In Vitro Osteogenic Potential of Human Mesenchymal Stem Cells Is Predicted by *Runx2/Sox9* Ratio

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Introduction: Runx2 is one of the most studied transcription factors expressed in mesenchymal stem cells (MSCs) upon their commitment toward an osteogenic differentiation. During endochondral bone formation *in vivo*, Sox9 directly interacts with Runx2 and represses its activity; however, the role of Sox9 in direct osteogenesis *in vitro* has been largely overlooked.

Methods: Bone marrow-derived human MSCs (hMSCs) were cultured *in vitro* either in the control or osteogenic medium supplemented with dexamethasone (DEX). To further investigate the role of Sox9 in direct osteogenesis *in vitro*, hMSCs were treated with Sox9 siRNA.

Results: We show here that Sox9 is the key early indicator during *in vitro* osteogenic differentiation of hMSCs. Osteogenic induction leads to a significant decrease of Sox9 gene and protein expression by day 7. Treatment of hMSCs with Sox9 siRNA enhanced mineralization *in vitro*, suggesting that downregulation of Sox9 is involved in direct osteogenesis. siRNA knockdown of Sox9 did not in itself induce osteogenesis in the absence of DEX, indicating that other factors are still required.

Conclusion: Screening of not preselected donors of different ages and gender ($n=12$) has shown that the *Runx2/Sox9* ratio on day 7 is correlated to the ⁴⁵Ca incorporation on day 28. The impact of Sox9 downregulation in the mineralization of human MSCs *in vitro* indicates a so far unprecedented role of Sox9 as a major regulator of direct osteogenesis. We propose that the *Runx2/Sox9* ratio is a promising, early, *in vitro* screening method for osteogenicity of human MSCs.

Introduction

INCREASING AGING POPULATIONS cause a growing need for new approaches to augment and repair bone tissue lost through trauma or disease. To meet this demand, a variety of cells have been utilized to heal these defects, including mesenchymal stem cells (MSCs). MSCs represent an attractive cell population for bone regeneration due to their expansion properties and osteogenic regeneration potential. During skeletal tissue formation, master genes have been identified; however, the rules by which they specify distinct tissue types are still unclear. The Sry-related transcription factor Sox9 is mainly described as the key regulator for chondrogenesis.¹ Sox9 is expressed in resting and proliferating chondrocytes, with a maximum of expression in prehypertrophic chondrocytes, but disappears completely from the hypertrophic zone.² It is proposed that Sox9 downregulation is required to allow the onset of cartilage–bone transition, such as cartilage resorption and formation of bone marrow during development.³ The Runt-related transcription factor Runx2 was identified initially as a positive

regulator of osteoblast differentiation.⁴ It is one of the most studied transcription factors expressed in MSCs upon their commitment toward an osteogenic differentiation.^{5,6} *In vivo*, the sufficiency of Runx2 to induce direct ossification is still hypothetical. Overexpression of Runx2 in chick embryos did not induce ectopic bone formation.⁷ MSC-derived osteochondroprogenitors express both transcription factors, Sox9 and Runx2, during condensation of the skeletal anlagen. Sox9 directly interacts with Runx2 and represses its activity. It was shown previously that osteochondroprogenitor cells differentiate toward osteogenic phenotypes, when Sox9 expression is lower than Runx2, during endochondral bone formation in mice.⁸ The inhibitory effect of Sox9 on osteoblast maturation through Runx2 repression is an essential mechanism for osteochondroprogenitor fate determination.⁹

Besides endochondral ossification as the most commonly occurring process in fracture healing, direct ossification is an important requirement for successful osseointegration of implants.¹⁰ Furthermore, in the area of craniomaxillofacial surgery, most of the fractures heal through direct ossification.¹¹ During *in vitro* differentiation into mature osteoblasts,

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the secretion of an extracellular matrix and mineralization is accompanied by stage-specific expression of bone-related genes.¹² *In vitro* systems for osteogenesis recapitulate events during direct osteogenic differentiation. Several hormonal and growth factors were shown to promote osteogenic differentiation of hMSCs *in vitro*.¹³ Dexamethasone (DEX) is routinely used to promote hMSC osteogenic differentiation and ensure mineralization *in vitro*.^{12,14,15}

