Short Communication

miR-125b Regulates Calcification of Vascular Smooth Muscle Cells

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Vascular calcification is a prominent feature of atherosclerosis and is closely linked to osteoporosis. Cellular differentiation is regulated by various microRNAs (miRs), including miR-125b, which is known to be involved in osteoblast differentiation. However, no specific miR has been defined that modulates vascular calcification. Herein, we assessed the impact of miR-125b in osteogenic transformation of vascular smooth muscle cells. Osteogenic transdifferentiation of human coronary artery smooth muscle cells was induced by osteogenic medium and enhanced the formation of mineralized matrix, resulting in a significantly higher mineral deposition after 21 days. Increased expression of miR-125b was time-dependent in human coronary artery smooth muscle cells and diminished during osteogenic transdifferentiation. At day 21, miR-125b was significantly reduced (–42%) compared with that in the untreated control. The expression of miR-processing enzymes, RNase III endonucleases DICER1 and DROSHA, was also decreased. Furthermore, inhibition of endogenous miR-125b promoted osteogenic transdifferentiation, as measured by increased alkaline phosphatase activity and matrix mineralization. Expression analysis revealed the osteoblast transcription factor SP7 (osterix) as a target of miR-125b. *In vivo*, miR-125b was decreased in calcified aortas of apolipoprotein E knockout mice. In conclusion, our results suggest that miR-125b is involved in vascular calcification *in vitro* and *in vivo*, at least partially by targeting SP7. Evaluating the role of miRs in arterial calcification *in vivo* may have important therapeutic implications. (Am J Pathol 2011, 179:1594–1600; DOI: 10.1016/j.ajpath.2011.06.016)

Atherosclerosis, vascular calcification, and osteoporosis are the most common diseases of the aging population and are associated with significant morbidity and mortality. Various clinical,¹ histological,² and animal³ studies support the coincidence of osteoporosis and vascular calcification and suggest that processes in vascular calcification are similar to those of bone remodeling.⁴ Hence, a better molecular understanding of common processes of these comorbidities may lead to more efficient therapeutic strategies against these age-related diseases. Vascular smooth muscle cells (VSMCs) may play an important role in vessel calcification via transdifferentiation toward an osteoblast-like state. Vascular calcification is an active, cell-regulated process in which SMCs can lose the expression of marker genes, such as α -smooth muscle actin, acquire osteogenic markers, and deposit a mineralized bonelike matrix.⁵

MicroRNAs (miRs) are small nonencoded RNAs serving as important post-transcriptional gene regulators. There are >1000 known regulatory miRs that control cell proliferation and differentiation of various cell types. In addition, specific miR profiles are associated with cardiovascular diseases. The dysregulation of individual miRs has been linked to many pathological processes of the cardiovascular system, including cardiac hypertrophy, heart failure, cardiac fibrosis, and vascular atherosclerosis.^{6,7} Circulating levels of vascular and inflammation-associated miRs were down-regulated in patients with coronary artery disease.⁸ Patients with type 2 diabetes mellitus had less circulating endothelial miR-126.⁹

Several studies^{10–12} have recognized miRs as important mediators for the modulation of the VSMC phenotype by targeting transcription factors that act as

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molecular switches for differentiation. miR-21, miR-221, miR-222, and miR-145 play a regulative role in aberrant VSMC proliferation, which is a key pathological process of proliferative vascular diseases, such as atherosclerosis or coronary heart disease. miR-26a inhibited VSMC differentiation and apoptosis while promoting proliferation and migration through a mechanism that targets the transforming growth factor- β -bone morphogenetic protein pathway.¹³ miR-146a and Krüppel-like factor 4 constitute a feedback loop to regulate VSMC proliferation and vascular neointimal hyperplasia.¹⁴

miRs also play a role in differentiation of different cell types. miR-181 controls mouse hematopoiesis,¹⁵ and B-cell differentiation is regulated by an *miR-17-92* cluster and miR-150.^{16,17} The differentiation of bone precursor cells to mature bone cells has also been controlled by miRs. Osteoblast differentiation is controlled by miR-125b by regulating cell proliferation¹⁸ and by miR-206 by regulating the accumulation of connexin 43.¹⁹ In addition, data suggest the role of miR-26a in the differentiation of adipose tissue-derived stem cells toward the osteogenic lineage by targeting the SMAD1 protein.²⁰

The role of miRs in osteogenic transformation of VSMCs in the process of vascular calcification is unknown. Thus, we aimed to investigate the role of miR-125b and miR-26a, which have been implicated in osteoblast differentiation, in the osteogenic transdifferentiation process of human coronary artery SMCs (HCASMCs).

