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Canonical WNT Signaling Pathway is Altered in Mesenchymal Stromal Cells From Acute Myeloid Leukemia Patients And Is Implicated in *BMP4* Down-Regulation[1]

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Abstract

Mesenchymal stromal cells (hMSCs) are key components of the bone marrow microenvironment (BMM). A molecular signature in hMSCs from Acute myeloid leukemia patients (hMSC-AML) has been proposed where *BMP4* is decreased and could be regulated by WNT signaling pathway. Therefore, the aim of this work was to verify whether the WNT signaling pathway can regulate the *BMP4* gene in hMSCs. The results showed differentially expressed genes in the WNT canonical pathway between hMSC-AML and hMSCs from healthy donors and a real-time quantitative assay corroborated with these findings. Moreover, the main WNT canonical pathway regulators were decreased in hMSC-AML, such as LEF-1, β-catenin and the β-catenin/TCF-LEF regulatory complex in the nucleus. This result, together with functional assays, suggests that the induction of *BMP4* expression by the WNT signaling pathway is decreased in hMSC-AML. Overall, the WNT canonical pathway is able to regulate the *BMP4* gene in hMSC-AML and its reduced activation could also lead to the lower expression of *BMP4* in hMSC-AML. Due to the important role of the BMM, changes in *BMP4* expression through the WNT canonical pathway may be a potential mechanism of leukemogenesis.

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Introduction

Acute myeloid leukemia (AML) is a hematological disease characterized by cellular differentiation arrest, decreased apoptosis levels, increases in proliferation and the accumulation of myeloid precursors in the bone marrow (BM) [1].

AML is extremely heterogeneous, and the cellular and molecular basis for this heterogeneity represents a fundamental problem. Despite this heterogeneity, Lapitop and coworkers described that AML has a unique origin: the malignant transformation of normal hematopoietic stem cells (HSCs) into leukemic stem cells (LSCs). Similar to normal HSCs, LSCs maintain the ability to self-renew and the potential to repopulate and produce progeny cells. However, these cells generate leukemia progenitors and leukemic blast cells, consequently perpetuating the leukemia population [2]. To date, other studies have also confirmed this proposed model [3–5]; nevertheless, the events related to AML initiation and progression remain unclear.

The idea that LSCs have stem cell characteristics suggests that HSCs undergo mutation(s), an intrinsic mechanism of tumor biology, that gives rise to LSCs [6]. In seeking to identify mutations present in LSCs from AML patients that could be related to leukemic transformation, Shlush and coworkers identified mutations in the *DNMT3A* and *NMP1* genes that were present in LSCs from several AML patients. However, not all LSCs presented these mutations [7]. The evidence suggests that other factors could play important roles in cancer progression. In this context, changes in signaling in the BM microenvironment, where HSCs are located, could promote malignant transformation [8].

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The BM microenvironment is complex and dynamic and has a cellular and molecular signaling network coordinated to maintain and regulate the functions of HSCs [9,10]. Alterations in the different components of the BM microenvironment, including fibroblasts, adipocytes, endothelial cells, the extracellular matrix and mesenchymal stromal cells (hMSCs), could play important roles in the context of leukemia initiation [11].

hMSCs are critical for regulating and maintaining HSCs [12,13]. hMSCs are multipotent cells that are present in the niche that generates most marrow stromal cell lineages, including osteoblasts, chondrocytes, fibroblasts, adipocytes, endothelial cells and myocytes [14]. These cells can regulate the balance between self-renewal and differentiation of HSCs through cell–cell interactions and paracrine secretion of cytokines and growth factors in the extracellular matrix [15].

Due to the importance of hMSC, the malignant transformation that generates LSCs could be related to changes in mesenchymal stromal cell signaling.

Based on this supposition, Binato et al. showed a molecular signature in AML mesenchymal stromal cells (hMSC-AML) that was different from that of hMSCs derived from healthy donors (hMSC-HD). Among the genes found in this molecular signature, *BMP4* presented decreased expression in hMSC-AML and in plasma from the same patients, indicating changes in the signaling of hMSC-AML [16].

BMP4 (Bone Morphogenetic Protein 4), a member of the superfamily of TGF-β growth factors, is a protein that is secreted into the BM microenvironment, and decreases in its expression can result in the alteration of HSC function [16,17], as Goldman and coworkers showed that BMP4 is able to regulate the number of HSCs [18]. Therefore, decreased *BMP4* expression in hMSC-AML can promote alterations in the maintenance of HSCs and, consequently, could be related to leukemic transformation.

In silico analyses have provided evidence that *BMP4* could be regulated by the WNT signaling pathway [16]. The interactions between the WNT and BMP4 signaling pathways are well described during embryonic development [19–21], the induction of myogenic differentiation [22] and in human colon cancer [23]. However, *BMP4* gene regulation by the WNT signaling pathway in hMSCs remains unclear.

In this context, the aim of this work was to verify whether the WNT signaling pathway can act in *BMP4* gene regulation in hMSCs. The data presented in this work provides evidence that the canonical WNT signaling pathway is less active in hMSC-AML than in hMSC-HD. We also suggest that the decrease in *BMP4* in hMSC-AML is associated with a reduction in β-catenin/TCF-LEF complex formation in the *BMP4* promoter region.

Materials and Methods

Patient and Donor Samples

BM-derived samples were obtained from patients with AML at diagnosis (without any treatment) and from healthy donors (HD) registered at the Bone Marrow Transplantation Unit, National Cancer Institute (INCA) (Rio de Janeiro, Brazil). The AML samples (mean age: 41.3) were morphologically characterized according to the FAB classification [24] (Table 1). The samples used as controls were obtained from HD with a mean age of 30.1 years (Table 2). These patients and donors were stratified into six cohorts (Tables 1 and 2). All samples were obtained in accordance with the guidelines of the local Ethics Committee and the Declaration of Helsinki. This study

Table 1. List of AML patients that participated in this study

Laboratory code	FAB subtype	% Blasts	Sex	Age	PCR array cohort	RT-qPCR cohort	WB cohort	IF cohort	IP cohort
005/12	M4/M5	80%	Male	43	X	X	X		
006/12	M3	85%	Male	68		X	X		
007/12	M1/M2	75%	Female	9		X			
011/12	M1/M2	64%	Female	13		X			
012/12	M1/M2	60%	Female	13		X			
008/13	M2	98%	Male	42		X			
009/13	M2	56%	Male	30		X			
010/13	M2	38%	Male	25	X	X	X		
014/13	LMA unclassified	60%	Male	55		X			
017/13	M3	71.8%	Female	21		X			
023/13	M3	53,2%	Male	38	X	X			
025/13	M4/M5	75%	Male	46		X			
026/13	LMA unclassified	34%	Female	86		X			
028/13	M2	17%	Male	26		X			
036/13	M3	84%	Female	29		X	X		
045/13	M3	75%	Male	31		X			
050/13	M2	25%	Male	28		X			
051/13	M1	67%	Male	50		X			
053/13	M1	90%	Male	32		X			
055/13	LMA unclassified	60%	Male	32		X			
056/13	M4/M5	90%	Female	60		X			
059/13	M3	98%	Female	47		X			
051/14	M1	67%	Male	52	X	X	X		
054/14	LMA unclassified	60%	Male	42		X			
017/16	M3	20%	Male	37		X		X	
018/16	M3	68%	Male	37		X	X		
019/16	M1/M2	-	Male	64		X			
022/16	M3	60%	Female	38		X		X	
031/16	LMA unclassified	65%	Male	53		X	X		
033/16	M3	55%	Male	32		X		X	X
035/17	LMA	20%	Male	61		X			
036/17	M3	35%	Male	60		X	X		
051/17	LMA	37%	Female	60		X		X	X
055/17	M4/M5	50%	Female	46		X		X	X

WB = Western Blot, IF- Immunofluorescence, IP- Chromatin immunoprecipitation.