The osteogenic potency of hMSCs is commonly characterized by the expression of early markers (alkaline phosphatase [ALP], collagen type I) followed by late markers (bone sialoprotein, osteocalcin).^{12,16} Recent studies have shown that levels of Runx2 during *in vitro* differentiation of primary human osteoblasts showed no major changes in the levels of Runx2 protein or mRNA during human osteoblast differentiation.¹⁶ Commonly used early *in vitro* assays, such as the activity of ALP, may not be proportional to the mineralization levels.¹⁷ However, little is known about the role of Sox9 in direct osteogenesis of hMSCs *in vitro*. Recently, Stockl *et al.* showed that Sox9 has a positive proliferative role and delays osteogenic differentiation in rat adipose stem cells.¹⁸

We hypothesized that Sox9 is an early key regulator during *in vitro* osteogenic differentiation of hMSCs. Moreover, we propose that the ratio between *Runx2* and *Sox9* gene expression can predict the osteogenic differentiation potential of hMSCs.

Materials and Methods

MSC culture

Human bone marrow was harvested from the iliac crest or vertebral body of twelve patients, after full ethical approval (Freiburg, EK-326/08) (mean age: 45.8 years; range: 20–75 years; male:female ratio: 6:6), to isolate MSCs using previously described protocols.¹⁹ P2 MSCs were seeded at 2×10^4 cells/cm² and grown in DMEM low glucose with 10% FBS (Life Technologies), 1% penicillin/streptomycin without (control) or with additives. Cells were incubated at 37°C/5% CO₂ and the medium was refreshed every second day.

Osteogenic differentiation of monolayer expanded cells

Osteogenic differentiation (differentiation medium [DEX]) was induced through supplementation of the control medium with 50 µg/mL ascorbic acid, 100 nM DEX, and 5 mM β-glycerolphosphate. Cells were cultured in control or DEX for 28 days and harvested on days 0, 2, 7, 14, 21, and 28 for analysis.

DNA assay for MSC proliferation

MSCs were digested in proteinase K (0.5 mg/mL) for 16 h at 56°C. The DNA content was measured using Hoechst 33258 (Polysciences) assay, as previously described.²⁰

ALP activity and alizarin red staining

The ALP activity was determined by measuring the formation of p-Nitrophenol (pNp) from p-Nitrophenyl phosphate, as described earlier.¹² Briefly, after extraction at 4°C for 1 h in 0.1% Triton-X in Tris-HCl, absorbance was measured at 405 nm using a Victor3™ plate reader (Perkin Elmer). The ALP activity of cell lysates was calculated according to a generated ALP standard curve. The obtained ALP activity was normalized to the total DNA amount. For alizarin red staining, cell–matrix layers were washed with phosphate-buffered saline (PBS), fixed with 4% formaldehyde, and stained with a 2% alizarin red solution with a pH of 4.3 (Sigma-Aldrich) for 1 h.

⁴⁵Calcium (⁴⁵Ca) radioisotope incorporation

Monolayer cultures were incubated for 16 h with ⁴⁵Ca (1.25 µCi/mL) radioisotope, and the radioactive counts were measured using a scintillation counter WinSpectral (Perkin Elmer). The obtained ⁴⁵Ca incorporation (counts per minute, CPMI) was normalized to the total DNA amount.

MSC optical density in monolayer cultures

MSCs cultured in 24-well plates were washed with PBS. Following the wash step, 500 µL fresh PBS was added to each well and the OD 450 nm was measured followed by the addition of a fresh medium to enable continuation of cell culture.

Quantitative PCR analysis

Total RNA was extracted using TriReagent and RNeasy Kit (Qiagen). Briefly, cells were lysed in TriReagent and 10% (v/v) BCP was added. After centrifugation, the upper phase containing RNA was collected and precipitated by adding of 70% ethanol. RNA was transferred to RNeasy columns and purified according to the manufacturer's instructions. RNA (400 ng) was reverse transcribed using the TaqMan Reverse Transcription kit. RT-PCR was performed using the AB7500 Real-Time PCR System (Applied Biosystems) according to previously described methods.²¹ The specific primer sequences used are listed in Table 1. Data analysis was performed using ddCT values, which were determined by normalization to 18S rRNA and samples harvested on day 0.

