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Epigenetic Signatures at the RUNX2-P1 and Sp7 Gene Promoters Control Osteogenic Lineage Commitment of Umbilical Cord-Derived Mesenchymal Stem Cells

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Wharton's Jelly mesenchymal stem cells (WJ-MSCs) are an attractive potential source of multipotent stem cells for bone tissue replacement therapies. However, the molecular mechanisms involved in their osteogenic conversion are poorly understood. Particularly, epigenetic control operating at the promoter regions of the two master regulators of the osteogenic program, RUNX2/P57 and SP7 has not yet been described in WJ-MSCs. Via quantitative PCR profiling and chromatin immunoprecipitation (ChIP) studies, here we analyze the ability of WJ-MSCs to engage osteoblast lineage. In undifferentiated WJ-MSCs, RUNX2/P57 P1, and SP7 promoters are found deprived of significant levels of the histone post-translational marks that are normally associated with transcriptionally active genes (H3ac, H3K27ac, and H3K4me3). Moreover, the RUNX2 P1 promoter lacks two relevant histone repressive marks (H3K9me3 and H3K27me3). Importantly, RUNX2 P1 promoter is found highly enriched in the H3K4me1 mark, which has been shown recently to mediate gene repression of key regulatory genes. Upon induction of WJ-MSCs osteogenic differentiation, we found that RUNX2/P57, but not SP7 gene expression is strongly activated, in a process that is accompanied by enrichment of activating histone marks (H3K4me3, H3ac, and H3K27ac) at the P1 promoter region. Histone mark analysis showed that SP7 gene promoter is robustly enriched in epigenetic repressive marks that may explain its poor transcriptional response to osteoblast differentiating media. Together, these results point to critical regulatory steps during epigenetic control of WJ-MSCs osteogenic lineage commitment that are relevant for future applications in regenerative medicine.

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Controlling specific lineage commitment of human mesenchymal stem cells (hMSCs) represents a principal task in biomedical research. hMSCs, a heterogeneous population consisting of cells with distinct morphologic and functional characteristics, have been isolated from almost all available post- natal and fetal tissue sources (Murray and Peault, 2015; Sheng, 2015). hMSCs can be obtained at significantly larger quantities of cells compared to embryonic stem cells making possible to establish potentially relevant, therapeutic-linked procedures (Todeschi et al., 2015). One relevant source of hMSCs is the umbilical cord Wharton's Jelly (Erices et al., 2000; Wang et al., 2004; Bieback and Kluter, 2007; Weiss et al., 2008). Wharton's Jelly (WJ) corresponds to the connective tissue found around the principal vein and arteries of the umbilical cord and represents the major source of mesenchymal cells in the whole tissue (Nekanti et al., 2010). Based on internationally accepted criteria, a subpopulation of these WJ mesenchymal cells (WJ-MSCs) are defined as bona fide hMSCs as they express specific surface antigens including CD29, CD73, and are negative for endothelial, CD31, and hematopoietic, CD45, CD34 markers (Murray et al., 2014). WJ-MSCs can be isolated from fresh umbilical cord tissue following reported protocols (Edwards et al., 2014) and cultured given their high proliferative

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properties, under conditions that preserve their multipotent stem cell potential for several passages (Edwards et al., 2014; Fong et al., 2016). It has been shown that WJ-MSCs are multipotent as they can be induced to differentiate toward the chondrogenic, osteogenic, and adipogenic lineages (La Rocca and Anzalone, 2013). In addition, these cells can differentiate to other cell types, including some that are of non-mesodermal origin like neuron- and oligodendroglial-like lineages (Leite et al., 2014).

Albeit these multipotent properties, recent reports also indicate that WJ-MSCs may possess a significantly higher ability to engage adipogenic and chondrogenic, instead of osteogenic, lineage commitment (La Rocca et al., 2009), thus raising concerns about their potential utilization in developing new cell-based therapies to treat human pathologies that include bone-forming deficiencies.

Bone formation mainly depends on osteoblast differentiation, a process that is tightly controlled by osteoblast master transcriptional regulators that in turn are expressed in response to a coordinated set of extracellular stimuli and developmentally regulated signaling pathways (Marie, 2008; Komori, 2011). Commitment of mesenchymal precursor cells to the osteoblast lineage requires the function of two key transcription factors: RUNX2 (Runt-related transcription factor 2) and SP7 (also known as Osterix). Both proteins control the expression of numerous critical bone-matrix protein coding genes, including BGLAP (Komori, 2011). Furthermore, independent ablation of RUNX2 or SP7 genes in vivo prevents formation of a normal skeleton (Komori et al., 1997; Otto et al., 1997; Nakashima et al., 2002).

Accumulating evidence indicates that epigenetic mechanisms are critical components during control of tissue-specific gene expression (Boland et al., 2014). Among these regulatory components are the histone post-translational modifications (PTMs) which are deposited and turned over through the activity of specific nuclear enzymes. These PTMs can function as docking sites on the chromatin fiber as they can be potentially recognized by specific domains shared among proteins that mediate transcriptional control (Yun et al., 2011). It has been extensively recognized that these epigenetic mechanisms regulate gene transcription during osteoblast lineage commitment (Montecino et al., 2015). Thus, we and others have shown that active expression and silencing of the bone-related RUNX2/P57 gene isoform are epigenetically regulated during osteoblast and myoblast differentiation in murine models (Cruzat et al., 2009; Yang et al., 2013; Tai et al., 2014; Rojas et al., 2015; Zhang et al., 2015). Mechanisms regulating RUNX2/P57 expression include enrichment of the histone H3 lysine 4 tri-methylation (H3K4me3) and H3K27 acetylation (H3K27ac) marks when transcription of the gene is activated during osteoblast differentiation. On the other hand, enrichment of the H3K4me1 and H3K27me3 marks occur when RUNX2 gene is repressed during myogenesis (Rojas et al., 2015). These results have prompted us to further evaluate the contribution of these epigenetic parameters on the ability of WJ-MSCs to undergo osteoblast lineage commitment in presence of osteogenic cultured medium.

