time RT-PCR. Scrambled (SCR)-shRNA- and MTA1-shRNA-transfected MSCs were seeded in six-well plates and grown under non-osteogenic growth conditions for the indicated time periods. Total RNA was extracted and measured by real-time RT-PCR for (a) bone sialoprotein (BSP), (b) osteopontin (OSP), (c) Runx2, (d) osteocalcin (OC), (e) collagen type I (Col1A) and (f) alkaline phosphatase (ALP) mRNA expression. mRNA expression levels were normalized to GAPDH. Results are expressed as mean arbitrary units $\pm$ SD ($n = 3$). Asterisks represent statistically significant differences between the groups (* $P < 0.05$; ** $P < 0.005$; *** $P < 0.0005$).

in cells grown under non-osteogenic conditions (Fig. 6c–e). In accordance with ALP activity measurements, ALP mRNA expression was slightly suppressed in cells grown under non-osteogenic conditions (Fig. 6f). However, ALP

mRNA expression was significantly increased on *MTA1* gene silencing on day 7 (2.1-fold) and day 14 (3.77-fold) when MSCs were grown in osteogenic differentiation medium (Fig. 7f).

Figure 7. Effect of *MTA1* gene silencing on osteogenic marker gene expression under osteogenic differentiation conditions assessed by quantitative real-time RT-PCR. Scrambled (SCR)-shRNA- and MTA1-shRNA-transfected MSCs were seeded in six-well plates and grown under osteogenic growth conditions for indicated time periods. Total RNA was extracted and measured by real-time RT-PCR for (a) bone sialoprotein (BSP), (b) osteopontin (OSP), (c) Runx2, (d) osteocalcin (OC), (e) collagen type I (Col1A) and (f) alkaline phosphatase (ALP) mRNA expression. mRNA expression levels were normalized to GAPDH. Results are expressed as mean arbitrary units $\pm$ SD ($n = 3$). Asterisks represent statistically significant differences between the groups (* $P < 0.05$; ** $P < 0.005$; *** $P < 0.0005$).

Discussion

MTA1 is an integral subunit of the Mi-2/NuRD complex. As the latter contains both histone deacetylase and chromatin remodelling ATPase activities, it is thought that such complexes are involved in hypoacetylation of core histones

and that HDAC-containing complexes participate in transcriptional repression. To date, most direct targets of MTA1 identified are nuclear proteins such as ER- α , HDAC1, MTA1-interacting coactivator (MICoA) and nuclear receptor-interacting factor 3 (NRIF3) (31). All together, studies have demonstrated that *MTA1* gene regulates divergent

cellular pathways including hormonal action, epithelial-to-mesenchymal transitions, differentiation, protein stability and development, and cell fate programmes, by modifying the acetylation status of crucial target genes (32). However, the role of the MTA family of chromatin modifiers in controlling cell and physiological functions of mesenchymal stem cells, particularly in the context of osteogenesis, has not been studied. Here, we have demonstrated that downregulation of *MTA1* expression affects osteogenic differentiation of immortalized MSCs.

There are several reports that show that HDACs act as regulators in bone formation. HDAC4 or HDAC5 is involved in TGF- β /Sma- and Mad-related protein (Smad) 3-mediated repression of Runx2 function in osteogenesis and mineralization (43). HDAC represses activity of Runx2, and overexpression of HDAC4 in chondrocytes *in vivo* inhibits chondrocyte hypertrophy and differentiation, indicating that HDAC4 would act as a key regulator of endochondral bone formation (36). In this context, it is tempting to presume that MTA1 should regulate osteogenic marker genes by inducing histone deacetylation.

To investigate the role of *MTA1* in osteogenic differentiation of MSCs, we established MSCs stably expressing shRNA to *MTA1*. MSCs were grown under non-osteogenic differentiation conditions as well as in osteogenic differentiation medium, containing classical inducers of osteogenesis: β -glycerolphosphate, dexamethasone and ascorbic acid. Silencing *MTA1* was effectively maintained in MSCs for indicated time periods under both growth conditions. We have been able to show that silencing *MTA1* strongly suppressed proliferation of MSCs. This result is in agreement with findings from other groups (35,44), demonstrating the critical importance of this coregulator for the proliferative response of immortalized MSCs. We have seen in our study that as expected, mineralized-matrix production was dramatically increased on culture of MSCs in osteogenic differentiation medium in comparison with non-osteogenic conditions, demonstrating the principal osteoblastic differentiation capacity of immortalized MSCs used in our experiments. Suppression of MTA1 by RNA interference in MSCs caused strong upregulation of OSP and BSP and consequently accelerated late-stage events in osteoblast differentiation, namely calcium deposition in the mineralized matrix. Mineralized-matrix production, as evaluated by von Kossa and alizarin-red staining procedures or by quantitative measurement of Ca^{2+} -levels, was significantly increased on *MTA1* gene silencing in MSCs grown under osteogenic differentiation conditions. Likewise, expression of all investigated osteogenic marker genes increased on *MTA1* gene silencing in MSCs grown under osteogenic differentiation conditions. Strong upregulation of OSP and BSP on *MTA1* silencing was also observed in MSCs grown

under non-osteogenic conditions, whereas mRNA expression of Runx2, OC, Col 1A and ALP remained almost unaffected under these conditions.

Our results indicate that osteogenic differentiation potential of MSCs was drastically enhanced by *MTA1* gene silencing when MSCs were committed to the osteoblastic lineage by treatment with classical osteogenic inducers, β -glycerolphosphate, dexamethasone and ascorbic acid. Our findings are in line with results of Liang *et al.*, who show that knockdown of *MTA1* induces ES cell differentiation (39). They also showed that MTA1-RNAi cells have much lower ALP activity during the early stage of differentiation, consistent with our findings. These data suggest that MTA1 may have a crucial role in MSC self-renewal or inhibition of differentiation.

Although histone modifications play crucial roles in transcriptional regulation of most eukaryotic genes, the relationship between specific cell type differentiation and histone modifications has not been completely understood. Overall, data presented in this study indicate that MTA1 is involved in immortalized MSC proliferation, and transcriptional repression of osteoblastic marker genes. Therefore, exact underlying mechanisms of the role of MTA1 in osteogenic differentiation is an area of great interest. As MTA1 suppression enhances osteoblast differentiation, it would be possible to develop tissue-selective MTA1 inhibitors for potential remedy of bone-related diseases, including osteoporosis.

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