TABLE 1. PRIMERS/PROBES USED FOR REAL-TIME POLYMERASE CHAIN REACTION

Gene	Forward primer	Reverse primer	Probe	Applied biosystem reference number
<i>Runx2</i>	AAG CAG TAT TTA CAA CAG AGG GTA CAA G	GGT GCT CGG ATC CCA AAA	CAT CAA ACA GCC TCT TCA GCA CAG TGA CAC	
<i>Sox9</i>				Hs00165814_m1
<i>18S</i>				431089E

RNA interference

The Neon Transfection System (Invitrogen) was used to transfect hMSCs (990V, 40 ms, 1 pulse) with 600 nM Sox9 (buffer concentration, OriGene). Cells were seeded at P2 24 h before electroporation at 50–70% confluence. Cells were harvested 48 h after electroporation and knockdown of Sox9 mRNA was assessed. Negative controls were transfected using scrambled siRNA and no siRNA. Sox9 knockdown cells showing at least 70% reduction of Sox9 gene expression were cultured in the control, DEX, or osteo-permissive medium without dexamethasone (OP) for 28 days. Final osteogenic differentiation on day 28 was assessed by OD 450 nm, ^{45}Ca incorporation, and alizarin red staining.

Protein extraction from human MSC culture and western blotting

hMSCs were harvested after 48 h and 7 days of culture in either the control or DEX medium. Cells were lysed in a hypotonic buffer containing 10 mM HEPES, 10 mM KCl, 0.1 mM EDTA, and 10% IGEPAL with proteinase inhibitor cocktail (PIC; Sigma Aldrich) and centrifuged for 3 min. The remaining cell pellets were resuspended with 20 mM HEPES, 0.4 mM NaCl, 1 mM EDTA, 10% glycerol, and PIC and kept at 4°C with vigorous shaking for 2 h. Nuclear protein extracts were collected from the supernatants after centrifugation for 5 min. The protein concentration was measured with Quick Start™ Bradford Protein Assay (Biorad).

Five micrograms of nuclear protein was used for western blotting assay. Specifically, after denaturation at 95°C for 5 min and addition of 2.5% β -mercaptoethanol, the proteins were loaded on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and then transferred to the nitrocellulose membrane (Bio-Rad). The membranes were blocked in 5% nonfat dried milk and then incubated with different primary antibodies (mouse anti-Sox9, 1:250, Abnova; rabbit anti-Laminin B1, 1:500; Thermo-Scientific) at 4°C overnight. Horseradish peroxidase-labeled secondary antibodies (anti-mouse, 1:20,000, abcam; anti-rabbit, 1:20,000, Amersham) were added for 1 h at room temperature. The Amersham ECL chemiluminescence Detection reagent was used for detecting membrane signals using ImageJ (NIH) software. Laminin B1 served as internal control.

Statistical analysis

All experiments were performed in triplicates. Data were analyzed using SPSS 16.0. The Leven's test for equality of variances and then the independent Student's *t*-test were performed. A difference of $p < 0.05$ was considered significant.

Results

DEX stimulation induces downregulation of Sox9 during early osteogenesis

As described earlier, hMSCs differentiate into osteoblasts in response to a supplementary cocktail of ascorbic acid, β -glycerolphosphate, and DEX.¹² Direct osteogenic differentiation of hMSCs *in vitro* was monitored by early osteogenic markers such as gene expression of *Runx2* and ALP activity, and late osteogenic markers ^{45}Ca incorporation and alizarin red staining.

hMSCs cultured in the control medium had a higher *Sox9* mRNA expression compared to DEX-stimulated MSCs on days 2, 7, and 14, whereas the osteogenic medium led to a downregulation of *Sox9* mRNA expression during early osteogenesis (Fig. 1A, B). Decreased *Sox9* mRNA expression in the DEX group was significant on day 2 and 7 of culture ($p < 0.05$) (Fig. 1A). On day 21 of culture, differences in *Sox9* gene expression of hMSCs in the control and DEX group diminished (Fig. 1B). Next, we determined protein expression of Sox9 in hMSCs on day 7 after osteogenic stimulation with DEX. In line with *Sox9* gene expression, the Sox9 protein signal was reduced in DEX-stimulated cells compared with hMSCs in the control medium (Fig. 1C). However, suppression of Sox9 protein signals is not as strong as *Sox9* gene expression (Fig. 1A). Laminin B1 served as an internal loading control (Fig. 1C).