Here, we report that although WJ-MSC respond to an osteoblast differentiating stimuli by transcribing RUNX2/P57 gene associated with an increase in the levels of histone H3 acetylation (including H3K27ac) and H3K4me3 at the RUNX2-P1 gene promoter, they also exhibit significant limitations to terminally differentiate toward the osteoblast lineage. This limitation is associated with the inability of these cells to activate SP7 gene expression under differentiating conditions as demonstrated by a robust enrichment of repressive histone epigenetic marks at the SP7 proximal promoter.

Together, these results indicate that RUNX2 and SP7 epigenetic regulation mechanisms are critical for the control of WJ-MSCs' commitment to the osteogenic lineage that are relevant for future applications in regenerative medicine.

Materials and Methods

Isolation and culture of Wharton's Jelly-derived mesenchymal stem cells (WJ-MSCs)

The protocols concerning the work with human-derived cells were approved by ethics committees from the Faculty of Sciences of Universidad de Chile and Universidad Andres Bello. Healthy pregnant women without regular medication, attending routine antenatal care at Hospital Dr. Luis Tisné Brousse, Santiago, Chile, were invited to participate in the study. Written consent from these patients was obtained. Umbilical cords were obtained immediately after delivery and transported from the maternity ward to the laboratory, where Whartońs Jelly-derived MSCs were isolated as described (Edwards et al., 2014). Briefly, pieces of umbilical cords were cut into 1 cm^2 medium pieces and blood vessels removed. The remaining after tissue was then cut into 2 mm^2 pieces and digested with collagenase I (1 μg/μl, Thermo Fisher, Waltham, MA). To obtain clean cellular pellets, several centrifugation steps (2,000 rpm, 10 min) were carried out. Cells were then suspended in Growth Medium: Dulbecco's modified Eagle's medium (DMEM, Thermo Fisher) supplemented with 10% fetal bovine serum (FBS, Biological Industries, Israel) and antibiotics (100 U/ml Penicillin/Streptomycin, Thermo Fisher). Cell cultures were maintained in a humidified atmosphere containing 5% CO2 at 37°C. Osteogenic and adipogenic induction assays were performed as previously described (Mosna et al., 2010). Briefly, WJ-MSCs were incubated with osteoblast differentiation media (DMEM supplemented with 10% fetal bovine serum, 50 μg/ml ascorbic acid, 1 mM β-glycerophosphate, and 100 nM dexamethasone and 300 ng/ml BMP2) or adipogenic differentiation media (DMEM supplemented with 1 μM dexamethasone, 500 μM IBMX, 60 μM indomethacin, and 10 μg/ml insulin) in cell culture dishes at a density of 4–5 × 105 cells/cm^2.

Saos-2 cells culture

Saos-2 cells (American Type Culture Collection HTB-85) were cultured in DMEM media (Thermo Fisher Scientific) supplemented with 10% FBS (Hyclone) and cultured at 37°C and 5% CO2.

Reverse transcriptase and quantitative real-time PCR (qPCR) analyses

Total RNA was extracted with TRIzol (Life Technologies) according to the manufacturer's instructions. An equivalent amount of each sample (2 μg) was used for reverse transcription analyses (PROMEGA, Madison, WI, #M610A). qPCR was performed using Brilliant II SYBR® Green QPCR Master Mix (Agilent Technologies, Santa Clara CA) in a Stratagene Mx3000P thermal cycler Agilent tech. Primer concentrations in qPCR reactions were previously standardized for 100% efficiency. Primers used were: RUNX2/P57 Fw: GGT TAA TCT CCG CAG GTC AC, Rev: GTC ACT GTG CTG AAG AGG CT; SP7/Osterix Fw: GCC AGA AGC TGT GAA ACC TC, Rev: TGA TGG GGT CAT GGT GTC TA; PPAR-γ Fw: GCC TTG CAG TGG GGA TGT CTC, Rev: CCT CGC CTT TGC TTT GGT CAG; AP-2 Fw: CCT TAG ATG GGG GTG TCC TGG TA, Rev: AAC GTC CCT TGG CTT ATG CTC TC; Oct4 Fw: GTA CTC CTC GGT CCC TTT CC, Rev: CAA AAA CCC TGG CAC AAA CT; ACTA-2 Fw: ACT GCC TTG GTG TGT GAC AA, Rev: CAC CAT CAC CCC CTG ATG TC; Desmin Fw: CCC TGA AGG GCA CTA ACG AT, Rev: TCC TTG AGG TGC CGG ATT TC; GAPDH Fw: AGA AGG CTG GGG CTC ATT TG, Rev: AGG GGC CAT CCA CAG TCT TC.

