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

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.

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 NPM1 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].
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
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% CO₂. 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 × 10³ cells/cm² (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 RT² First Strand Kit (Qiagen), and the cDNA was subsequently loaded into a Human WNT Signaling Pathway RT² Profiler PCR Array according to the manufacturer’s instructions (PAHS-0432, Qiagen). Data were normalized to the average expression of GAPDH, B2M and ACTB. The data were analyzed using the platform GeneGlobe (www.qiagen.com/bi/shop/gene-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 DNase1 (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 Digit 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 20×10³ cells/cm² (passage 1). These processes were repeated until the hMSCs were removed from the plates by treatment with 0.05% trypsin (Invitrogen™) for 5 min at 37°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 Digit software v3.1 with a LI-COR instrument (Uniscience Co.), after chemiluminescence reaction (Pierce™ ECL Plus Western Blotting Substrate, Thermo Scientific).

**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'−CTTTGAG−3'; 5'−CTTTAG−3') [28,29].

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

| Gene      | Forward            | Reverse               |
|-----------|--------------------|-----------------------|
| BMP4      | CCATGATTTCCGCAGGCATAGGAA | CCGATCATCCGCAGACCTTT | |
| KREMEN1   | CGTGCTCTGGAACCTCTCTCTT | CCGGCATTCCGCAGACCTTT | |
| LEF1      | CAGACATACCTGCAGCTCTGGATAT | CGGTGAGCAGATACCTCT | |
| PORCN     | CCCTCTTACTGATGCTTCAGTT | CCGGCTTACTCAGATGCTGAC | |
| PRICKLE1  | GTGCTCTCAGCAGCTGAGATAA | GCAGATTTAGGCGAGATGAGTAC | |
| TCF7      | ACTTCTCCGCAGAATACTCCC | TCGACACTTCAAGCGGAGGATAC | |
| GAPDH     | ATTTACCCACTGCGGAAATTTC | CGGTGAGTCAGGGTCTTTCA | |
| B2M       | AGTGTAGTATCCGCCGTGTAAG | CGCGATCTTCAAACCTCCTCAGT | |

**Primer sets for RT-qPCR analysis**

For RT-qPCR analysis, the primers were designed as follows: 5′-ACGTAAAGAAGTGGCCCATCAAGC-3′; 5′-TCCTGTTCGACCACGTAGAC-3′; 5′-CTTTAG-3′; 5′-CTTTAG-3′.

**Primers used for RTq-PCR after chromatin immunoprecipitation assays**

| Primer Set 1 | Primer Set 2 | Primer Set 3 | Primer Set 4 | Primer Set 5 | Primer Set 6 |
|--------------|--------------|--------------|--------------|--------------|--------------|
| S1 ChipBMP4  | ACTGTAATACATCCGCAGGAGGATAC | ACCCTGAGATCTCCGAGGATACAGAT | CCAATGGTATCTCCGAGGATACAGAT | CCAATGGTATCTCCGAGGATACAGAT | CCAATGGTATCTCCGAGGATACAGAT |
| S2 ChipBMP4  | GATGTGATTTAGGCGCTCTATTCCAG | CCAATGGTATCTCCGAGGATACAGAT | CCAATGGTATCTCCGAGGATACAGAT | CCAATGGTATCTCCGAGGATACAGAT | CCAATGGTATCTCCGAGGATACAGAT |
| S3 ChipBMP4  | TTGGAACATCTGAGCTCTGGTGAAGTC | CCAATGGTATCTCCGAGGATACAGAT | CCAATGGTATCTCCGAGGATACAGAT | CCAATGGTATCTCCGAGGATACAGAT | CCAATGGTATCTCCGAGGATACAGAT |
| S4 ChipBMP4  | GATGTGAGGACAGAATTAAGCTGCTCC | CCAATGGTATCTCCGAGGATACAGAT | CCAATGGTATCTCCGAGGATACAGAT | CCAATGGTATCTCCGAGGATACAGAT | CCAATGGTATCTCCGAGGATACAGAT |
| S5 ChipBMP4  | GGACCCAGGAGGTCTGCAATTTCA | CCAATGGTATCTCCGAGGATACAGAT | CCAATGGTATCTCCGAGGATACAGAT | CCAATGGTATCTCCGAGGATACAGAT | CCAATGGTATCTCCGAGGATACAGAT |
| S6 ChipBMP4  | GAGGAGAGATCTGAGCTTGGTGC | CCAATGGTATCTCCGAGGATACAGAT | CCAATGGTATCTCCGAGGATACAGAT | CCAATGGTATCTCCGAGGATACAGAT | CCAATGGTATCTCCGAGGATACAGAT |
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 RT-q-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, chromatin 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.

### 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 | E- cadherin 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_012308 | Peroxisome proliferator-activated receptor delta | -1.54 |
| PRICKLE1 | NM_022768 | 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 |
| CREMEN | NM_013570 | Kringle containing transmembrane protein 1 | 1.76 |
| VANGL2 | NM_020335 | Frizzled-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_004243 | Matrix metallopeptidase 7 (matriptase, uterine) | 3.11 |
| SFRP1  | NM_003012 | Secreted frizzled-related protein 1 | 3.2 |
| FZD9   | NM_003506 | 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 |
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 them (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.

**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.

**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.
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.

**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.

**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.
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 the predicted TCF/LEF sites in the BMP4 promoter region.

**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.

**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.
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, −821860, −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
The decreased expression of BMP4. The suppression of normal hematopoiesis, increase the number of circulating blasts and can cause alterations in the HSCs. 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; nevertheless, the regulatory mechanism in hMSCs remains unclear. In silico analyses from our group suggested that BMP4 in hMSCs could be regulated by the WNT signaling pathway. 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. Moreover, these pathways have been described in the development of several hematological malignancies.

The WNT signaling pathway has already been described as dysregulated in AML-HSC. 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. In epidermal stem cell differentiation, increased β-catenin expression is accompanied by increased BMP4 expression. In rat mesenchyme, β-catenin together with LEF1 and TCF1 is required to activate BMP4 expression during incisor development. However, in Xenopus embryos, WNT signaling inhibits BMP4 expression and activates neural development.

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. 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. 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.

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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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