The *Runx2/Sox9* gene expression ratio is an indicator for osteogenic responsive/sensitive MSC donors

A striking observation in our study was that DEX treatment also induced *Runx2* mRNA downregulation on day 7, although not significant (Fig. 2A). No differences between the control and DEX groups were seen on day 2 (Fig. 1A), while at later time points, *Runx2* was slightly upregulated compared to the control group (Fig. 2B).

Since *Sox9* was downregulated in the DEX group on days 2 and 7 (Fig. 1A) with little change in *Runx2* expression (Fig. 2A), we addressed the balance between *Runx2* and *Sox9*. It is well known that the osteogenic differentiation potential can show huge variations among donors. *Runx2/Sox9* ratios in the DEX group showed significant higher values on day 2 ($p < 0.05$) (Fig. 2C). Noteworthy, *Runx2/Sox9* ratios on day 14 and 21 are much lower compared to day 2 and 7 (Fig. 2C, D).

However, a detailed analysis of each particular donor revealed a correlation between the *Runx2/Sox9* ratio on day 7 and the osteogenic performance. To rule out the possibility that the interplay of *Runx2* and *Sox9* plays a role in early osteogenesis, we have chosen a population of 12 not pre-selected donors.

For better comparison, we first classified MSC donors into either high osteogenic potential or low osteogenic potential. High osteogenic potential was defined as ^{45}Ca incorporation above 100,000 CPM/ μg DNA on day 28 ($n = 4$) and low osteogenic potential as ^{45}Ca incorporation below 80,000 CPM/ μg DNA on day 28 ($n = 4$). This classification revealed that high osteogenic potential MSC donors had a significantly increased *Runx2/Sox9* ratio on day 7 compared with donors with low osteogenic potential (Fig. 3A). Accordingly, MSC donors with higher *Runx2/Sox9* ratios had a significant enhanced ^{45}Ca incorporation on day 28 compared with MSC donors with a low *Runx2/Sox9* ratio (Fig. 3B).

To determine whether *Runx2/Sox9* is correlated to early osteogenic markers, the peak of ALP activity on day 14 (Fig. 3D) was chosen. Although MSC donors in the high osteogenic potential group showed higher ALP activities on day 14, this trend was not significant (Fig. 3C), suggesting that ALP alone is not a sufficient early marker of the calcification potential.

Including the entire population of unselected donors, a positive correlation between the *Runx2/Sox9* ratio on day 7

FIG. 1. *Sox9* gene expression of (A) on day 2 and 7 ($n=12$); (B) on day 14 and 21 ($n=4$), based on expression fold change to day 0 (mean $\pm$ SEM $*p<0.05$); (C) *Sox9* protein analysis: western blot and image analysis on day 7 either in control or dexamethasone (DEX) medium. Laminin B 1 served as internal control; one representative donor ($n=3$).

and ^{45}Ca incorporation on day 28 can be displayed (Fig. 3E). However, on the basis of 12 donors, a *Runx2/Sox9* ratio of above 2 is required to reliably predict an increase in ^{45}Ca incorporation (Fig. 3E).

To address the wide range of osteogenic response of MSC donors upon DEX stimulation, we have chosen ^{45}Ca incorporation on day 28 as the most reliable osteogenic assay.