Chromatin immunoprecipitation (ChIP) assays

Chromatin immunoprecipitation (ChIP) assays were performed in cross-linked chromatin samples as described earlier (Soutoglou and Talianidis, 2002; Grandy et al., 2011), with the following modifications: Confluent cell cultures (100-mm diameter plates) were incubated for 10 min with 1% formaldehyde (FA) in gentle agitation at room temperature (RT). The cells were washed with 10 ml of PBS three times, re-suspended in five volumes of cell lysis buffer (CLB; 5 mM Hepes, pH 8.0, 85 mM KCl, 1% Triton X–100, and a mixture of proteinase inhibitors), and homogenized with a Dounce homogenizer (~25 times using a tight pestle). The cell extract was collected by centrifugation at 3,000*g* for 5 min, re-suspended in 0.5 ml of sonication buffer (50 mM Hepes, pH 7.9, 140 Mm NaCl, 1 mM EDTA, 1% Triton X–100, 0.1% deoxycholate acid, 0.1% SDS, and a mixture of proteinase inhibitors), and incubated for 10 min on ice. Chromatin was sheared in a water bath Sonicator Bioruptor (Diagenode Inc., Denville, NJ) to obtain fragments of 200–500 bp. Extracts were sonicated at high power for three pulses of 5 min each, and centrifuged at 16,000*g* for 15 min at 4°C. Supernatant was collected, aliquoted, frozen in liquid nitrogen, and stored at −80°C; one aliquot was used for A260 measurements. Chromatin size was confirmed by electrophoretic analysis. Cross-linked chromatin extracts (25 μg) were re-suspended in sonication buffer to a final volume of 500 μl; samples were pre-cleared by incubating with 2–4 μg of normal IgG and 50 μl of protein A/G-agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at 4°C with agitation. Chromatin was centrifuged at 4,000*g* for 5 min and the supernatant immunoprecipitated with specific antibodies (see list below for antibodies used) for 12–16 h at 4°C. The immune complexes were recovered with addition of 50 μl of protein A or G-agarose beads, followed by incubation for 1 h at 4°C with gentle agitation. Immunoprecipitated complexes were washed once with sonication buffer, twice with LiCl buffer (100 mM Tris–HCl, pH 8.0, 500 mM LiCl, 0.1% Nonidet P40, and 0.1% deoxycholic acid), and once with Tris–EDTA (TE) buffer pH 8.0 (2 mM EDTA and 50 mM Tris–HCl, pH 8.0), each time for 5 min at 4°C; this was followed by centrifugation at 4,000*g* for 5 min. The protein–DNA complexes were eluted by incubation with 100 μl of elution buffer (50 mM NaHCO3 and 1% SDS) for 15 min at 65°C. Extracts were centrifuged at 10,000*g* for 5 min, and the supernatant was collected and incubated for 12–16 h at 65°C, to reverse the cross-linking. Proteins were digested with 100 μg/ml of proteinase K for 2 h at 50°C, and the DNA was recovered by phenol/chloroform extraction and ethanol precipitation using glycogen (20 μg/ml) as a precipitation carrier. The qPCR primers used to evaluate the promoter regions were: RUNX2/P57 (−171/+10) Fw: GTG GTA GGC AGT CCC ACT TTA, Rev: AGA AAG TTT GCA CCG CAC TTG; SP7 (−165/+28) Fw: CAG CAA ATG GAG CAG GAA AT, Rev: AAG GGA GAG GGA GGG AGA AT; GAPDH (−191/−125) Fw: CGG GAT TGT CTG CCC TAA T, Rev: GCA CGG AAG GTC ACG ATG T. The following antibodies were used in ChIP assays: Total H3 (ab1791, Abcam, Cambridge, MA), Acetyl H3 (06–599, Millipore, Billerica, MA), H3K4me1 (ab8895, Abcam), H3K4me3 (ab8580, Abcam), H3K9me3 (ab8898, Abcam), H3K27ac (ab4729, Abcam), H3K27me3 (07–449, Millipore), normal rabbit IgG (12–370, Millipore).

Statistical analysis

We used a one-way analysis of variance (ANOVA) analysis followed by the Dunnett post-test to compare significant changes with respect to controls. In all figures, error bars represent the mean ± Standard Deviation; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Results

We have previously established a standardized procedure to isolate and culture WJ-MSCs ex vivo, demonstrating that under these experimental conditions the cells are capable to retain at least for up to five passages their ability to differentiate toward various cell types (Edwards et al., 2014). Primary cultures obtained from three independent umbilical cords were initially characterized by evaluating the expression of a set of mRNAs coding for pluripotency- and osteoblast-related markers. As shown in Figure 1, WJ-MSCs express mRNAs encoding for OCT4 (Fig. 1A), a critical transcription factor required for pluripotent stem cell identity (Sun et al., 2006). This result is in agreement with the accepted notion that WJ-MSCs exhibit an intermediate potency between embryonic and adult MSCs. In agreement with previous findings, WJ-MSCs show elevated ACTA2 and DESMIN mRNA expression (Fig. 1B and C) (Toma et al., 2002), confirming the MSC identity of our samples. Importantly, WJ-MSCs do not exhibit significant mRNA expression from the RUNX2/P57, SP7, and BGLAP genes (Fig. 1D–F, respectively). As control, consistently with previous reports we show that under our experimental conditions RUNX2/P57, SP7, and BGLAP genes are abundantly expressed in the human osteoblastic cell line SaOS-2 (Fig. 1C–F, respectively) (Sudhakar et al., 2001; Mitsui et al., 2006).

In order to evaluate commitment to the osteogenic or adipogenic lineages, we next determined the ability of WJ-MSCs to respond to specific and well-established differentiation media (see Materials and Methods). Following incubation of the cells with osteoblastic (OB) media for 2 or 4 days, there is a significant increase in RUNX2/P57 mRNA expression (Fig. 2A). These same incubation periods, however, did not activate the expression of other osteoblastic genes like SP7 and BGLAP (Fig. 2B and C). Moreover, expression of these two genes is not induced even after 14 days of culture with OB media (data not shown). As it has been shown that expression of SP7 and BGLAP occurs downstream of RUNX2/P57 expression during osteoblast differentiation in vivo (Nakashima et al., 2002), these results indicate that although WJ-MSCs readily respond to the differentiating stimuli and express the master regulator gene of osteogenesis RUNX2/P57, they are unable to fully reproduce the physiological gene expression program that leads to terminally differentiated osteoblast in vivo. To clarify if this limitation was simply related to a generally reduced differentiating potential in these cells, we evaluated their ability to respond to a well-established adipogenic (AD) media. It has been shown that WJ-MSCs readily respond to this media and reproduce the adipogenic differentiation process observed in vivo, including the expression of early and late phenotypic markers (Mosna et al., 2010). We found that in response to this AD media our WJ-MSCs are capable of robustly express early PPARG and late AP2 adipogenic genes (Fig. 2D and E, respectively). As expected, we determined that while WJ-MSCs engage osteogenic and adipogenic differentiation, the expression of the pluripotency gene marker OCT4 is reduced (Fig. 2F). Taken together, these results indicate that WJ-MSCs can engage initial stages of osteoblast differentiation in response to OB media, reflected by the expression of the master regulator of osteogenesis RUNX2/P57. On the other hand, and in agreement with previous reports (Amable et al., 2014), WJ-MSCs grown in AD media acquire the adipogenic phenotype in accordance with the differentiation potential reported for these cells.