Materials and Methods

Cell Culture

The HCASMCs were obtained from Promocell (Heidelberg, Germany). HCASMCs were grown in Smooth Muscle Cell Growth Medium 2 (Promocell) supplemented with epidermal growth factor (0.5 ng/mL), insulin (5 μ g/mL), basic fibroblast growth factor-B (2 ng/mL), and 5% fetal bovine serum. The cells were maintained at 37°C (5% CO₂, 90% humidity) and were used between passages 4 and 8. Cells isolated from three to five independent donors were used.

Osteogenic Transdifferentiation

HCASMCs were cultured for up to 21 days in the presence of osteogenic medium (OM), which consisted of regular medium with 0.1% fetal bovine serum supplemented with 10 nmol/L dexamethasone, 10 mmol/L β -glycerol phosphate, and 100 μ mol/L L-ascorbate phosphate. The medium was changed three times per week.

Mineralization Assay and Activity of ALP

Mineralized matrix formation was assessed by Alizarin Red S staining. HCASMCs were fixed in 4% paraformaldehyde and stained with 40 mmol/L Alizarin Red S (pH 4.2, Sigma-Aldrich, Munich, Germany) for 30 minutes at room temperature. Excess dye was removed by washing the plates with distilled water. Incorporated calcium was eluted with 100 mmol/L cetylpyridinium chloride and

measured at 540 nm. Alkaline phosphatase (ALP) activity was measured in cell cultures, as previously described.²¹ Cells were lysed using 1% Triton X-100-containing Tris-HCl buffer (pH 7.0). Aliquots of each sample were incubated with ALP substrate buffer (100 mmol/L diethanolamine, 150 mmol/L NaCl, 2 mmol/L MgCl₂, and 2.5 μ g/mL *p*-nitrophenylphosphate) for 10 minutes at 37°C, measured at 405 nm, and normalized to the total protein content determined by the bicinchoninic acid method from the same protein extracts.

Transfection

To inhibit the function of miR-125b, a miR inhibitor (anti-miR-125b; Ambion, Darmstadt, Germany) and a negative control (anti-negative control; Ambion) were used. An miR precursor (pre-miR-125b; Ambion) and a negative control (pre-negative control; Ambion) were used to promote the function of miR-125b. Transfection of 50 nmol/L RNAs was performed by lipofection using siPORT NeoFX Transfection Agent (Ambion) twice per week during the entire cell culture period, or HCASMCs were transfected once at the start of the experiment. Incubation with the transfection reagent alone served as a control.

miR Preparation and Real-Time PCR

miR from cultured HCASMCs was isolated using the miR-Neasy Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's protocols. RNA, 500 ng, was reverse transcribed using the miScript Reverse Transcription Kit (Qiagen). Subsequently, 2 μ L of the product was used for detecting miR expression by real-time PCR (ABI 7500 fast, Applied Biosystems, Darmstadt, Germany) using an miScript SYBR Green PCR kit and miScript Primer Assays (Hs_miR-125b_1 miScript Primer Assay and Hs_miR-26a_1 miScript Primer Assay; Qiagen). Values were normalized to RNU6B. The mRNA expression was determined by SYBR Green-based real-time PCRs using a standard protocol (Applied Biosystems). The primer pairs used are as follows: human SP7, forward-5'-TC-CCTTTTCCCACTCATTCC-3' and reverse-5'-GGGCA-GACAGTCAGAAGAGC-3'; human ALP, forward-5'-CA-ACCCTGGGGAGGAGAC-3' and reverse-5'-GCATTGGT-GTTGTACGTCTTG-3'; human RUNX2, forward-5'-CAGA-TGGGACTGTGGTTACTG-3' and reverse-5'-TGGGGAG-GAATTTGTGAAGAC-3'; human α -SMC actin, forward-5'-GAATGCAGAAGGAGATCACG-3' and reverse-5'-CTG-GAAGGTGGACAGAGAGG-3'; mouse SP7, forward-5'-AG-GCACAAGAAGCCATACG-3' and reverse-5'-GCCC-AGGAAATGAGTGAGG-3'; and mouse RUNX2, forward-5'-CCCAGCCACCTTTACCTACA-3' and reverse-5'-TATG-GAGTGCTGCTGGTCTG-3'. Expression was normalized to β -actin (human, forward-5'-CCAACCGCGAGAAGATGA-3' and reverse-5'-CCAGAGGCGTACAGGGATAG-3'; mouse, forward-5'-GATCTGGCACCACACCTTCT-3' and reverse-5'-GGGGTGTGAAGGTCTCAA-3'). The relative expression was calculated using the $\Delta\Delta C_T$ method [relative gene expression = $2^{-(\Delta C_T \text{ sample} - \Delta C_T \text{ control})}$] and is presented in fold increase relative to control.