Downregulation of *Sox9* triggers direct osteogenesis of hMSCs

After demonstrating that *Sox9* downregulation occurs during osteogenic differentiation *in vitro*, we next silenced *Sox9* expression. *Sox9* mRNA after 48 h was reduced by at least 70% by *Sox9* siRNA when normalized to nonelectroporated cells (Fig. 4A). Cells transfected with a scrambled negative control or without siRNA showed no reduction of *Sox9* gene expression (Fig. 4A). Accordingly, *Sox9* siRNA-treated cells showed reduced *Sox9* protein signals after 48 h compared to the control group without siRNA treatment (Fig. 4B). Depending on *Sox9* siRNA concentrations, *Sox9* protein expressions could be reduced by 50% (200 nM) to 62% (600 nM) compared to the control group without siRNA (Fig. 4B). Therefore, for suppression of *Sox9* gene and protein expression by at least 60%, a 600 nM *Sox9* siRNA buffer concentration was used for the following experiments.

Runx2 gene expression was not significantly changed (data not shown). As expected, silencing *Sox9* gene ex-

pression leads to a vastly increased *Runx2/Sox9* gene expression ratio (Fig. 4B).

To investigate the effect of *Sox9* silencing on direct osteogenic differentiation, cells were cultured in the control and DEX medium, respectively. A third group of cells was maintained in the osteo-permissive medium without dexamethasone (OP) to investigate if a knockdown of *Sox9* alone can induce mineralization *in vitro*. ^{45}Ca incorporation, alizarin red staining, and OD 450 nm were chosen as appropriate assays.

^{45}Ca incorporation after 28 days strongly increased when *Sox9* was silenced compared to control groups in the DEX medium, with significance for scrambled and nonelectroporated controls (Fig. 4D). Alizarin red staining on day 28 confirmed an enhanced calcification after *Sox9* silencing (Fig. 4E). OD 450 nm values during the entire period further underlined an increase in mineralization in the DEX medium if *Sox9* was silenced (Fig. 4F). Cells cultured in the OP medium without DEX showed an increase in ^{45}Ca incorporation and enhanced alizarin red stain on day 28 compared with cells cultured in the control medium (data not shown). However, between the groups in the OP medium, no differences in ^{45}Ca incorporation could be observed (data not shown).

Discussion

It is known that the coordination of *Runx2* and *Sox9* is highly important for the process of cartilage and bone development. *Sox9* decreases *Runx2* binding to its target

FIG. 2. Runx2 gene expression (A) on day 2 and 7 ($n=12$); (B) on day 14 and 21 ($n=4$); Runx2/Sox9 ratio (C) on day 2 and 7 ($n=12$), (D) on day 14 and 21 ($n=4$), based on expression fold change to day 0 (mean $\pm$ SEM * $p < 0.05$).

sequences and drastically inhibits Runx2 either through preventing transactivation of osteoblast-specific enhancers or direct protein degradation of the gene itself.^{9,22} The complete absence of Sox9 from hypertrophic chondrocytes suggests that Sox9 downregulation is required to allow the onset of subsequent events, for example, endochondral bone formation.³ This indicates the dominance of Sox9 function over Runx2 during skeletogenesis. In endochondral ossification, the formation of hypertrophic cartilage is a prerequisite step in bone development.^{23,24}

The present study indicates that Sox9 downregulation is required for direct osteogenesis of hMSCs (Fig. 1A). It was shown recently that isolated MSCs from Sox9 transgenic mice showed decreased osteoblast differentiation,²⁵ thereby confirming the importance of inhibiting Sox9 activity in osteoblast differentiation. Yet, the regulatory role of Sox9 in direct osteogenesis of human MSCs has not yet been identified.

Furthermore, Runx2 and Sox9 mRNA expression during direct osteogenesis might be reflective of the interplay between these two transcription factors. Our data imply that a critical factor in this interplay is the decrease in Sox9 expression that leads to a vastly increased Runx2/Sox9 ratio. In fact, we suggest that the ratio between Runx2 and Sox9 gene expression is a key marker of osteogenic potential.