We and others have shown that transcriptional activation and repression of the RUNX2/P57 gene is accompanied by selective deposition and elimination of histone post-translational modifications during differentiation of mesenchymal cells to the osteogenic and myogenic lineages (Cruzat et al., 2009; Yang et al., 2013; Tai et al., 2014; Rojas et al., 2015; Zhang et al., 2015). Similarly, SP7 gene regulation has been shown to be controlled by specific epigenetic mechanisms (Gordon et al., 2015; Zhang et al., 2015). Therefore, we next determined the relative enrichment of

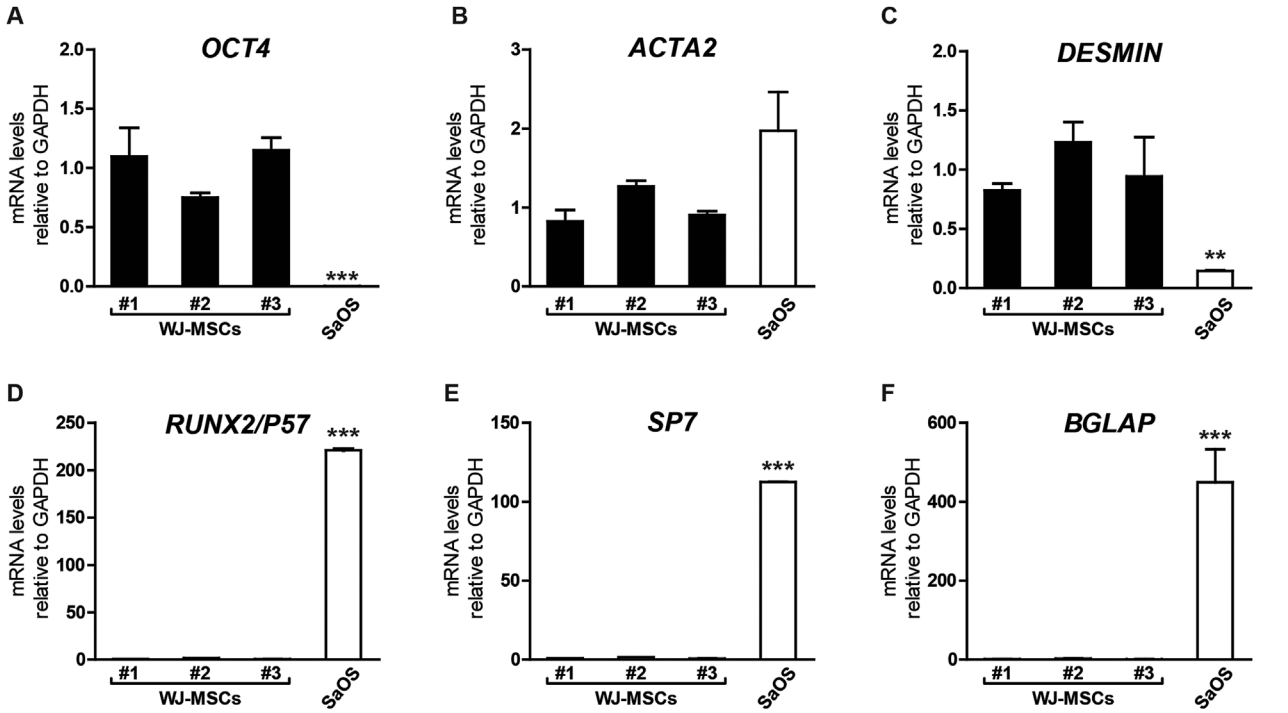

Fig. 1. Reduced expression of osteoblastic genes in Wharton's Jelly-derived Mesenchymal Stem Cells (WJ-MSCs). Total RNA was obtained from WJ-MSCs isolated from three independent umbilical cords (#1, #2, and #3, as indicated). mRNA levels were analyzed by qRT-PCR using specific primers for the indicated genes. We evaluated pluripotent- (A), mesenchymal- (B and C), and osteoblastic-associated (D–F) gene markers. Samples from human osteosarcoma SaOS-2 cells were used as positive controls for expression of bone-related genes. Results were normalized against GAPDH and expressed as fold change ± SD with respect to average levels detected in samples of WJ-MSCs. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

these histone epigenetic marks at the RUNX2/P57 osteoblast-specific P1 and the SP7 proximal promoter regions in WJ-MSCs via ChIP assay. Our results indicate that both promoters lack significant enrichment of histone marks that are normally associated with transcriptional activity, including H3Ac (Fig. 3B), H3K4me3 (Fig. 3D), and H3K27Ac (Fig. 3F). As a control we show that in the same samples these three histone marks are robustly enriched at the transcriptionally active GAPDH gene promoter. Interestingly, both osteoblast-specific promoters exhibit striking differences in the enrichment levels of histone marks associated with gene repression. Thus, the repressive marks H3K9me3 and H3K27me3 that are highly enriched at the SP7 proximal promoter region are not strongly detected at the RUNX2-P1 promoter (Fig. 3E and G, respectively). These two repressive histone marks are not detected at the active GAPDH gene promoter. The differences in H3K9me3 and H3K27me3 enrichment between both osteoblastic promoters are not due to variations in the total histone H3 enrichment as both promoter regions exhibit comparable levels of this critical nucleosomal histone (Fig. 3A). Importantly, the H3K4me1 mark, normally found associated with enhancers (Heintzman et al., 2007) as well as with active, repressed, and poised genes (Cheng et al., 2014), is shown to be highly enriched on both RUNX2-P1 and SP7 gene promoters (Fig. 3C). Together, these results indicate that albeit the RUNX2-P1 gene promoter region is transcriptionally inactive in WJ-MSCs, the promoter is not significantly associated with the two principal epigenetic histone marks that have been shown to mediate gene repression. Rather, this promoter exhibits a histone mark that has been shown to maintain gene promoters in a poised state. On the other hand, the SP7 gene promoter exhibits an epigenetic configuration that is in accordance with its transcriptionally silent status.