Western Blot Analysis

Total protein was separated by 10% SDS-PAGE and transferred to a Protran membrane (Schleicher & Schuell BioScience GmbH, Dassel, Germany). Primary antibodies against human SP7 (1:1000; Santa Cruz, Heidelberg, Germany) and human β -actin (1:2000; Cell Signaling, Frankfurt/Main, Germany) were used. Protein expression was detected using Western Lightning Chemiluminescence Reagent (PerkinElmer Life Sciences, Rodgau, Germany).

Proliferation

The proliferation rate was monitored by quantifying DNA synthesis through the incorporation of methyl- ^3H thymidine (2.22 to 3.33 TBq/mmol; Hartmann Analytic GmbH, Braunschweig, Germany). At indicated points, medium was supplemented with 37 kBq methyl- ^3H thymidine/mL and cells were incubated for 24 hours. Then, the medium was removed, and cells were washed with PBS and treated twice with 10% trichloroacetic acid at 4°C for 10 minutes to precipitate DNA. After washing with ethanol, cells were lysed in a solution containing 100 mmol/L NaOH, 22 mmol/L Na_2CO_3 , and 1% SDS, followed by scintillation counting in a Trilux 1450 Microbeta counter (Wallac, Turku, Finland).

Animals

Male apolipoprotein E knockout (ApoE $^{-/-}$) mice (strain B6.129P2-Apoetm1Unc/J; Jackson Laboratories, Bar

Harbor, ME), aged 4 weeks, were fed a high-fat diet (FD EF R/M TD88137; Ssniff GmbH, Soest, Germany) or a control diet for 6 or 26 weeks. After the feeding period, mice were euthanized by carbon dioxide anesthesia and aortas were prepared. All experiments were performed in accordance with the *Guide for the Care and Use of Laboratory Animals*, published by the US NIH. The animal research Ethics Committee of the Dresden University of Technology and the Regional Board of Dresden approved the experiments (AZ: 24-9168.24-1 2009-14). RNA isolation was performed using the RNeasy Micro Kit (Qiagen).

Statistical Analysis

Data are given as the mean $\pm$ SEM, and n indicates the number of independent experiments. Statistical analysis for time-response curves was performed using a one-way analysis of variance with Bonferroni's post hoc test and single-group comparisons using a Student's t -test. $P < 0.05$ was considered statistically significant.

Results

miR Modulation during Osteogenic Transdifferentiation of HCASMCs

As expected, HCASMCs cultured in OM resulted in a time-dependent increase in matrix mineralization compared with cells cultured in control medium (Figure 1A). miR-125b was increased within 21 days in the cells that

Figure 1. Regulation of miR and its processing enzymes during osteogenic transdifferentiation of HCASMCs. The HCASMCs were cultured for 21 days in control medium (control) or OM. **A:** Matrix mineralization. Results of one-way analysis of variance are shown at top. **B:** miR-125b expression. Results of one-way analysis of variance are shown at top. **C:** miR-26a expression after 21 days. Dicer (**D**) and Drosha (**E**) mRNA expression was determined after 7 and 21 days by real-time PCR. $n = 4$. * $P < 0.05$ versus day 0. † $P < 0.05$ for control versus OM at day 21.