A high ratio of Runx2/Sox9 gene expression on day 7 maintains a high osteogenic potential for the related donor as shown by the extended ALP activity on day 14 and ⁴⁵Ca incorporation on day 28 (Fig. 3B, C). In contrast, human MSCs with a lower osteogenic potential show a very low

ratio of Runx2/Sox9 gene expression (Fig. 3A). Although the ALP activity peaks around day 14 of DEX stimulation (Fig. 3D), a potential shift of very high/low osteogenic MSC donors may mask the actual ALP activity peak.¹⁷ Therefore, we assume that the ALP activity is not optimal for a correlation with the Runx2/Sox9 gene expression profile. We suggest that the Runx2/Sox9 ratio is correlated to the ⁴⁵Ca incorporation on day 28 (Fig. 3E), thus linking mRNA expression levels with cell function. Even though ⁴⁵Ca incorporation on day 21 showed a good correlation to the ratio of Runx2/Sox9 (data not shown), MSC donors with delayed osteogenic differentiation are rather included after 28 days (Fig. 3B, E).

We screened various donors of different ages and gender who were not preselected ($n=12$) either with or without DEX stimulation to confirm our hypothesis (Fig. 3E). It has never been demonstrated that the Runx2/Sox9 ratio implicates the osteogenic potential of human MSCs.

This might be useful in evaluating freshly isolated hMSCs with regard to their osteogenic differentiation capacity. *In vitro* culturing of MSCs for scientific questions usually requires time- and cost-intensive procedures. Our data assume that the ratio of Runx2/Sox9 at the earliest points of *in vitro* culture can predict if the particular donor has osteogenic potential. Although we could demonstrate that Sox9 is already downregulated after 2 days of DEX stimulation, we suggest day 7 to be the most reliable time point (Fig. 1A). A period of 7 days may include MSC donors with a potentially delayed proliferation/differentiation profile and minimizes the risk of stress-induced effects on the gene expression

FIG. 3. (A) *Runx2/Sox9* ratio on day 7 (mean $\pm$ SEM * $p < 0.05$); (B) ^{45}Ca incorporation on day 28 (mean $\pm$ SEM * $p < 0.05$), (C) alkaline phosphatase (ALP) activity on day 14, for 8 representative donors with low ($n = 4$) and high ($n = 4$) osteogenic potential (high osteogenic potential defined as above 100,000 CPMI/ μ g DNA on day 28; low osteogenic potential as below 80,000 CPMI/ μ g DNA on day 28); (D) ALP activity for donors with both low and high osteogenic potential ($n = 8$); (E) correlation of ^{45}Ca incorporation on day 28 with *Runx2/Sox9* ratio on day 7; for unselected population of donors ($n = 12$).

FIG. 4. (A) Electroporation of hMSCs with Sox9 siRNA, gene expression after 48 h based on expression fold change to day 0; *indicates significant difference between Sox9 siRNA and controls ($n=6$, mean $\pm$ SEM, $*p<0.05$), (B) protein analysis: western blot and image analysis of Sox9 protein after Sox9 siRNA electroporation (48 h) in control medium. Laminin B 1 served as internal control; one representative donor ($n=3$); (C) Runx2/Sox9 ratio after 48 h, one representative donor; (D) ^{45}Ca incorporation on day 28 either in control or DEX medium; *indicates significant difference between Sox9 siRNA treatment and scrambled control and control, respectively ($n=6$, mean $\pm$ SEM, $*p<0.05$); (E) staining of alizarin red S on day 28 after Sox9 siRNA treatment, either in control or DEX medium; representative images (scale bar: 200 μm); (F) optical density graphs in DEX medium; *indicates significant difference between Sox9 siRNA and control groups ($n=6$, mean $\pm$ SEM $*p<0.05$). Color images available online at www.liebertpub.com/tea

level. Since the average of *Runx2* gene expression was rather downregulated on day 7 (Fig. 2A), the *Runx2/Sox9* on day 7 may be even more sensitive to predict the osteogenic potential of each particular donor (Fig. 3E). In addition to the consolidated findings on gene expression levels, we could further demonstrate downregulation of Sox9 protein expression on day 7, when cells are osteogenic stimulated (Fig. 1C). Although the effect on protein levels was less distinctive, it supports the impact of Sox9 downregulation during early osteogenic differentiation *in vitro*. However, aiming for an early *in vitro* screening and evaluation system for the osteogenic potential of hMSCs, quantitative mRNA expression levels may be superior.