We next addressed if incubation of WJ-MSCs with OB media results in changes in the histone marks profile at the RUNX2-P1 promoter that parallel transcriptional activation of the RUNX2/P57 gene. As shown in Figure 4, culturing these cells with OB media for 4 days resulted in a significant increase in the enrichment of the transcriptionally active associated marks H3Ac (Fig. 4B), H3K4me3 (Fig. 4D), and H3K27Ac (Fig. 4F) at the RUNX2-P1 promoter, reaching values that are comparable with those found at the transcriptionally active GAPDH gene. Changes of this magnitude are not due to enrichment of histone H3 at the P1 promoter region (Fig. 4A) and were not detected at the SP7 gene promoter, that remained transcriptionally silent under this treatment (see Fig. 2B) and maintained the predominance of silencing epigenetic marks. Collectively, these results indicate that culturing of WJ-MSCs in the presence of media containing well known osteogenic inductors (including BMP-2 and ascorbic acid, among others) leads to an enrichment of active epigenetic histone marks on the P1 promoter that accompanies transcriptional activity of the RUNX2/P57 gene. Nevertheless, this stimulatory effect is not extended to the SP7 gene that is maintained silent. Accordingly, the expression of downstream late bone markers as BGLAP was not induced under these conditions (Fig. 1F), suggesting that an epigenetic barrier in these cells severely limits an efficient progression of the osteoblast differentiation process. This conclusion also raises when the differentiation of WJ-MSCs to the osteoblast lineage

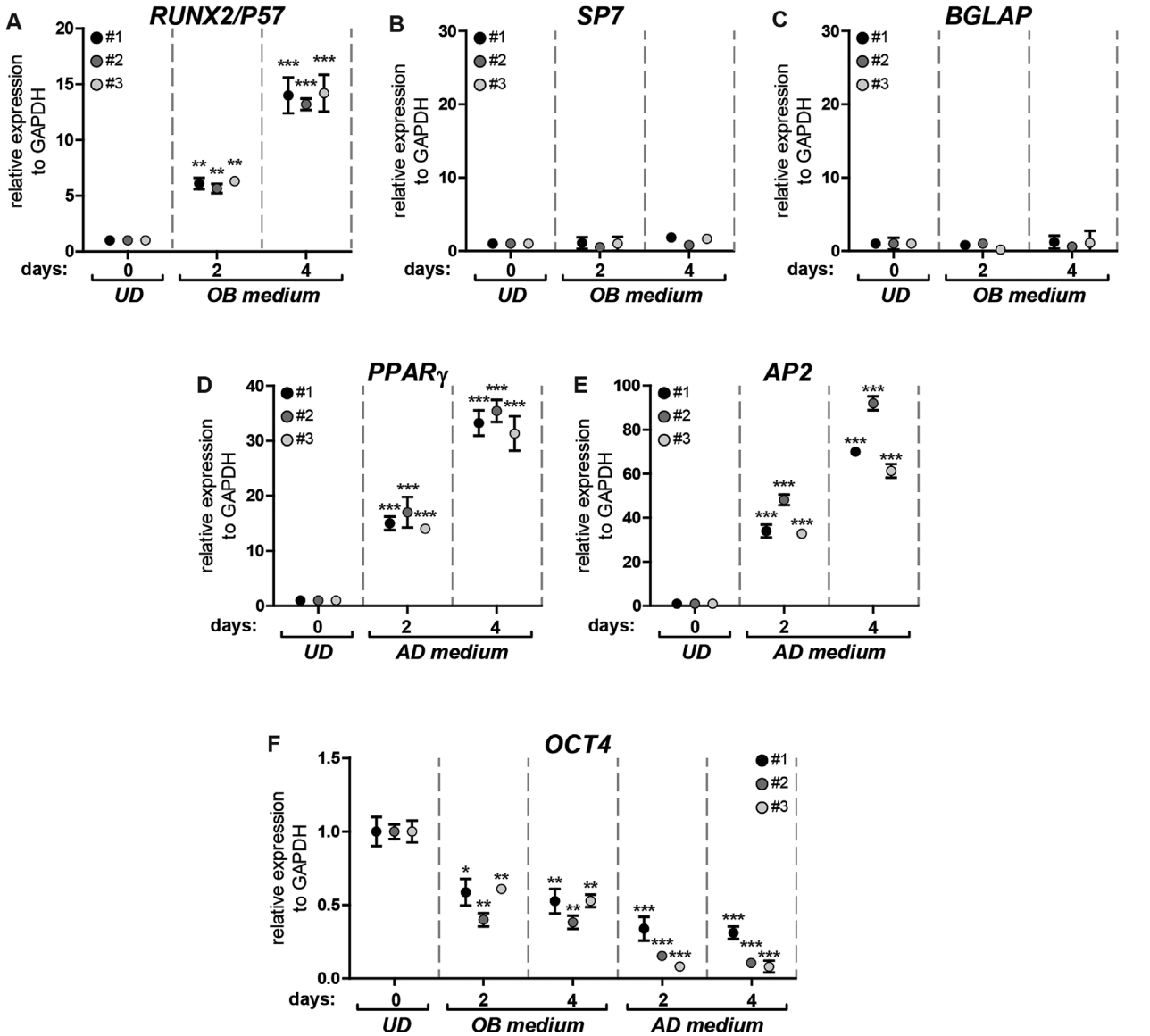

Fig. 2. WJ-MSCs induced to differentiate to osteoblasts do not express late bone phenotypic markers. Undifferentiated WJ-MSCs (UD) isolated from three independent umbilical cords (#1, #2, and #3, as indicated in each panel) were induced to differentiate by incubation with osteoblastic (OB) or adipocytic (AD) differentiation media for up to 4 days. Total RNA was obtained and mRNA levels of RUNX2/P57 (A), SP7 (B), BGLAP (C), PPAR-γ (D), AP2 (E), and OCT4 (F) genes were evaluated by qRT-PCR using specific primers. Results were normalized against GAPDH and expressed as fold change ± SD with respect to undifferentiated cells (UD). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

is evaluated in situ by detecting Alkaline Phosphatase activity (APA) associated with the extracellular matrix. As shown in Figure 5, only a small number of cells expresses this differentiation marker following 8 days in the presence of OB media. This result indicates that although cells with true osteogenic potential are present in our WJ-MSCs, they only represent a very small proportion of the total cell population.

Discussion

To understand the underlying regulatory mechanism of WJ-MSC osteogenic potential, we have analyzed epigenetic post-translational modifications at histone proteins associated with regulatory promoter regions of two critical osteoblast master transcriptional regulators, RUNX2/P57 and SP7 in WJ-MSCs. We found that upon induction of osteoblast differentiation WJ-MSCs mainly engage the initial stages of the process, reflected by the expression of the RUNX2/P57 gene but not of the SP7 gene. As it has been established that SP7 expression represents a molecular event that occurs downstream of expression of RUNX2/P57 (Nakashima et al., 2002), our results suggest that WJ-MSCs grown in the presence of osteoblast differentiating media exhibit the potential of turning toward this lineage. However, the ability of these cells to progress through this differentiation process is limited, likely due to the presence of suppressing epigenetic signatures at the SP7 gene promoter.

