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Stathmin-like 2, a developmentally-associated neuronal marker, is expressed and modulated during osteogenesis of human mesenchymal stem cells

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ABSTRACT

Stathmin-like 2 (STMN2) protein, a neuronal protein of the stathmin family, has been implicated in the microtubule regulatory network as a crucial element of cytoskeletal regulation. Herein, we describe that STMN2 expression increases at both mRNA and protein levels during osteogenesis of human mesenchymal stem cells derived from adipose tissue (hMADS cells) and bone marrow (hBMS cells), whereas it decreases to undetectable levels during adipogenesis. STMN2 protein is localized in both Golgi and cytosolic compartments. Its expression appears modulated in osteoblasts by nerve growth factor, dexamethasone or RhoA kinase inhibitor Y-27632 which are known effectors of osteogenesis. Thus STMN2 appears a novel marker of osteogenesis and osteoblast *per se*, that could play a role in the regulation of the adipocyte/osteoblast balance.

Introduction

Numerous studies indicate that multipotential mesenchymal stem cells differentiate into several lineages, including osteoblasts, adipocytes, myocytes and chondrocytes [1–3]. The use of such stem cells allow a better understanding of cellular and molecular events involved in the commitment to different cell lineages. Osteoblasts and adipocytes share the same mesenchymal precursor and recently the transcription factor TAZ has been demonstrated to influence cell fate, modulating the cross-talk between PPARγ2 and CBFA1 [4]. Commitment and differentiation toward a cell lineage leads to changes in cell shape that can alter the differentiation of pre-committed mesenchymal lineages [5–7]. While the late events regulating lineage specification and differentiation are well known, our knowledge at the molecular level of the early steps remains scant.

Recently, we have isolated mesenchymal stem cell from human adipose tissue (hMADS cells) at both population and clonal level. hMADS cells which exhibit normal karyotype, and high self-renewal ability. Similarly to mesenchymal stem cells from human bone marrow, they are able to differentiate into various lineages including adipocytes and osteoblasts and to support *in vivo* regenerative processes [2,8–10]. In order to gain better insights into the early steps of osteoblast and adipocyte differentiation, we performed RNA analysis of hMADS cells at different time points of osteogenesis or adipogenesis using a previously developed microarray [11]. We identified 1606 differentially expressed candidate genes (data deposited in Array Express, accession number A-MARS 3 and E-MARS 10) and focused our attention on genes, the expression of which had never been so far reported in either differentiation process, among which we identified stathmin-like 2 gene. Stathmin family phosphoproteins have been assumed so far to play an integrating role within intracellular signaling networks and recently shown to be phosphorylation-dependent tubulin binding proteins involved in the control of microtubule assembly and dynamics [12,13]. This family includes STMN1 (op18), STMN2 (SCG10), STMN3 (SCLIP), RB3 and its splicing variants RB3′ and RB3″, which share a conserved stathmin-like tubulin binding domain and possess, with the exception of STMN1, additional N-terminal subcellular targeting sequences [14]. STMN1 is cytosolic and ubiquitously expressed, whereas the other members of the family are mainly found in the nervous system and localized within the Golgi complex, vesicle-like structures and in neuronal growth cones [14–17]. Stathmin family members are known to inhibit microtubule polymerization through their microtubule-destabilizing activity that is regulated by phosphorylation [13,18]. In particular, STMN2 has been characterized as a neuronal marker at an early stage of

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neural development, playing a regulatory role in the control of neuronal differentiation [19–21].

We report herein that STMN2 is not confined to neural cells. It is expressed in human mesenchymal stem cells undergoing osteogenesis—but not adipogenesis—and its expression is modulated by known effectors of osteogenesis.

Materials and methods

Materials. Cell culture media were purchased from Lonza and fetal calf serum (FCS) from Dutscher. Human FGF-2, EGF and NGF were from Euromedex. Reverse transcriptase was from Promega and all the other products were from Sigma–Aldrich.