Table 2. List of healthy donors that participated in this study

Laboratory code	Sex	Age	PCR array cohort	RT-qPCR cohort	WB cohort	IF cohort	IP cohort
DOD 2	Female	25		X	X		
DOD 3	Female	32	X	X			
DOD 5	Male	23	X	X	X		
DOD 08/16	Male	32		X		X	
DOD 09/16	Male	25		X		X	
DOD 10/16	Male	18		X		X	
DOD 3/17	Female	30		X	X	X	X
DOD 4/17	Male	28		X	X	X	X
DOD 5/17	Male	45		X		X	X
DOD 6/17	Female	22		X			
DOD 48	Female	32		X			
DOD 50	Male	29		X			
DOD 55	Female	55		X			
DOD 60	Male	61		X			
DOD 61	Male	32		X			
DOD 96	Male	25		X	X		
DOD 97	Male	18		X			
DOD 100	Male	19	X	X	X		
DOD 101	Female	24	X	X	X		
DOD 102	Female	60	X	X			
DOD 103	Male	26		X			
DOD 104	Female	33		X	X		
DOD 106	Female	19		X			
DOD 107	Male	25		X			
DOD 108	Female	35	X	X			

WB = Western Blot, IF- Immunofluorescence, IP- Chromatin immunoprecipitation.

was approved by the INCA Ethics Committee (no.034/06), and all participants signed informed consent forms.

Isolation and Culture of hMSCs

hMSCs derived from BM samples from AML patients and HD were cultured as previously described [16]. The cells were maintained at 37°C in a humidified atmosphere with 5% CO_2. When the hMSC cultures reached 80% confluence, the hMSCs were removed from the plates by treatment with 0.05% trypsin (Invitrogen™) for 5 min at 37°C and then replated in another culture flask at a density of 2×10^3 cells/cm^2 (passage 1). These processes were repeated until passage 3, when the hMSCs were used for all experiments.

Confirmation of hMSCs

To characterize the hMSCs, experiments were performed as previously described [16], in accordance with the minimal criteria for defining multipotent mesenchymal stromal cells as defined by the International Society for Cellular Therapy (ISCT) [25].

WNT Signaling Pathway Analysis

RNA was isolated using a RNeasy® mini kit (Qiagen) according to the manufacturer's instructions. A total of 800 ng of high-quality RNA was then reverse transcribed using the RT^2 First Strand Kit (Qiagen), and the cDNA was subsequently loaded into a Human WNT Signaling Pathway RT^2 Profiler PCR Array according to the manufacturer's instructions (PAHS-043Z, Qiagen). Data were normalized to the average expression of *GAPDH*, *B2M* and *ACTB*. The data were analyzed using the platform GeneGlobe (www.qiagen.com/br/shop/genes-and-pathways/data-analysis-center-overview-page/).

Real-Time Quantitative PCR (RT-qPCR) Analysis

RNA was extracted using TRIzol reagent (Invitrogen™) according to the manufacturer's instructions.

RT-qPCR analyses were performed using 4 μg of mRNA treated with amplification-grade DNaseI (Invitrogen™) and reverse transcribed with SuperScriptIII Reverse transcriptase® (Invitrogen™) following the manufacturer's protocol. Each reaction was performed with 5 μL of SYBR Green PCR Master Mix® (Applied Biosystems), 2.5 μL of cDNA (10 ng) and 2 μM of each primer. Reactions were performed in a Rotor-Gene 6000 thermocycler (Qiagen) as follow: 95°C for 10 min, followed by 40 cycles of 95°C for 20s and 60°C for 30s with a final extension at 72°C for 30s.

The relative quantification was performed according to a standard curve-based method [26]. The expression levels of specific genes (Table 3) were estimated and *B2M* and *GAPDH* were used as normalization genes.

Western Blot Analysis

Protein extracts were obtained as previously described [27]. Protein extracts (30 μg) were separated with 10% SDS-PAGE gel electrophoresis, transferred to nitrocellulose membranes (Bio-Rad), and incubated with anti-LEF1 antibody (Santa Cruz Biotechnology) at 4°C overnight, followed by incubation with the appropriate secondary antibody at room temperature (RT) for 2 h. Rouge Ponceau staining was used as the loading control. Signal was acquired using Image Studio Digits software v3.1 with a LI-COR instrument (Uniscience Co.), after chemiluminescence reaction (Pierce™ ECL Plus Western Blotting Substrate, Thermo Scientific).

Immunofluorescence Assay

hMSCs plated on coverslips were fixed with 4% paraformaldehyde, permeabilized with 0.3% Triton X-100 diluted in PBS (T-PBS) and incubated for 75 min at RT with 1% bovine serum albumin (BSA) and 0.1% T-PBS. The coverslips were rinsed with 0.3% T-PBS and incubated with appropriately diluted primary antibodies (anti-β-catenin and anti-LEF1 antibodies-Santa Cruz Biotechnology) in 0.05% Tween and 0.1% BSA diluted in PBS for 3 days at 4°C in a humid chamber. After incubation, the coverslips were rinsed with 0.3% T-PBS and incubated with Alexa Fluor® 488-conjugated anti-mouse IgG or Alexa Fluor® 555-conjugated anti-goat IgG (Molecular Probes) for 2 h at RT. The coverslips used as controls were incubated only with the secondary antibody or the diluted primary antibodies. The coverslips were mounted with VECTASHIELD antifade medium containing DAPI (Vector Labs). Proteins were evaluated for their expression and location with a Leica TCS SP5 confocal laser scanning microscope (Leica®) to capture representative images of each sample.

Prediction of TCF/LEF Binding Sites in the BMP4 Promoter

To screen for putative TCF/LEF consensus binding sites, 3 kb upstream of the transcription start site of the *BMP4* gene was acquired from the NCBI database. Next, we identified the consensus binding sequence for TCF/LEF transcription factors (5′-CTTTGA-3′; 5′-CTTTAG-3′) [28,29].

The consensus binding sites were confirmed using online tools: TRANSFAC (http://www.gene-regulation.com), Tfsitescan (www.