We and others have previously demonstrated that the expression of the RUNX2/P57 master regulator of osteoblast

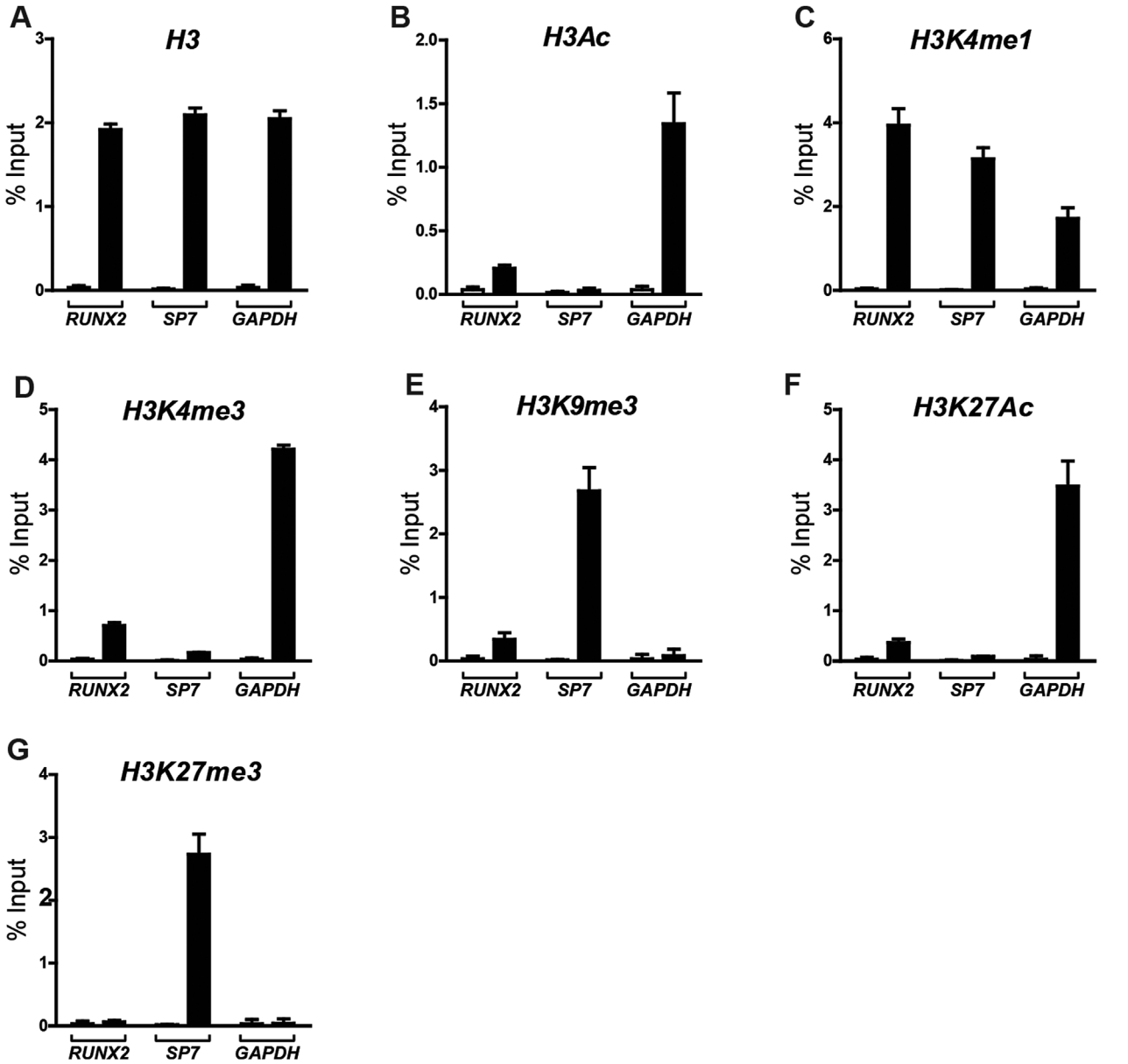

Fig. 3. Epigenetic histone modifications at the osteoblast RUNX2/P57 and SP7 master gene promoters in undifferentiated WJ-MSCs. Chromatin immunoprecipitation (ChIP) assays were performed in samples obtained from undifferentiated WJ-MSCs. Chromatin was incubated with antibodies against total histone H3 (A), acetylated H3 (B), H3K4me1 (C), H3K4me3 (D), H3K9me3 (E), H3K27ac (F), and H3K27me3 (G). Enrichment levels (% Input ± SD) obtained for normal unspecific IgG (white bars) or for the specific histone marks indicated in each graph (black bars) were determined by qPCR using specific primers against RUNX2/P57 (−171/+10), SP7 (−165/+28), and GAPDH (−191/−125) gene promoter regions.

lineage commitment is epigenetically controlled (Cruzat et al., 2009; Yang et al., 2013; Tai et al., 2014; Dudakovic et al., 2015; Rojas et al., 2015; Zhang et al., 2015; Aguilar et al., 2016). Particularly, it has been shown that enrichment of histone marks normally associated with gene repression accompany RUNX2/P57 transcriptional silencing gene during hippocampal maturation (Aguilar et al., 2016). Moreover, it has been demonstrated that complementary epigenetic mechanisms progressively and efficiently silence critical osteoblastic genes during non-osteoblastic differentiation (Rojas et al., 2015; Aguilar et al., 2016). Analyses of the RUNX2 P1 promoter in undifferentiated WJ-MSCs reveal a very low enrichment of repressive histone marks like H3K9me3 and H3K27me3, which contribute to RUNX2/P57 silencing in other cell types (Yang et al., 2013; Tai et al., 2014; Rojas et al., 2015; Zhang et al., 2015; Aguilar et al., 2016). This surprising result indicates that additional regulatory components are mediating silencing at the RUNX2/P57 P1 promoter in un-stimulated WJ-MSCs. Because of the elevated enrichment of the H3K4me1 mark detected at this regulatory region, it is tempting to speculate about a potential inhibitory role of this modification. Recent studies demonstrate that maintaining H3K4me1 elevated at regulatory promoter regions can be critical for inhibiting transcriptional activation (Cheng et al., 2014). This H3K4me1-dependent