Cell culture. The establishment and characterization of the multipotency and self-renewal of hMADS cells have already been described [2,8–10]. In this work, hMADS-2 cells, established from the pubic region fat pad of a 5-year-old male donor, were used at passages between 16 and 35 corresponding to 35–100 population doublings. Cells were seeded at a density of 4500 cells/cm^2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS, 2.5 ng/ml hFGF2, 60 μg/ml penicillin, and 50 μg/ml streptomycin. The medium was changed every other day and hFGF2 was removed when cells reached confluence. At day 2 post-confluence (designated as day 0), cells were induced to differentiate either to adipocytic or osteoblastic lineage. For osteoblast differentiation, cells were maintained in α-MEM medium containing 1% FCS, 10 ng/ml EGF, 10 nM 1,25-dihydroxyvitamin D3, 100 nM dexamethasone (DEX), 50 μg/ml L-ascorbic acid phosphate and 10 mM β-glycerophosphate. For adipocyte differentiation, cells were cultured in DMEM/Ham's F12 media supplemented with 10 μg/ml transferrin, 0.85 μM insulin, 0.2 nM triiodothyronine, 1 μM DEX, 500 μM isobutyl-methylxanthine. Three days later, the medium was changed (DEX and isobutyl-methylxanthine were omitted) and 100 nM rosiglitazone added. The media were then changed every other day and cells were used at the indicated days.

Human bone marrow mesenchymal stromal (hBMS) cells were used as recommended by the manufacturer (Lonza).

Isolation and analysis of RNA. Total RNA was extracted using TRI-Reagent kit (Euromedex) according to manufacturer's instructions. Reverse transcriptase reactions and quantitative RT-PCR (qRT-PCR) assays were performed as already described [8,9]. qRT-PCR assays were run on an ABI Prism 7000 real-time PCR machine (PerkinElmer Life Sciences) using SYBR green PCR Master Mix according to the manufacturer's instructions (Applied Biosystems). Gene expression was normalized to that of the TATA-binding protein (TBP) encoding gene and quantified using the comparative-ΔC_t method. The oligonucleotides, designed using Primer Express software, are shown in Supplementary Table 1.

Western blot analysis. Cell were washed twice with cold PBS (pH 7.4) and then scraped into lysis buffer containing 1% SDS and 60 mM Tris–Cl (pH 6.8). Total cellular lysates were sonicated for 15 s, clarified by centrifugation, and subjected to immunoblotting. Cell extracts were used for Western-blotting as previously described [8,9]. Primary antibodies were rabbit anti-SCG10-BR [22], anti STMN1 and anti-TBP (Santa Cruz Biotechnology). Secondary horseradish peroxidase-conjugated antibodies were from Promega. Enhanced ChemiLuminescence (Millipore) was used for detection.

Immunostaining. hMADS cells were cultured on poly-D-lysine coated coverslips and fixed in 4% formaldehyde for 20 min at room temperature and rinsed with PBS. Free aldehydes were quenched with 100 mM Tris, pH 8.5, 150 mM NaCl for 5 min. Fixed cells were then permeabilized in 0.05% digitonin for 15 min and unspecific reactions were blocked with 1% normal goat serum and 1% bovine serum albumin in PBS for 30 min at room temperature. Cells were then incubated with primary antibodies diluted in 0.1% carrageenan overnight at 4 °C. Primary antibodies were rabbit anti-SCG10-BR and γ-adaptin (Sigma). The cells were washed and incubated for 1 h at room temperature with Alexa-594-conjugated goat anti-rabbit antibody or Alexa-488-conjugated goat anti-rabbit antibody (Molecular Probes) in the presence of 0.5 μg/ml Hoechst 33258 (Invitrogen) for nuclei counterstaining. Coverslips were mounted on slides in Mowiol 4-88 solution (Sigma). Images were taken on an LSM510 META confocal microscope (Zeiss).

Data analysis. qRT-PCR and Western blot results were analyzed using Student's *t*-test. Statistical significance was assumed at P level <0.05.

Results and discussion

STMN2 gene expression increases during osteogenesis of hMADS and hBMS cells

We performed a microarray analysis of adipocyte and osteoblast differentiation of hMADS cells (data deposited in Array Express, accession number A-MARS 3 and E-MARS 10, manuscript submitted). Among the 1606 differentially expressed genes, we focused our interest on SCG10 also named stathmin-like 2 (STMN2), as its expression and function were originally described to be confined to neurons [19,20,23].