Table 3. List of primers used in this study

Gene	Forward	Reverse
Primers used for RT-qPCR analysis		
BMP4	CCATGATTCCTGGTAACCGA	CCTGAATCTCGGCGACTT
KREMEN1	CGTCTCTCTGGACTTCGTCATCTT	CCTGTGGCAGTTCTTCCTTGA
LEF1	CAGACATCCTCCAGCTCCTGATAT	CGTGATGGGATATACAGGCTGAC
PORCN	CCCTCCTACATGGCTTCAGTT	CCGCTTTGACAAGACACAGG
PRICKLE1	GGTGCTCAGCATGTGACGAGATAA	TCACACTCAAGGCAGCAGAAGT
TCF7	ACTCTTCCCGGACAAACTTCC	GCAGATTGAAGGCGGAGTAGAC
GAPDH	ATTCCACCCATGGCAAATTC	GGCGTGGATGGGTCTTTCA
B2M	ATGAGTATGCCTGCCGTGTGA	CGGCATCTTCAAACCTCCATG
Primers used for RTq-PCR after chromatin immunoprecipitation assays		
S1 Chip*BMP4*	ACTGTAAAAAACGTGGCCCCAGC	ACCCTGAGGTAGACCCCAGTAAAT
S2 Chip*BMP4*	GTGATTGATTTAGGGGCTCAGTGA	CCTAATGTTTCTCCTGCAGCATCAG
S3 Chip*BMP4*	TTGGAAACTCCTGGACTGTGAGTG	GCACCAATGTCATTTCGGGGT
S4 Chip*BMP4*	GTGGGGAGAGAAATAAAGCTGTCC	CGACATACCATGTTTAGACCCCTG
S5 Chip*BMP4*	GGACCAGGAAGTCTGCATTTCA	TAGCTGGTCAATAGCCTGTCTGC
S6 Chip*BMP4*	GCCCAGCATATTCTTTGCCTGT	CAAGGTTCTCTGGTCATTCTGAGC

ifti.org/Tfsitescan/), Genomatix (www.genomatix.de) and GenAtlas (genatlas.medecine.univ-paris5.fr/).

The alignment among sequences from mammals was performed using the Ensembl orthology tool (https://www.ensembl.org). The primers presented in Table 3 were used for RTq-PCR after chromatin immunoprecipitation assays.

Chromatin Immunoprecipitation (ChIP) Assays

ChIP assays were conducted using the SimpleChIP® Enzymatic Chromatin IP kit according to the manufacturer's instructions (Cell Signaling Technology). Briefly, chromatins that had been previously prepared and digested was incubated with 2 μg of LEF-1 antibody (Santa Cruz Biotechnology) or with normal anti-IgG rabbit antibody (negative control). Then, the DNA was purified, and RT-qPCR assays were performed using the specific primers for each putative *BMP4* binding site listed above. The reactions were performed in a Rotor-Gene 6000 thermocycler (Qiagen) using the following program: 95°C for 10 min, followed by 40 cycles at 95°C for 20s and 60°C for 30s with a final extension at 72°C for 30s. Changes in LEF1 binding to DNA were calculated in relation to that of the IgG-precipitated control, normalized to the input.

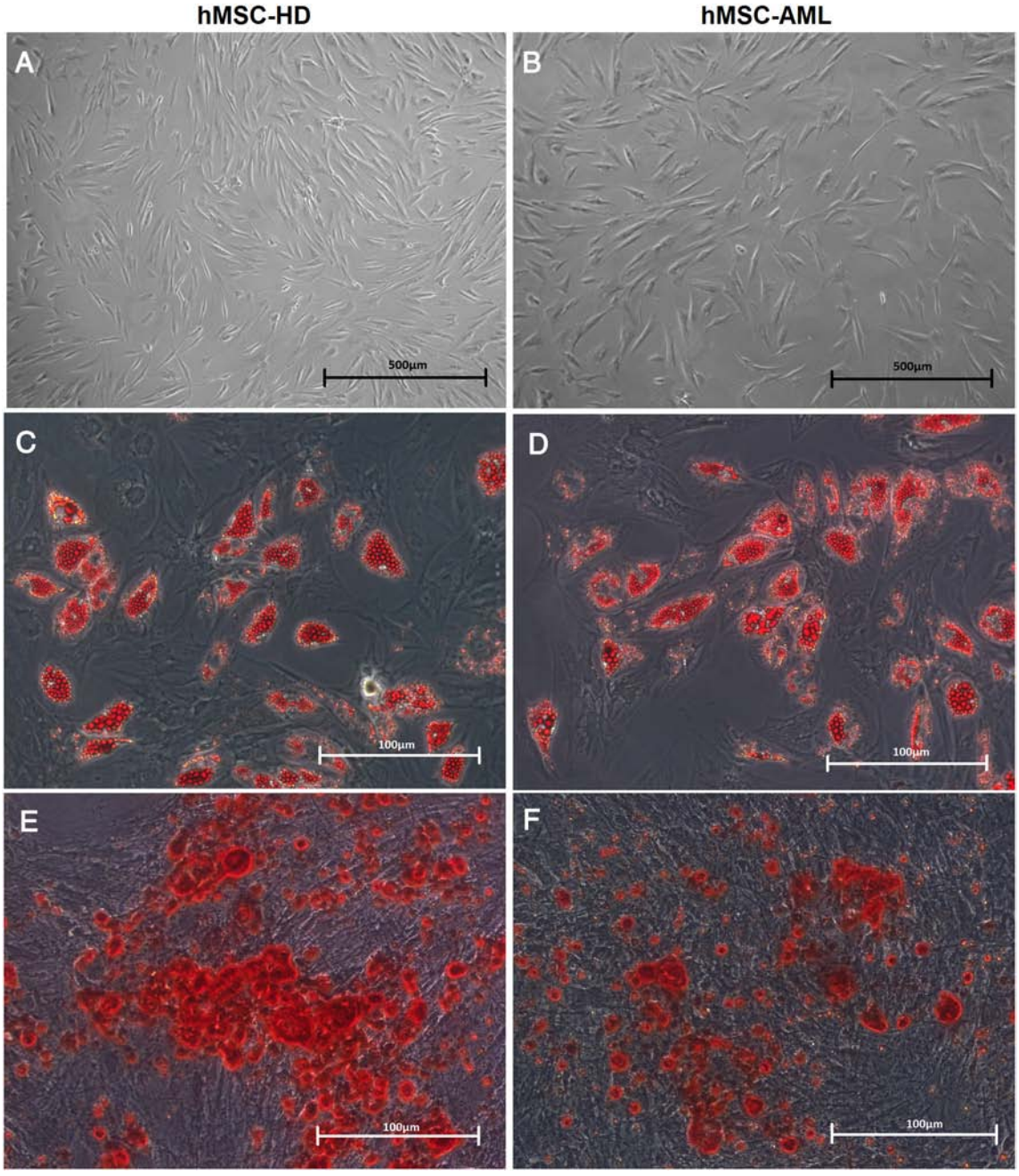

Figure 1. hMSC multipotency capacity. (**A** and **B**) Undifferentiated hMSC-HD and hMSC-AML, respectively (200× magnification). (**C** and **D**) Adipogenic differentiation of hMSC-HD and hMSC-AML, respectively. The accumulation of neutral lipid vacuoles stained with Oil Red O indicates cell differentiation (200× magnification). (**E** and **F**) Osteogenic differentiation of hMSC-HD and hMSC-AML, respectively. The calcium deposition stained with Alizarin Red indicates cell differentiation (50× magnification). hMSC-HD: mesenchymal stromal cells derived from healthy donors; hMSC-AML: mesenchymal stromal cells derived from AML patients.

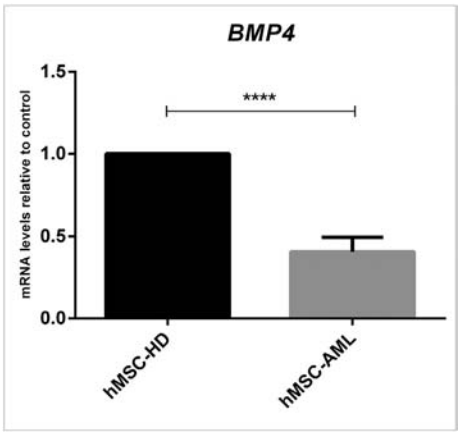

Figure 2. *BMP4* is down-regulated in hMSC-AML. To verify the *BMP4* expression, we used RT-qPCR assay to determine changes in the mRNA expression obtained from hMSC-AML and from hMSC-HD cultures. Data normalization was performed using the endogenous genes *B2M* and *GAPDH*. The bars indicate the mean mRNA levels (± standard deviation). **** P <0 .0001. hMSC-HD: mesenchymal stromal cells derived from healthy donors; hMSC-AML: mesenchymal stromal cells derived from AML patients.