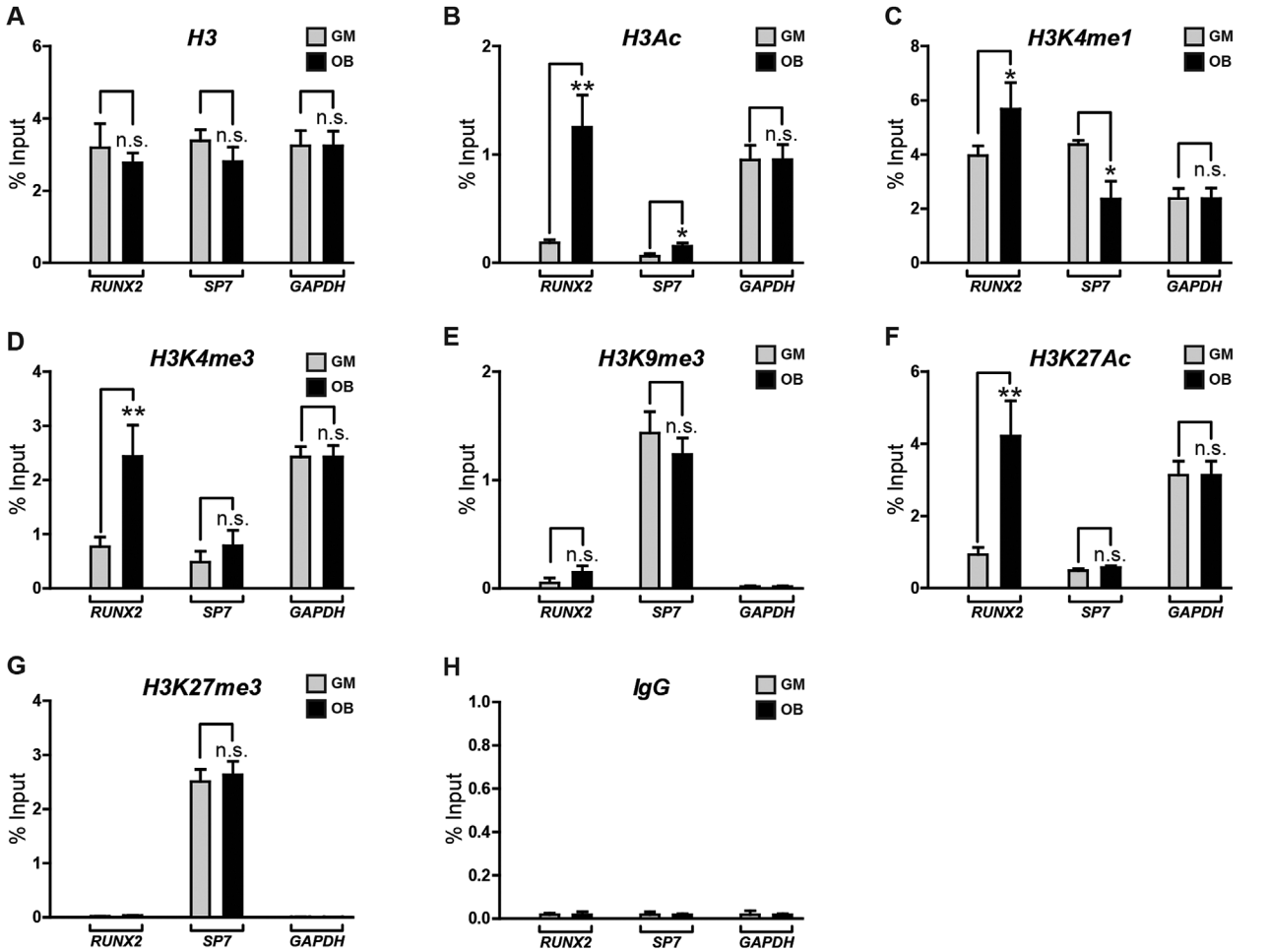

Fig. 4. Osteoblastic-induction of WJ-MSCs results in enrichment of activating histone marks at the RUNX2/P57 gene P1 promoter but does not change the repressive epigenetic state at the SP7 promoter. ChIP assays were performed in chromatin samples obtained from WJ-MSCs incubated with osteoblastic differentiation medium (OB) for 4 days. Samples from cells maintained in growing media (GM) for the same period of time, were also collected and used as controls. Chromatin was incubated with antibodies against total histone H3 (A), acetylated H3 (B), H3K4me1 (C), H3K4me3 (D), H3K9me3 (E), H3K27ac (F), H3K27me3 (G), and normal unspecific IgG (H). The precipitated DNA was quantified using specific primers against RUNX2/P57 (−171/+10), SP7 (−165/+28), and GAPDH (−191/−125) promoters. Results are shown as % Input ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

repressive state is mostly maintained by MLL3/4-containing COMPASS-like complexes which do not mediate further di- and tri-methylation of the H3K4 residue (Cheng et al., 2014). Interestingly, it was further shown that depletion of the expression of either MLL3 or MLL4 methyltransferases results in both transcriptional activation of the target genes and enrichment in the H3K4me3 mark, in a process mediated by the SET1-methylase-containing complex. Hence, H3K4me1 can serve as a mechanism to maintain a gene silent and at the same time poised for rapid activation following appropriate signaling. Whether an equivalent complex mechanism is also operating at the RUNX2 P1 promoter is currently under investigation.