As assessed by qRT-PCR, STMN2 mRNA levels remained very low in hMADS cells undergoing adipogenesis, while and as expected the expression of the adipocyte Fatty Acid Binding Protein (FABP4) increased dramatically (Fig. 1A). By contrast, STMN2 mRNA levels increased during osteogenesis in parallel to those of alkaline phosphatase (AP) (Fig. 1A) in agreement with microarray data. Osteogenesis was also assessed by Alizarin red staining and CBFA1 and podoplanin gene expression (not shown). Similarly, STMN2 mRNA levels increased during osteogenesis of hBMS cells

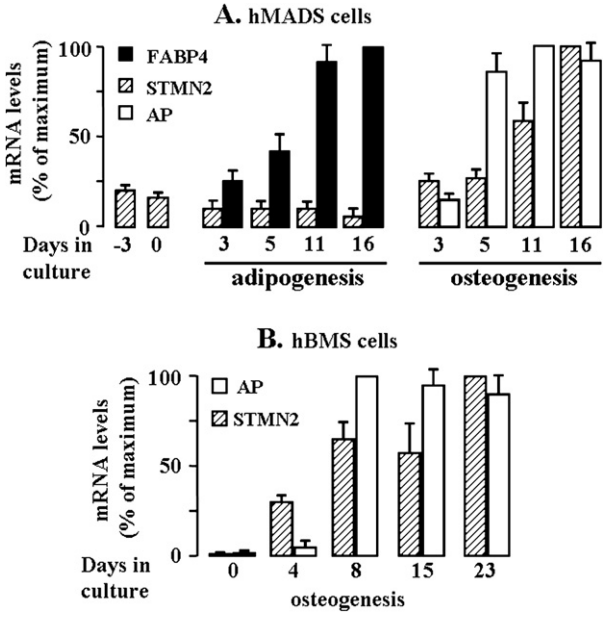

Fig. 1. Expression of STMN2 mRNA during osteogenesis of hMADS and hBMS cells. (A) qRT-PCR analysis of STMN2 expression in hMADS cells during adipocyte and osteoblast differentiation. Total RNA was extracted at the indicated times. The expression of FABP4 and AP was determined by qRT-PCR as markers of adipogenesis and osteogenesis, respectively. (B) STMN2 and AP gene expression were analyzed during osteogenesis of hBMS cells. The results, expressed as % of maximum, are representative of independent experiments performed on three different series of cells.

(Fig. 1B), indicating that STMN2 expression during osteogenesis was not restricted to human adipose-derived stem cells.

The expression of STMN1, an ubiquitously expressed member of the stathmin family, was also analyzed during osteogenesis of hMADS cells. STMN1 mRNA was expressed in proliferating cells but became down-regulated as soon as the cells reached confluence and subsequently induced to differentiate (Fig. 2A). Similar observations were made with hBMS cells (Fig. 2B). STMN1 expression analyzed by conventional RT-PCR had been reported in transformed human osteoblast-like cells, human osteosarcoma cells (Saos-2), and rat osteoblast cell lines (ROS 17/2.8, ROS 25/1) [24], but neither quantitative analysis nor differentiation-dependent expression had been performed in these experiments.

STMN2 protein expression and localization in hMADS cells

Western blot analysis of total cell extracts showed that STMN2 protein was not detected in confluent hMADS cells and derived adipocytes, whereas it was highly expressed in hMADS osteoblasts, as shown by a clear band at 24 kDa (Fig. 3A). A lower band of ~19 kDa, corresponding to STMN1 protein, was also observed in confluent hMADS cells but disappeared in cells differentiated into adipocytes or osteoblasts (Fig. 3A). These data are in agreement with those of mRNA levels (Figs. 1 and 2) and indicate that STMN1 expression was down-regulated in both differentiation processes whereas STMN2 protein expression was confined to osteoblasts. The localization of STMN2 protein has been described as peri-nuclear within the Golgi complex [25,26]. In the developing cortex of mice, STMN2 was shown to be present within the *trans* face Golgi complex and associated with vesicular structures in putative growth cones [27]. In order to gain insights into the subcellular localization of STMN2 during osteogenesis of hMADS cells, immunostaining experiments were performed, using the anti-SCG10-polyclonal antibody [22]. Fig. 3B shows a rather widespread staining of STMN2 in confluent hMADS cells, which increases in differentiated osteoblasts, consistent with gene expression and Western blot analyses, suggesting that STMN2 protein is present as both a membrane-associated and a cytosolic protein. Immunostaining with anti-γ-adaptin, a ubiquitous protein localized at the *trans*-Golgi network, was used as a positive control and showed a typical vesicular staining (Fig. 3C). As expected, no signal was detected in hMADS cells differentiated into adipocytes (data not shown). Further studies will be necessary to characterize more precisely the subcellular localization of STMN2 in hMADS cell-derived osteoblasts in response to various effectors (*vide infra*).