To achieve a better understanding of the underlying molecular pathway, we analyzed the osteogenic differentiation of hMSCs after suppressing *Sox9* mRNA (Fig. 4). Sox9 silencing successfully reduced Sox9 protein signals (Fig. 4B). The chosen Sox9 siRNA concentration allowed a reduction of *Sox9* mRNA and protein by at least 60% (Fig. 4A, B).

To elucidate the role of Sox9 in direct osteogenesis, we cultured the electroporated hMSCs of six not preselected donors in the control, DEX, or OP medium without DEX.

If Sox9 is regulatory in direct osteogenesis, decreased Sox9 may potentiate the effect of DEX or be a direct stimulus. It is conceivable that cells in the control medium will not mineralize even with a downregulated Sox9, due to lack of phosphates in the medium¹² (Fig. 4D). Although our data have shown that Sox9 does not solely regulate direct osteogenesis *in vitro*, downregulation of Sox9 enhances the mineralization process¹⁸ (Fig. 4D, E). An increased absorbance in the measurement of the optical density at 450 nm supported our findings (Fig. 4F). This assay is useful as an online method, where calcified areas appear darker because of a higher refractive index.²⁶ The analysis of absorbance at 450 nm is a quick and inexpensive approach to evaluate osteogenic differentiation of monolayer cultured cells.

Previously, Stockl *et al.* showed that Sox9 has a positive proliferative role and delays osteogenic differentiation in rat adipose stem cells.¹⁸ Our results clearly demonstrated a drop of *Sox9* gene expression after 7 days of osteogenic culture in a large number of donors (Fig. 1A) that is further supported by lower Sox9 protein expression on day 7 (Fig. 1C). However, upstream events resulting in Sox9 downregulation during early osteogenesis remain unknown. Additionally, it remains unclear if osteogenic induction induces a drop of Sox9 gene expression or rather a slow decrease over time. Therefore, correlating mRNA and protein levels should consider the importance of kinetics and a steady-state comparison might be misleading due to the dynamics of genetic information processing across transcription and translation.²⁷

Our data further imply that downregulation of Sox9 before osteogenic induction may be too early to take over the whole mineralization process. A better understanding of the regulatory network would further elucidate if knockdown of Sox9, in turn, may switch on inhibitory genes.

However, the ratio of *Runx2* and *Sox9* on day 7 can predict the osteogenic potential of each particular donor, suggesting that the balance of both genes is the result of earlier regulatory events. A more detailed analysis of the interplay between *Runx2* and *Sox9* during direct osteogenesis *in vitro* is required for a better understanding of this process.

To our knowledge, this is the first report that the *Runx2/Sox9* ratio correlates with direct osteogenic differentiation of hMSCs *in vitro*. Although the complex dynamics and the regulation control at many levels have not been elucidated in this study, the aim is to present a powerful early screening tool for *in vitro* osteogenic differentiation. Further work is required to establish any correlation between the *Runx2/Sox9* ratio and *in vivo* outcome.

Conclusion

The impact of Sox9 downregulation in mineralization of human MSCs *in vitro* indicates a so far unprecedented role of Sox9 as a major regulator of direct osteogenesis. This supports the hypothesis that Sox9 leads to a direct or indirect suppression of *Runx2*. Rather than focusing on *Runx2* as a crucial factor of direct osteogenesis, Sox9 seems to be the key to better understand *in vitro* osteogenesis of hMSCs. We propose the *Runx2/Sox9* ratio to be a promising early screening method for osteogenicity of human MSCs.

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Author Contribution

CL: Study conduct, data analysis and interpretation, and manuscript writing. EMC: Study conduct, data analysis and interpretation, and manuscript writing. BM: Study conduct, data analysis and interpretation, and manuscript writing. GS: Study conduct, data analysis and interpretation, and final approval of manuscript. MA: Conception and design, data analysis and interpretation, manuscript writing, and final approval of manuscript. MJS: Conception and design, data analysis and interpretation, manuscript writing, and final approval of manuscript. MJS takes responsibility for the integrity of the data analysis.

Disclosure Statement

None of the authors has anything to declare.

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