Our team has recently demonstrated that reduced levels of the H3K27me3 mark at the RUNX2 P1 promoter that follows EZH2 methyltransferase knock-down in pre-myoblastic cells does not prevent RUNX2/P57 repression during myogenic differentiation (Rojas et al., 2015). Instead, we determined that RUNX2 repression during this process is mainly mediated through the presence and activity of the H3K4me3 demethylase JARID1B/KDM5B. Accordingly, knock-down of JARID1B/KDM5B results in elevated H3K4me3 at the P1 promoter in murine pre-myoblastic cells and prevents RUNX2/P57 gene silencing after induction of myogenesis (Rojas et al., 2015). Together, these results further-support a model where reaching appropriate high enrichment of H3K4me3 at the RUNX2 P1 promoter region represent a key step during transcriptional activation of this osteogenic master regulator.

We find that the inability of WJ-MSCs to activate SP7 transcription is directly associated with the presence of the repressive histone marks H3K9me3 and H3K27me3, as well as with an elevated enrichment of the H3K4me1 mark, at the SP7 proximal promoter region. Upon induction of osteogenic differentiation, the presence of these repressive epigenetic marks H3K9me3 and H3K27me3 are not significantly altered, hence indicating that under these experimental conditions the mechanisms leading to their removal at the SP7 promoter cannot be activated. Further studies are aimed to investigate the identity of the enzymes involved in the epigenetic modifications at the SP7 promoter.

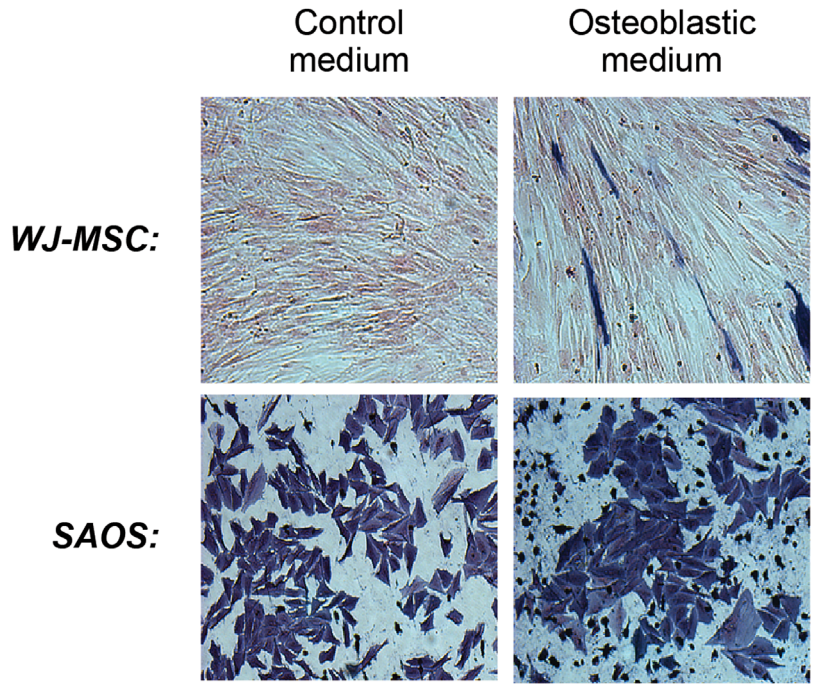

Fig. 5. A reduced number of WJ-MSCs retain the potential to fully differentiate to osteoblasts. Alkaline Phosphatase (ALP) activity associated with the extracellular matrix (blue staining) was determined in situ to evaluate the ability of WJ-MSCs to engage osteoblast differentiation. The results indicate that only a reduced number of cells within the population expresses this bone-related marker after 8 days of incubation in OB media.

Alternatively, other additional repressive epigenetic mechanisms may be operating at the SP7 gene further preventing its transcriptional activation in WJ-MSCs. Among them is the well-established DNA methylation that accompanies SP7 gene silencing in several non-osteoblastic cell types (Lee et al., 2006). DNA CpG methylation is well correlated with the enrichment of the H3K9me3 mark at many silent gene regulatory regions (Fuks et al., 2003; Lehnertz et al., 2003), functioning therefore simultaneously as complementary mechanisms to repress gene expression. Studies leading to address the contribution of SP7 gene methylation to transcriptional silencing in this system are currently underway in our team.

Manipulating WJ-MSCs' cell fate will be of crucial importance for future biomedical applications. For this, it is critical to fully understand the molecular mechanisms controlling their differentiation potentials. RUNX2 levels, alkaline phosphatase activity and calcium deposit formation has widely been used to demonstrate WJ-MSCs osteogenic potential (Penolazzi et al., 2009; Kouroupis et al., 2013; Loebel et al., 2015). Therefore, WJ-MSC with true osteogenic potential are present in the heterogeneous culture but appear to constitute only a very small proportion of cells. To our knowledge, neither are reports of SP7 expression in WJ-MSC during osteoblastic differentiation nor has the absence of its expression been proposed to explain the reduced osteogenic lineage differentiation of WJ-MSCs. hMSCs derived from different tissues show significant variations in their differentiation potential, which depend on the tissue they originate from as they receive inputs that directly affect their specification (Fernandez-Moure et al., 2015). It has been shown that WJ-MSCs exhibit reduced osteogenic potential compared to bone marrow MSCs (Kouroupis et al., 2013). Hence, the results showed here may constitute a molecular explanation to those differences. Furthermore, SP7/Osterix overexpression has been demonstrated to increase hMSC osteogenic potential (Lee et al., 2011; Wang et al., 2013).

It is tempting to hypothesize that WJ-MSC are more primed to an alternative phenotype. As multipotent stem cells, WJ-MSCs are able to undergo three mesodermal lineages whose are mutually repressing each other (Lee et al., 2014; Chen et al., 2016). Unraveling the molecular mechanisms controlling WJ-MSC osteogenic/adipogenic/chondrogenic determination will be of key importance to suppress the influence of adipogenic or chondrogenic signals to enhance the efficacy of WJ-MSCs osteoblastic differentiation. Although, our results begin revealing the basis WJ-MSC limitations to efficiently engage osteogenic lineage commitment, we identify critical epigenetic regulatory steps that need to be overcome in order to drive these cells through osteoblast differentiation for tissue replacement.

Authors' Contribution

H.S. and R.A. designed and performed most of the experiments and analyzed the data. C.P. and F.B. performed experiments and analyzed data. B.v.Z. provided technical and conceptual advice and analyzed the results. V.P. and M.M. supervised the research, analyzed the data, and wrote the main parts of the manuscript.

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