Figure 2. Effect of inhibition and overexpression of miR-125b on matrix mineralization in calcifying HCASMCs. The HCASMCs were cultured in OM and transfected twice per week during the entire cell culture period of 21 days with miR-125b inhibitor (anti-125b), precursor (pre-125b), the corresponding controls (anti-con and pre-con, respectively), or the lipid control (lipid con). **A:** miR-125b expression at days 4, 7, and 21. **B:** Matrix mineralization at day 21. **C:** ALP activity at day 14. **D:** ALP mRNA expression. **E:** RUNX2 mRNA expression. **F:** α-SMC actin mRNA expression. **G:** HCASMCs were cultured for 21 days in control medium (con) or OM. SP7 mRNA expression was determined by real-time PCR. **H:** SP7 mRNA expression after modulation of miR-125b ($n = 3$ to 5). **I:** SP7 protein expression. A representative Western blot analysis was used ($n = 2$). * $P < 0.05$, rel., relative.

proliferated naturally without osteogenic induction (Figure 1B). This suggests that miR-125b might be involved in the proliferation of HCASMCs, as shown for other cell types. In calcifying HCASMCs, the increased level of miR-125b was significantly reduced at day 21 (Figure 1B). miR-26a was not altered after 21 days in normal or calcifying HCASMCs (Figure 1C).

Expression of miR Processing Enzymes

Because the two RNase III endonucleases DROSHA and DICER are involved in the maturation of miRs, we assessed the regulation of both enzymes in calcified HCASMCs. DROSHA and DICER were increased within 21 days in the cells that proliferated naturally without osteogenic induction (Figure 1, D and E). Osteogenic transdifferentiation caused a significant attenuation of the induction of DICER (Figure 1D) and DROSHA (Figure 1E).

Effect of Inhibition and Overexpression of miR-125b on Matrix Mineralization in Calcifying HCASMCs

To analyze the role of miR-125b during osteogenic transdifferentiation of HCASMCs, we altered the functional

level of miR-125b in HCASMCs. We first tested whether transfection of precursor (pre-miR125b) and inhibitor (anti-miR-125b) could change the level of cellular miR-125b. Inhibition of miR-125b (transfection twice a week) completely inhibited miR-125b expression over 21 days, whereas overexpression induced endogenous miR-125b levels 5- to 10-fold (Figure 2A).

Inhibition of endogenous miR-125b in calcified HCASMCs promoted osteogenic transdifferentiation, measured by increased matrix mineralization (Figure 2B), ALP activity (Figure 2C), and mRNA expression of ALP (Figure 2D) and RUNX2 (Figure 2E). Overexpression of miR-125b had no effect. In addition, the smooth muscle cell lineage marker α-smooth muscle actin was decreased in miR-125b-deficient HCASMCs (Figure 2F). The inhibition of miR-125b had no effect on the proliferation of HCASMCs (data not shown).

Regulation of Predicted Targets of miR-125b Known To Be Involved in Vascular Calcification

Targets of miR-125b were searched using the GeneMir Web Database (<http://www.macinolab.org/Unionlist.asp>, last accessed March 1, 2011) and identified SP7 as a possible target. The HCASMCs cultured in OM resulted in

Figure 3. miR-125b plays a role in the early phase of osteogenic transdifferentiation of HCASMCs and is inhibited during atherosclerotic plaque formation. The HCASMCs were cultured in OM and transfected once with miR-125b inhibitor (anti-125b), precursor (pre-125b), the corresponding controls (anti-con and pre-con, respectively), or the lipid control (lipid con) and cultivated over 21 days. **A:** miR-125b expression at days 4, 14, and 21. **B:** Matrix mineralization at day 21. **C:** ALP activity at day 14. **D–F:** The 10- and 30-week-old ApoE knockout (KO) mice were fed a control diet or a high-fat diet (HFD). Expression levels of miR-125b (**D**), SP7 (**E**), and RUNX2 (**F**) were determined in the aorta. $n = 6$ to 8 . $*P < 0.05$ by analysis of variance. rel., relative.

a significant increase in SP7 expression compared with cells cultured in control medium (Figure 2G). Interestingly, long-term inhibition of miR-125b significantly up-regulated the SP7 mRNA level by 1.9-fold compared with the negative control (Figure 2H). This was confirmed at the protein level (Figure 2I).

miR-125b Plays a Role in the Early Phase of Osteogenic Transdifferentiation of HCASMCs