Statistical Analysis

All experiments were carried out in triplicate, and the data are expressed as the mean ± standard error of the mean. The data were compared using unpaired Mann–Whitney tests, and a p-value <0.05 was considered statistically significant (*P <0.05, **P < 0.01, ***P < 0.001, and ****P <0 .0001). Statistical analysis was performed and graphical representations were created using GraphPad Prism™ software (GraphPad Software Inc.).

Results

In Vitro Differentiation Potential of hMSC Cultures and Down-regulation of BMP4

To verify the hMSC multipotent differentiation capacity from all cultures used in this study, we induced hMSC-HD and hMSC-AML in passage 3 to differentiate into adipogenic and osteogenic cells *in vitro*. Undifferentiated hMSC-HD and hMSC-AML were used as controls (Figure 1, *A* and *B*). Our results showed that both cultures were able to differentiate, indicating preservation of their multipotent capacity according to the criteria of the ISTC. However, it was interesting to observe that while no differences between cultures were observed after adipogenic differentiation (Figure 1, *C* and *D*), the calcium accumulation observed after osteogenic differentiation presented some differences in hMSC-AML compared to hMSC-HD (Figure 1, *E* and *F*). Both cultures were able to differentiate into osteogenic cells, but hMSC-AML presented a reduced potential for osteogenic differentiation (Figure 1, *E* and *F*). This reduced potential could be related to decreased expression of the *BMP4* gene, which was found to be down-regulated in hMSC-AML, as this gene is essential for osteogenic differentiation.

We also verified the *BMP4* expression in our hMSC cultures. For this, we performed RT-qPCR assay with all hMSC cultures used in this study (Tables 1 and 2). As shown in Figure 2, *BMP4* was decreased in all hMSC-AML cultures compared to hMSC-HD, corroborating with Binato et al. [16].

The Differentially Expressed Genes Mainly Participate in The WNT Canonical Signaling Pathway

Before evaluating whether the WNT signaling pathway could act in *BMP4* gene regulation, we determined the expression profiles of the WNT signaling pathway in hMSC-AML cultures and compared them with the WNT signaling pathway expression in hMSC-HD cultures to verify whether this signaling pathway differed between the hMSC cultures.

Table 4. List of the 26 differentially expressed genes identified by WNT signaling pathway PCR array assay

Symbol	RefSeq	Description	Fold Change
WNT7B	NM_058238	Wingless-type MMTV integration site family, member 7B	**–23,75**
WNT11	NM_004626	Wingless-type MMTV integration site family, member 11	**–3,4**
WIF1	NM_007191	WNT inhibitory factor 1	**–2,99**
CXXC4	NM_025212	CXXC finger protein 4	**–2,44**
TCF7	NM_003202	Transcription factor 7 (T-cell specific, HMG-box)	**–2,29**
PORCN	NM_022825	Porcupine homolog (Drosophila)	**–2,05**
LEF1	NM_016269	Lymphoid enhancer-binding factor 1	**–1,86**
WNT16	NM_057168	Wingless-type MMTV integration site family, member 16	**–1,83**
WNT5B	NM_032642	Wingless-type MMTV integration site family, member 5B	**–1,73**
PITX2	NM_000325	Paired-like homeodomain 2	**–1,61**
RHOA	NM_001664	Ras homolog gene family, member A	**–1,57**
TCF7L1	NM_031283	Transcription factor 7-like 1 (T-cell specific, HMG-box)	**–1,56**
PPARD	NM_006238	Peroxisome proliferator-activated receptor delta	**–1,54**
PRICKLE1	NM_153026	Prickle homolog 1 (Drosophila)	**1,52**
WNT10A	NM_025216	Wingless-type MMTV integration site family, member 10A	**1,56**
BCL9	NM_004326	B-cell CLL/lymphoma 9	**1,57**
FZD3	NM_017412	Frizzled family receptor 3	**1,64**
KREMEN1	NM_1039570	Kringle containing transmembrane protein 1	**1,76**
VANGL2	NM_020335	Vang-like 2 (van gogh, Drosophila)	**1,85**
FRZB	NM_001463	Frizzled-related protein	**2,39**
FZD1	NM_003505	Frizzled family receptor 1	**2,42**
MMP7	NM_002423	Matrix metallopeptidase 7 (matrilysin, uterine)	**3,11**
SFRP1	NM_003012	Secreted frizzled-related protein 1	**3,2**
FZD9	NM_003508	Frizzled family receptor 9	**3,56**
SFRP4	NM_003014	Secreted frizzled-related protein 4	**3,61**
NKD1	NM_033119	Naked cuticle homolog 1 (Drosophila)	**9,56**