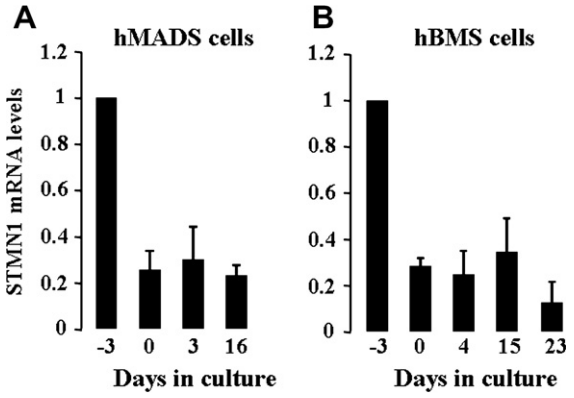

Fig. 2. Expression of STMN1 mRNA during osteogenesis of hMADS and hBMS cells. qRT-PCR analysis of STMN1 mRNA expression in hMADS (A) and hBMS cells (B) during osteoblast differentiation. The results are representative of independent experiments performed on three different series of cells.

STMN2 mRNA and protein regulation in hMADS osteoblasts

As various agents have been reported to modulate osteogenesis, we anticipated that the expression of both STMN2 gene and protein were regulated by the same effectors. NGF had been described to regulate STMN2 expression in neurons and PC12 cells [28,29]. With respect to osteoblastic cells, NGF stimulates their differentiation and acts as an autocrine survival factor of MC3T3-E1 cells [30] whereas immortalized hFOB cells express trk receptors and produce significant amounts of NGF [31]. First, hMADS osteoblasts expressed the NGF high-affinity trk-A receptor (RT-PCR, data not shown). Second, upon 24 h of NGF treatment, hMADS osteoblasts exhibited a significant up-regulation of STMN2 mRNA and protein levels compared to vehicle-treated cells (Fig. 4A). Thus STMN2, the expression of which can be modulated by NGF, could participate in the regulation and/or maintenance of osteogenesis.

Substantial evidence indicates that glucocorticoid-induced bone loss occurs in both humans and mice due to excessive bone resorption and to inadequate bone formation [32,33]. Glucocorticoid-induced osteoporosis is due to direct inhibition of osteoblast activity and/or to increase in osteoclast life span [33,34]. Paradoxically, glucocorticoids are required *in vitro* for bone nodule formation and mineralization, as well as for osteogenesis of bone marrow and adipose tissue-derived mesenchymal stem cells [10,35,36]. Therefore, we examined the effects of DEX, a synthetic glucocorticoid, on STMN2 expression in hMADS cells. Cells were induced to differentiate for 17 days in osteogenic medium with or without DEX. Osteoblast differentiation occurred only upon exposure to DEX, whereas the lack of differentiation was ascertained by the absence of alkaline phosphatase activity and no Alizarin red staining (data not shown). At both mRNA and protein levels, STMN2 expression increased in chronically DEX-treated cells, with faint levels detected in untreated cells (Fig. 4B). The removal of DEX at day 7 led to a low expression of STMN2 measured at day 17, indicating a reversible regulation. However, re-exposing the cells to DEX between day 14 and 17, only was sufficient to re-induce STMN2 mRNA and protein expression (Fig. 4B). These observations indicate that STMN2 expression is under the control of glucocorticoids and suggest that STMN2 could play a role in early events of differentiation associated with morphological changes and/or the fine regulation of later events.

RhoA signaling, which induces changes in cell shape, has been involved in the osteoblast/adipocyte balance of human mesenchymal stem cells [6]. We analyzed whether early treatment of hMADS cells (from day −3 to day 3) by compound Y-27632, an inhibitor of RhoA kinase, could inhibit subsequent STMN2 expression. At both mRNA and protein levels, a 65% decrease was observed at day 18 in drug-treated compared to untreated cells (Fig. 4C), and this decrease was associated with reduced osteogenesis (data not shown). Therefore it cannot be excluded that STMN2 interferes through the RhoA pathway for the control of osteoblast morphology in a way similar to other members of the stathmin family implicated in the cytoskeleton microtubule organization of neural cells.