Next, we assessed in which phase of differentiation miR-125b plays a role. We transfected HCASMCs with the miR-125b inhibitor only once at the beginning of osteogenic transdifferentiation (Figure 3A). This enhanced matrix mineralization (Figure 3B) and ALP activity (Figure 3C), indicating that miR-125b plays a role at the early stages of osteogenic transdifferentiation.

miR-125b Is Inhibited during Atherosclerotic Plaque Formation

We evaluated the expression of miR-125b in ApoE^{-/-} mice, an established model of atherosclerosis. ApoE^{-/-} mice fed a Western-type diet for 6 weeks significantly increased aortic miR-125b (Figure 3D) compared with mice fed a control diet, suggesting that early inflammatory processes increase miR-125b. In calcified aortas of 30-week-old ApoE^{-/-} mice, 1.5-fold less miR-125b was

expressed (Figure 3D). The expression levels of RUNX2 and SP7, two markers of osteogenic transdifferentiation, were twofold and threefold higher, respectively, in 30-week-old compared with 10-week-old ApoE^{-/-} mice fed a high-fat diet (Figure 3, E and F).

Discussion

miRs are involved in several physiological and pathophysiological processes. Although miRs act as regulators in the differentiation of different cell types (eg, osteoblasts, osteoclasts, and adipocytes), there is no report describing a role of miRs in osteogenic transdifferentiation of VSMCs in the process of vascular calcification.

In this study, we defined a novel miR-dependent mechanism during the progression of vascular calcification. We report that miR-125b regulates the transdifferentiation of VSMCs into osteoblast-like cells by targeting the transcription factor SP7. miR-125b was involved in osteoblastic differentiation through the regulation of cell proliferation.¹⁸ Our data provide further evidence that processes in vascular calcification are similar to those of osteoblastogenesis. We did not detect any difference in miR-26a expression in calcified versus noncalcified HCASMCs. However, miR-26a promoted VSMC proliferation while inhibiting cellular differentiation and apoptosis.¹³ Furthermore, a role for miR-26a during the late differentiation stage of human adipose tissue-derived

stem cells toward the osteogenic lineage was described by targeting the SMAD1 protein.²⁰ The results shown herein suggest that miR-125b, but not miR-26a, is involved in the early stage of osteogenic transdifferentiation of VSMCs, from proliferation to differentiation.

In arteries, miR-125b is one of the most abundant miRs, in addition to miR-145, miR-23, and miR-143.¹⁰ This could be one explanation why overexpression of miR-125b was only mildly induced above baseline levels compared with knockdown, which almost completely inhibited miR-125b expression and had no effect on mineralization of VSMCs in our setup. Moreover, the suggestion that miRs fine-tune gene expression, rather than induce dramatic changes,²² would explain the small, but significant, changes in cell function by inhibition of miR-125b.

miRs play a role in atherosclerotic plaque formation, including inflammation,^{23,24} apoptosis,²⁵ and angiogenesis.^{26,27} By using atherosclerotic mice, we showed that miR-125b was down-regulated in calcified vessels. Our data are in line with several studies that showed a down-regulation of miR in pathophysiological vascular processes. miR-125b was down-regulated during vascular neointima formation.¹⁰ In addition, miRs (ie, miR-26a, miR-30b, and miR-195) in aortic valves of patients with aortic stenosis were reduced compared with patients with aortic insufficiency.²⁸ The detected increase in miR-125b expression in ApoE^{-/-} mice fed a high-fat diet for 6 weeks suggests that miR-125b might be increased during atherosclerotic inflammation processes. This is in accordance with previous findings showing that miR-125b plays a role in the epigenetic regulation of inflammatory genes in VSMCs of diabetic mice²⁹ and that miR-125b is induced in response to lipopolysaccharide and tumor necrosis factor- α .³⁰ Recently, miRs were found to be present in the circulating bloodstream and were down-regulated in patients with coronary artery diseases⁸ and type 2 diabetes.⁹ It is conceivable that vascular miRs can act as biomarkers for the development of coronary artery diseases.^{6,31}

To our knowledge, no report has concentrated on the effect of miRs on vascular calcification. In this study, we demonstrate that miR-125b is involved in the osteogenic transdifferentiation of VSMCs, at least in part by targeting SP7, and implicate miRs as a novel link for the common mechanisms of vascular calcification and bone remodeling.

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