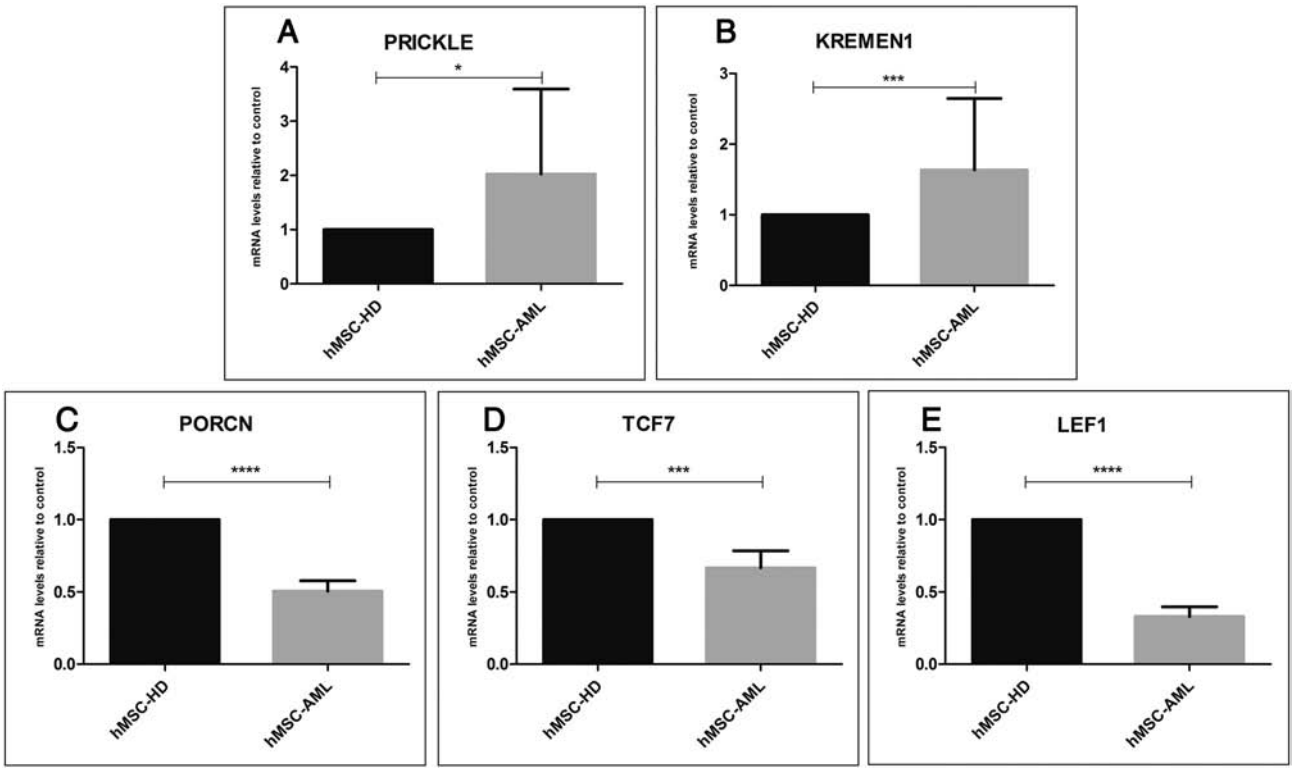

Figure 3. WNT signaling pathway components were differentially expressed in hMSC-AML. To confirm the PCR array results, we used RT-qPCR assays to determine changes in the mRNA expression of some differentially expressed genes using 30 samples obtained from hMSC-AML and 19 samples obtained from hMSC-HD. Data normalization was performed using the endogenous genes *B2M* and *GAPDH*. The RT-qPCR analyses of *PRICKLE* (A) and *KREMEN1* (B) (overexpressed in hMSC-AML) and *PORCN* (C), *TCF7* (D) and *LEF1* (E) (down-regulated in hMSC-AML) confirmed the PCR array assay results. The bars indicate the mean mRNA levels (± standard deviation). * $P < 0.05$/** $P < 0.01$/*** $P < 0.001$/**** $P < 0.0001$. hMSC-HD: mesenchymal stromal cells derived from healthy donors; hMSC-AML: mesenchymal stromal cells derived from AML patients.

We performed a PCR array assay to evaluate the expression profile of 84 genes related to the WNT signaling pathway (Supplementary file 1). Using a ≥ 1,5-fold change as a cutoff to define overexpression or down-regulation, 26 genes were identified to be differentially expressed between hMSC-AML and hMSC-HD (Table 4), suggesting a potential relationship with leukemic transformation.

To confirm the results obtained in the PCR array assay, RT-qPCR analysis was performed with a larger number of hMSC-AML derived from different subtypes (n = 30) and with hMSC-HD (n = 19) (Tables 1 and 2). The genes selected included *KREMEN1* and *PRICKLE1*, which were overexpressed in hMSC-AML cultures and which act as inhibitors of the WNT pathway [30,31], and the genes *TCF7*, *LEF1*, and *PORCN*, which were down-regulated in hMSC-AML cultures. The *PORCN* gene is responsible for the secretion of WNT protein and is thus essentially associated with WNT protein processing [32–34]. Moreover, the TCF and LEF proteins act as transcription factors responsible for the transcriptional activation of target genes [28]. The results presented in Figure 3 confirmed the PCR array findings, indicating that the differentially expressed genes are altered in all hMSC-AML cultures.

To verify in which of the WNT pathways our 26 differentially expressed genes were involved, we performed *in silico* analysis using KEGG software. The results showed that among the 26 differentially expressed genes, 61.5% of then (16 genes) were related to the canonical or Wnt/β-catenin-dependent pathway. Based on this result, our subsequent experiments focused on this β-catenin-dependent pathway.

The Levels of β-Catenin Are Decreased in hMSC-AML Nuclei, and LEF1 Protein Expression and a β-Catenin/TCF-LEF Regulatory Complex are Also Decreased in hMSC-AML

Although PCR array results did not show any alteration of β-catenin expression (mRNA), the location of β-catenin is essential to promote transcriptional regulation [35]. To determine whether there was any difference in the localization of β-catenin between hMSC-AML and hMSC-HD, we performed an immunofluorescence assay. As shown in Figure 4, a lower accumulation of β-catenin in the nucleus was observed in hMSC-AML, indicating that even if we did not find any differences in β-catenin expression, the levels of this protein were decreased in the nucleus of hMSC-AML.

Since we observed a decrease in the accumulation of β-catenin in the nucleus and a down-regulation of *TCF7* and *LEF1* mRNA expression in hMSC-AML, we evaluated whether the LEF1 protein expression profile was also altered. For this, we performed Western blot analysis. As shown in Figure 5, LEF1 protein expression was also decreased in hMSC-AML compared to that in hMSC-HD, corroborating the PCR array and RT-qPCR results. These results suggest that less β-catenin/TCF-LEF regulatory complexes responsible for the regulation of target genes are formed in the nuclei of hMSC-AML.

To confirm this hypothesis, we performed immunofluorescence assays with colocalization of β-catenin and LEF proteins. As shown in Figure 6, there was less overlap of the β-catenin and LEF1 proteins in hMSC-AML than in hMSC-HD, indicating a reduction in β-catenin/TCF-LEF complex formation.

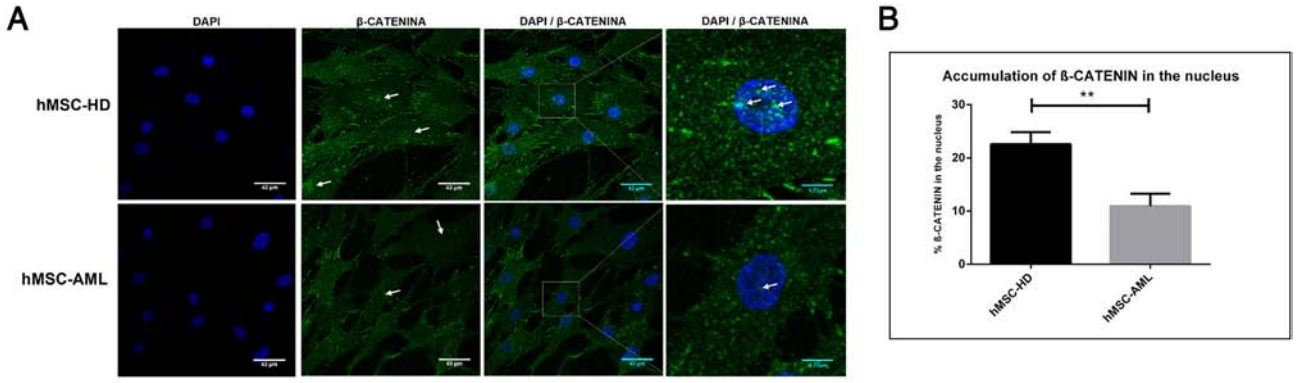

Figure 4. The levels of β-catenin were decreased in the nucleus of hMSC-AML. (A) Through immunofluorescence assays and confocal microscopy, we observed differences in the nuclear localization of β-catenin in hMSC-AML (n = 5) compared with that in hMSC-HD (n = 6). The nuclei were stained with DAPI (blue) and an antibody for β-catenin (green-labeled) (63× magnification). (B) Quantitative representation of β-catenin accumulation in the nucleus from LAS AF software (Leica, Hessen, Germany). The bars indicate the localization of β-catenin in the nucleus (± standard deviation). ** P <0 .01. hMSC-HD: mesenchymal stromal cells derived from healthy donors; hMSC-AML: mesenchymal stromal cells derived from AML patients.

Taken together, these results suggested that there is a decrease in β-catenin/TCF-LEF complex formation in hMSC-AML compared to that in hMSC-HD, which may result in compromised target gene regulation in hMSC-AML.