Altogether, our observations indicate that STMN2, a member of the stathmin family, is expressed in hMADS and hBMS osteoblasts and that its expression appears under the control of factors reported to modulate osteogenesis. The analysis of the adipocyte/osteoblast balance is of great interest for developing pharmacological tools for bone- and fat mass-related disorders such as osteoporosis and obesity. Various reports have suggested an inverse relationship in the expression of osteoblast and adipocyte phenotypes in marrow stroma in osteoporosis, reflecting the ability of a single or a combination of factors in modulating the commitment to either pathway [37–40]. As we have recently detected the expression of STMN2 in mouse humerus, it is anticipated that

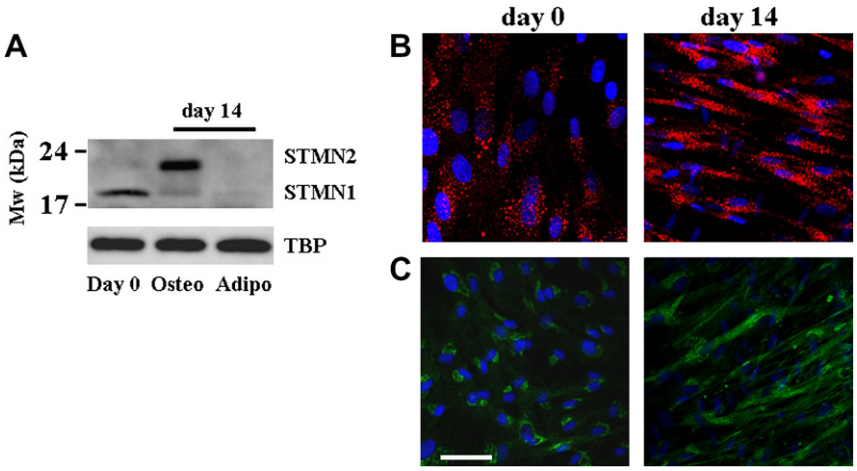

Fig. 3. STMN2 protein expression and localization in hMADS cells. A-STMN2 protein levels were analyzed by Western blot in hMADS cells at day 0 and at day 14 of osteogenesis (osteo) and adipogenesis (adipo). TBP was used as the internal standard. Proteins (30 μg) were loaded on 12% SDS–polyacrylamide gels. (B) Immunolocalization of STMN2 expressed in hMADS cells at day 0 and day 14 of osteoblast differentiation. Cells were fixed and stained with STMN2 antibodies. (C) Immunolocalization of γ-adaptin in hMADS cells at day 0 and day 14 of osteoblast differentiation, as a marker of the *trans*-Golgi compartment. Bar 20 μm.

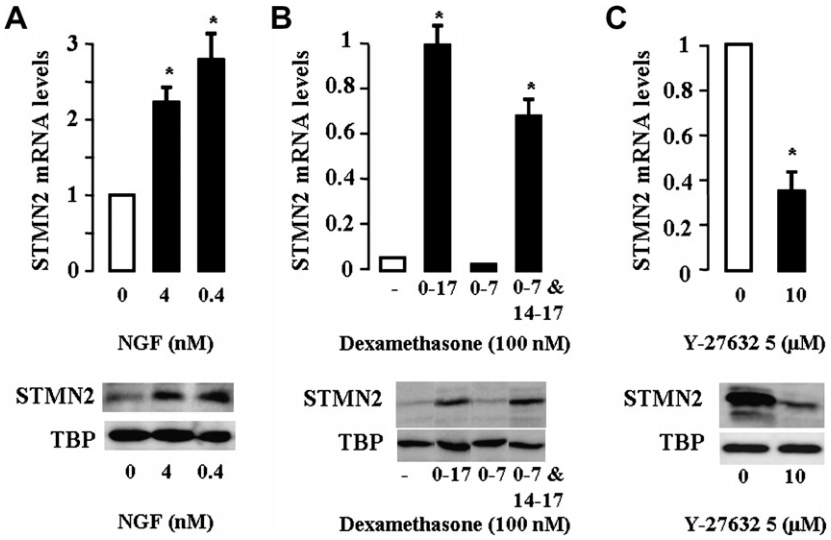

Fig. 4. Regulation of STMN2 mRNA and protein expression in hMADS cell-derived osteoblasts. A qRT-PCR and Western blot analysis of STMN2 expression in hMADS osteoblasts at day 9 of differentiation, after 24 h-treatment with NGF (10 and 100 ng/ml). (B) STMN2 levels were analyzed by qRT-PCR and Western blot in hMADS cells at day 17 of osteogenesis, treated or not for the indicated time period with 100 nM DEX. (C) qRT-PCR and Western blot analysis of STMN2 expression in hMADS osteoblasts at day 18, after pre-treatment with Y-27632 (10 μM) between day −3 and day 3. TBP was used as internal standard in Western blot experiments, using 30 μg of proteins. The results are representative of independent experiments performed on two different series of cells. $^{*}P < 0.05$.

studies on its regulation in bones of osteoporotic animals and humans should shed light on its role *in vivo*.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.06.121.

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