Decrease in LEF1 Binding to the BMP4 Gene Promoter in hMSC-AML

If the *BMP4* gene is regulated by the canonical WNT signaling pathway, it is necessary that the β-catenin/TCF-LEF complex recognizes

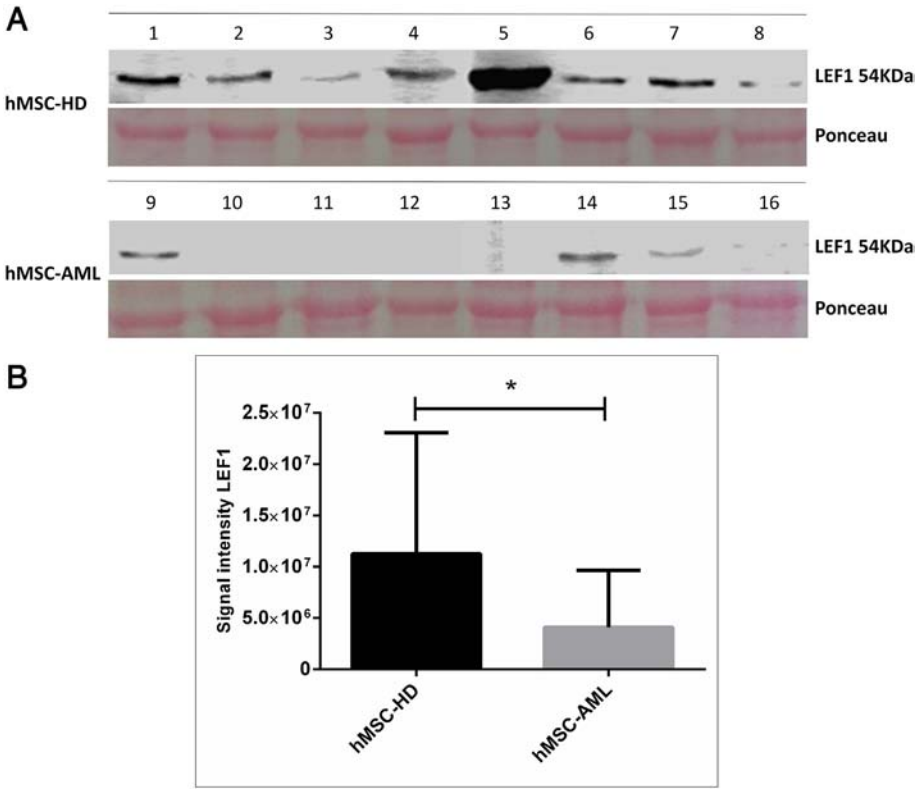

Figure 5. LEF1 protein expression is decreased in hMSC-AML. (A) Western blot analysis of LEF1. Protein extracts (30 μg) from hMSC-HD (1–8) and hMSC-AML (9–16) were separated by SDS-PAGE and probed with an LEF1 antibody. Ponceau staining was used as a loading control. (B) Representative graphic of the electrophoresis results confirming the decrease in LEF1 expression in hMSC-AML compared to that in hMSC-HD. The bars indicate the mean protein levels (± standard deviation). * P <0 .05. hMSC-HD: mesenchymal stromal cells derived from healthy donors; hMSC-AML: mesenchymal stromal cells derived from AML patients.

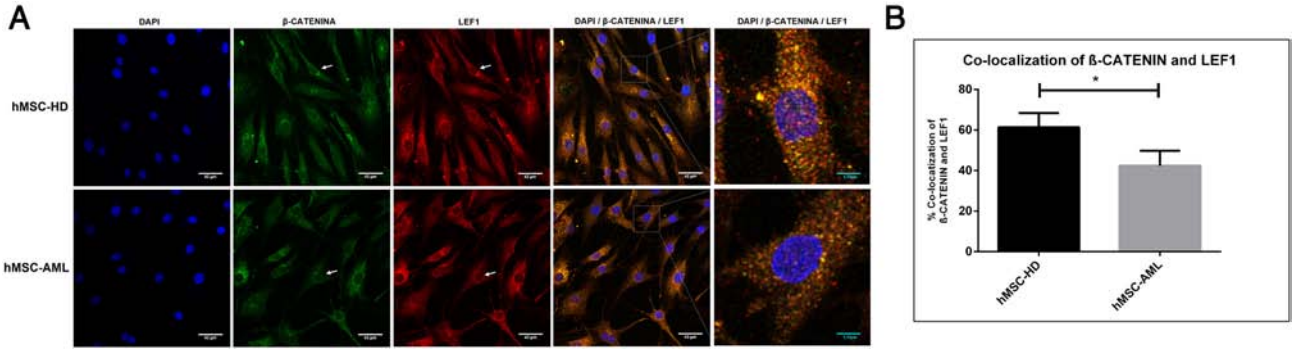

Figure 6. Formation of the β-catenin/TCF-LEF complex is decreased in hMSC-AML. (A) Through immunofluorescence assays and confocal microscopy, we observed differences in β-catenin/TCF-LEF complex formation in hMSC-AML (n = 5) and hMSC-HD (n = 6). The nuclei were stained with DAPI (blue); LEF1 is labeled with red, and β-catenin is labeled with green (63× magnification). (B) Quantitative representation of the colocalization of β-catenin and LEF1 from LAS AF software (Leica, Hessen, Germany). The bars indicate the colocalization of β-catenin and LEF1 (± standard deviation). $^{**}P < 0.01$. hMSC-HD: mesenchymal stromal cells derived from healthy donors; hMSC-AML: mesenchymal stromal cells derived from AML patients.

a specific region in the *BMP4* gene promoter and consequently activates its transcription [29]. For this, the presence of consensus binding sites for TCF-LEF in the *BMP4* promoter region is required.

To verify the TCF-LEF consensus binding sites, we analyzed 3 kb of the *BMP4* gene promoter. As shown in Figure 7, six TCF/LEF consensus binding sites were identified in the analyzed region (–613, –1860, –2100, –2240, –2680 and –2810 pb). Alignment analyses were also performed and revealed that these consensus binding sites were highly conserved among mammalian species.

To address whether LEF1 binds directly to the predicted sites in the *BMP4* promoter, we performed ChIP assay using hMSC-HD and hMSC-AML. Our results showed specific binding of LEF1 for five of

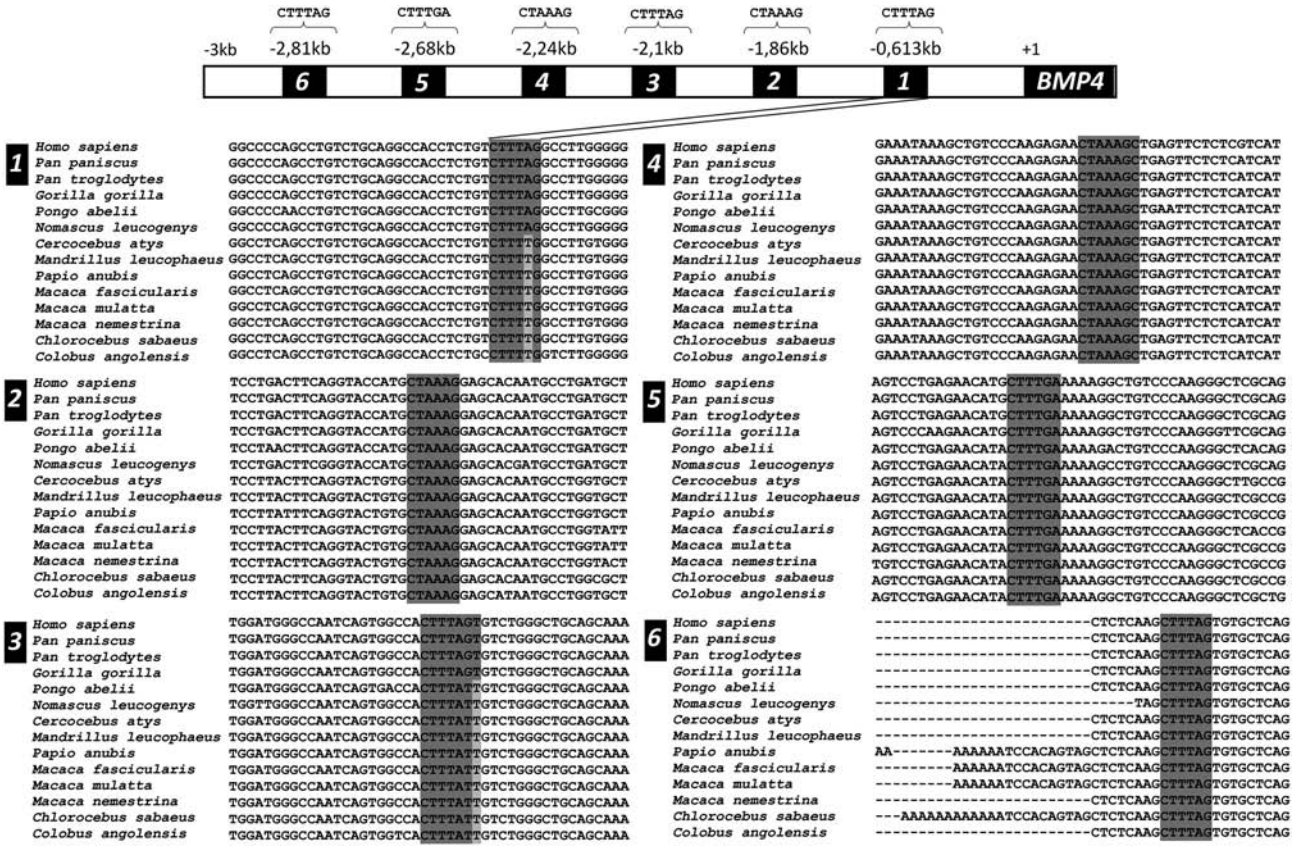

Figure 7. The *BMP4* gene promoter presents 6 putative TCF/LEF sites in 3 kb. Schematic representation of putative TCF/LEF consensus binding sites in 3 kb of the *BMP4* gene promoter region predicted manually and with the TRANSFAC, Tfsitescan, Genomatix and GenAtlas bioinformatics tools. Six TCF/LEF consensus binding sites were identified in the 3 kb region of the BMP4 promoter (5′-CTTTGA-3′; 3′-TCAAAG-5′ or 5′-CTTTAG-3′; 3′-CTAAAG-5′). An alignment of the DNA region showed evolutionary conservation among mammalian species. Identical nucleotides are in bold. The gray lines indicate regions investigated by chromatin immunoprecipitation. +1: transcription start site.

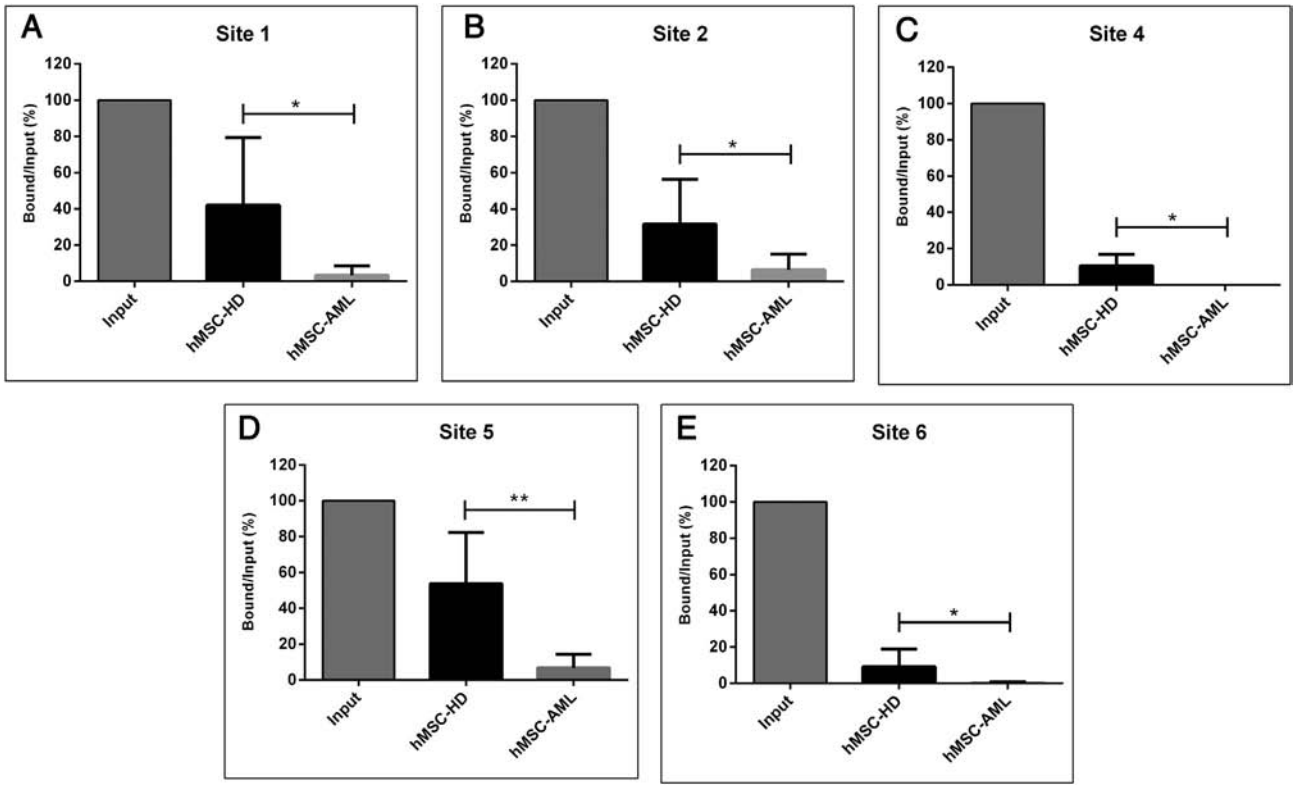

Figure 8. Decrease in LEF1 binding to the *BMP4* gene promoter in hMSC-AML. Through a chromatin immunoprecipitation assay (ChiP) with LEF1 followed by RT-qPCR of the predicted TCF/LEF binding sites (A-E) in the *BMP4* gene promoter, we observed reduced binding of LEF1 to the TCF/LEF in all consensus binding sites in hMSC-AML compared to that in hMSC-HD. The bar graphs show changes in LEF1 binding to DNA were calculated in relation to that of the IgG-precipitated control, normalized to the input the fold-change at each site compared to the binding of the input control. The data are expressed as the mean (± standard deviation). * $P < 0.05$/** $P < 0.01$. hMSC-HD: mesenchymal stromal cells derived from healthy donors; hMSC-AML: mesenchymal stromal cells derived from AML patients.

the six TCF/LEF consensus binding sites in both hMSC-HD and hMSC-AML. Moreover, consistent with all the previous results, a significant decrease in LEF1 binding was observed in all TCF/LEF consensus binding sites in hMSC-AML compared to that in hMSC-HD (–613, –1860, –2240, –2680 and –2810 bp sites) (Figure 8).

Overall, these results indicate that there are not only fewer β-catenin/TCF-LEF complexes formed in hMSC-AML but there is also less binding at the *BMP4* gene promoter, suggesting that the canonical WNT signaling pathway could be responsible for the lower expression of *BMP4* in hMSC-AML. The reduced activation of the canonical WNT signaling pathway in hMSC-AML could be related to the down-regulation of *BMP4* gene expression.

Discussion

Although AML is considered extremely heterogeneous, it is known that AML has a unique origin, and changes in signaling between the microenvironment and HSCs may be responsible for the leukemic transformation [36]. It has been observed that perturbations in microenvironment components are directly associated with hematopoietic insufficiency [13]. Among the components, mesenchymal stromal cells are fundamental in the maintenance of HSCs, regulating their self-renewal, quiescence and mobilization in BM [4] and providing essential signals to support hematopoiesis [15].

Several studies have shown that hMSC-AML, compared to hMSC-HD, present differences that may be related to the development of AML [4]. Geyh and coworkers verified that hMSC-AML are molecularly and functionally altered and contribute to hematopoietic insufficiency [13]. Chandran and colleagues showed that the ability of hMSC-AML to support the expansion of committed hematopoietic progenitors is impaired and that the expression of genes related to hematopoietic quiescence is increased in hMSC-AML [37]. In addition, a molecular signature capable of distinguishing hMSC-AML from hMSC-HD, has been identified. Among the differentially expressed genes presented in the molecular signature, *BMP4* shows decreased mRNA expression in hMSC-AML and plasma from AML patients [16].

Goldman and coworkers were the first group that highlighted *in vivo* the importance of *BMP4* in HSC maintenance, specifically in the regulation of the differentiation and proliferation of HSCs [18]. *BMP4* regulates the number and function of HSCs, which directly influences hematopoiesis [38] and has the ability to induce osteogenic differentiation in hMSCs [39,40].

One of the characteristics of hMSCs is their potential for adipogenic, chondrogenic and osteogenic differentiation. Osteogenic cells are an important component of the BM microenvironment that play an essential role in regulating normal hematopoiesis [41], and BMP4 signaling is one of the central signaling pathways involved in the induction of osteogenic differentiation and the regulation of bone formation. Disrupting the osteoblastic compartment results in aberrant hematopoiesis [18,38].

Thus, expectedly, we found in our study a decrease in osteogenic differentiation potential in hMSC-AML, which corroborates the

descriptions in some studies of a reduced capacity of hMSC-AML for osteoblast formation [13,42]. This capacity reduction can promote the suppression of normal hematopoiesis, increase the number of circulating blasts [13,43] and can cause alterations in the HSCs [44]. The decreased expression of *BMP4* in hMSC-AML may be responsible for the reduction in osteogenic differentiation potential. If fewer cells of the osteogenic lineage are present in BM, an imbalance among osteoblasts and osteoclasts is likely to occur, producing an environment favorable for LSC proliferation.

The contribution of *BMP4* produced by the microenvironment to the pathogenesis of hematological tumors has been discussed [45]; nevertheless, the regulatory mechanism in hMSCs remains unclear [46]. *In silico* analyses from our group suggested that *BMP4* in hMSCs could be regulated by the WNT signaling pathway [16]. In hMSCs, the WNT signaling pathways have been implicated in the regulation of hematopoiesis, which is necessary for the maintenance and self-renewal of HSCs [47]. Moreover, these pathways have been described in the development of several hematological malignancies [48,49].

The WNT signaling pathway has already been described as dysregulated in AML-HSC [50,51]. However, the expression profile of this pathway in hMSC-AML is still unknown.

The regulation of *BMP4* through the WNT signaling pathway has been described in colon cancer cells, and it has been observed that increases in *BMP4* expression are related to the activation of the canonical β-catenin-dependent WNT pathway [23]. In epidermal stem cell differentiation, increased β-catenin expression is accompanied by increased BMP4 expression [52]. In rat mesenchyme, β-catenin together with LEF1 and TCF1 is required to activate *BMP4* expression during incisor development [53]. However, in Xenopus embryos, WNT signaling inhibits *BMP4* expression and activates neural development [19].

In our study, 26 genes from the WNT signaling pathway were differentially expressed between hMSC-AML and hMSC-HD, suggesting a dysregulation of the WNT pathway in hMSC-AML. Thus, altered expression of these genes indicates that the WNT signaling pathway is altered in hMSC-AML. Interestingly, most of these differentially expressed genes are related to the WNT canonical or Wnt/β-catenin-dependent pathway. We also observed that the main components responsible for the WNT canonical pathway regulation were found to be decreased in hMSC-AML, such as LEF-1, β-catenin and the β-catenin/TCF-LEF regulatory complex in the nucleus.

For gene activation, the β-catenin/TCF-LEF complex must bind to specific sequences in the target gene promoter [28]. With several bioinformatic tools, it was possible to identify six consensus binding sites for the TCF/LEF transcription factors in a 3 kb sequence from the *BMP4* gene promoter region [28,29]. All six predicted TCF/LEF binding sites were highly conserved among mammalian species, indicating the biological relevance of these sites throughout evolution. In the ChIP experiments, we observed a significant decrease in LEF1 binding at five of the six TCF/LEF consensus binding sites in the *BMP4* gene promoter in hMSC-AML compared to that in hMSC-HD.

These results indicate that the WNT canonical signaling pathway is altered in hMSC-AML and that this alteration could influence *BMP4* expression in hMSC-AML. The decrease in β-catenin/TCF-LEF complex formation could be related to the decrease in *BMP4* expression, because this complex is required for the activation of *BMP4* expression. This altered regulation could influence the dysregulation of osteogenic differentiation and consequently the decrease of osteoblasts formation, generating an imbalance among osteoblasts and osteoclast, favoring LSC proliferation.

In conclusion, the current study shows that the WNT signaling pathway is altered in all hMSC-AML. These changes in the canonical WNT signaling pathway could influence *BMP4* expression. The decrease in β-catenin/TCF-LEF complex formation and the reduction in *BMP4* gene promoter binding suggest that the canonical WNT signaling pathway is essential for the activation of BMP4 expression. Therefore, the decreased BMP4 expression in hMSC-AML could be related to reduction in β-catenin/TCF-LEF complex formation. Moreover, changes in the expression of components of both the WNT and BMP4 signaling pathways could be important factors in the leukemic transformation process.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tranon.2019.01.003.

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Authorship

Contribution: P.L.A., isolated, cultivated and confirmed hMSC cultures, performed the experiments, analyzed data, prepared the manuscript draft, wrote the paper and contributed intellectual content.; N.C.A.C isolated, cultivated and confirmed hMSC cultures, S.C., performed WB experiments and contributed intellectual content. M.T.L.C-B, performed immunofluorescence experiments, E.A., participated in the study design and contributed intellectual content, R.B., designed the study, analyzed data, prepared the manuscript draft, wrote the paper and contributed intellectual content. All authors provided critical review of the manuscript.

Conflict-of-interest disclosure

The authors declare no competing financial interests.

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