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BRIEF REPORT

Reduced Osteogenic Differentiation Potential *In Vivo* in Acute Myeloid Leukaemia Patients Correlates with Decreased *BMP4* Expression in Mesenchymal Stromal Cells

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The osteogenic differentiation potential of mesenchymal stromal cells (hMSCs) is an essential process for the haematopoiesis and the maintenance of haematopoietic stem cells (HSCs). Therefore, the aim of this work was to evaluate this potential in hMSCs from AML patients (hMSCs-AML) and whether it is associated with *BMP4* expression. The results showed that bone formation potential *in vivo* was reduced in hMSCs-AML compared to hMSCs from healthy donors (hMSCs-HD). Moreover, the fact that hMSCs-AML were not able to develop supportive haematopoietic cells or to differentiate into osteocytes suggests possible changes in the bone marrow microenvironment. Furthermore, the expression of *BMP4* was decreased, indicating a lack of gene expression committed to the osteogenic lineage. Overall, these alterations could be associated with changes in the maintenance of HSCs, the leukaemic transformation process and the development of AML.

Keywords: Mesenchymal stromal cells (hMSCs), Acute myeloid leukaemia (AML), Osteogenic differentiation potential, *BMP4* gene expression

Introduction

Although acute myeloid leukaemia (AML) is a heterogeneous disease, it is known to have a unique origin from the malignant transformation of normal haematopoietic stem cells (HSCs) into leukaemic stem cells (LSCs) (1). However, what leads to this transformation is still unclear. Several studies suggest that HSCs undergo mutation(s), which gives rise to LSCs. However, not all LSCs present these mutations (2). In this context, changes in signalling in the bone marrow (BM) microenvironment, specifically in mesenchymal stromal cell (hMSC) signalling, could promote malignant transformation (3).

hMSCs play a key role, as they provide essential signals for maintaining and regulating HSCs (3). Various studies, including work from our group, have shown that hMSCs derived from AML patients (hMSCs-AML) are molecularly and functionally altered and that the *in vitro* osteo-

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genic differentiation potential is decreased (4-6). The ability of hMSCs to differentiate into osteoblasts is essential for the regulation of HSCs, and genes related to osteogenic differentiation are decreased in hMSCs-AML, such as *BMP4*, which is necessary for maintaining functional HSCs *in vivo* (7). However, it is not known if decreased *BMP4* expression *in vitro* is associated with alterations in osteogenic differentiation potential and if these findings are reflected in *in vivo* assays.

In this sense, the aim of this study was to evaluate whether the osteogenic differentiation potential *in vivo* is reduced in hMSCs-AML and whether it is associated with the expression level of *BMP4*. For this, we performed a xenotransplantation assay with MSCs-HD and hMSCs-AML to induce *in vivo* bone formation and compared the newly formed tissue, as well as the gene expression of important markers for osteogenic differentiation. With these results, we can improve our knowledge of the mechanisms related to the development of AML.

Materials and Methods

Patient and healthy donor samples

BM-derived samples were collected from adult patients with AML at diagnosis (without any treatment) and from adult healthy donors (HDs) registered at the National Cancer Institute (Rio de Janeiro, Brazil). 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 (CAAE 06281419.0.0000.5274), and all participants signed informed consent forms.

Isolation, culture and confirmation of hMSCs

hMSCs at passage 3 derived from BM samples were isolated and cultured as previously described (4) and were characterized as defined by the International Society for Cellular Therapy (8).

Real-time quantitative PCR (RT-qPCR)

mRNA was extracted from hMSCs at the end of the process of inducing osteogenic differentiation *in vitro*, and RT-qPCR was performed as previously described in Azevedo (9). The expression levels of *BMP4* were estimated, and B2M and GAPDH were used as normalization genes (Supplementary Table S1).

Subcutaneous xenotransplantation assay

The subcutaneous xenotransplantation assay with AML-hMSCs and HD-hMSCs was performed as previously reported (10). All animal procedures were performed following the guidelines of the Institutional Animal Care and Use Committee (010/2020).

Implant histology and immunohistochemistry

The implants obtained were processed for histology and immunohistochemistry analyses as previously reported (10). Rabbit anti-OSX antibody (sc-393325, Santa Cruz Biotechnology, USA) diluted 1 : 100 and goat anti-BMP-2/4 antibody (sc-6267, Santa Cruz Biotechnology, USA) diluted 1 : 100 were used.

X-ray microtomography (micro-CT) and morphometric quantification

MicroCT acquisition was performed as described in Dias (10). The deep learning segmentation tool available on DragonFly (11) was used to separate the newly formed bone from hydroxyapatite. Quantification was performed according to previously described methods (12).

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. Statistical analysis was performed, and graphical representations were created using GraphPad Prism^{TM} software (GraphPad Software Inc.).

Results and Discussion

All hMSCs used in this study were confirmed by the minimum criteria established by Dominici et al., 2006 (8) (Fig. 1). We observed a reduction in the osteogenic differentiation potential of hMSCs-AML *in vitro* (Fig. 1E and 1F), corroborating other studies (4, 5). The findings of conventional *in vitro* differentiation assays are important and partially predictive of the *in vivo* physiologic functions of hMSCs, but these cultures do not necessarily reflect the intrinsic physiological potential of the cells. Therefore, to accurately assess the *in vivo* functional properties of hMSCs in a physiological environment, we used a xenotransplantation assay (13).

Histological examination of the implants revealed that only hMSC-HD cultures formed ectopic ossicles with similar trabecular bone architecture and were able to support haematopoietic stroma (Fig. 2A and 2B). This reconstituted marrow stroma was filled with haematopoietic cells, which is an indicator of the multipotent capacity of hMSCs.

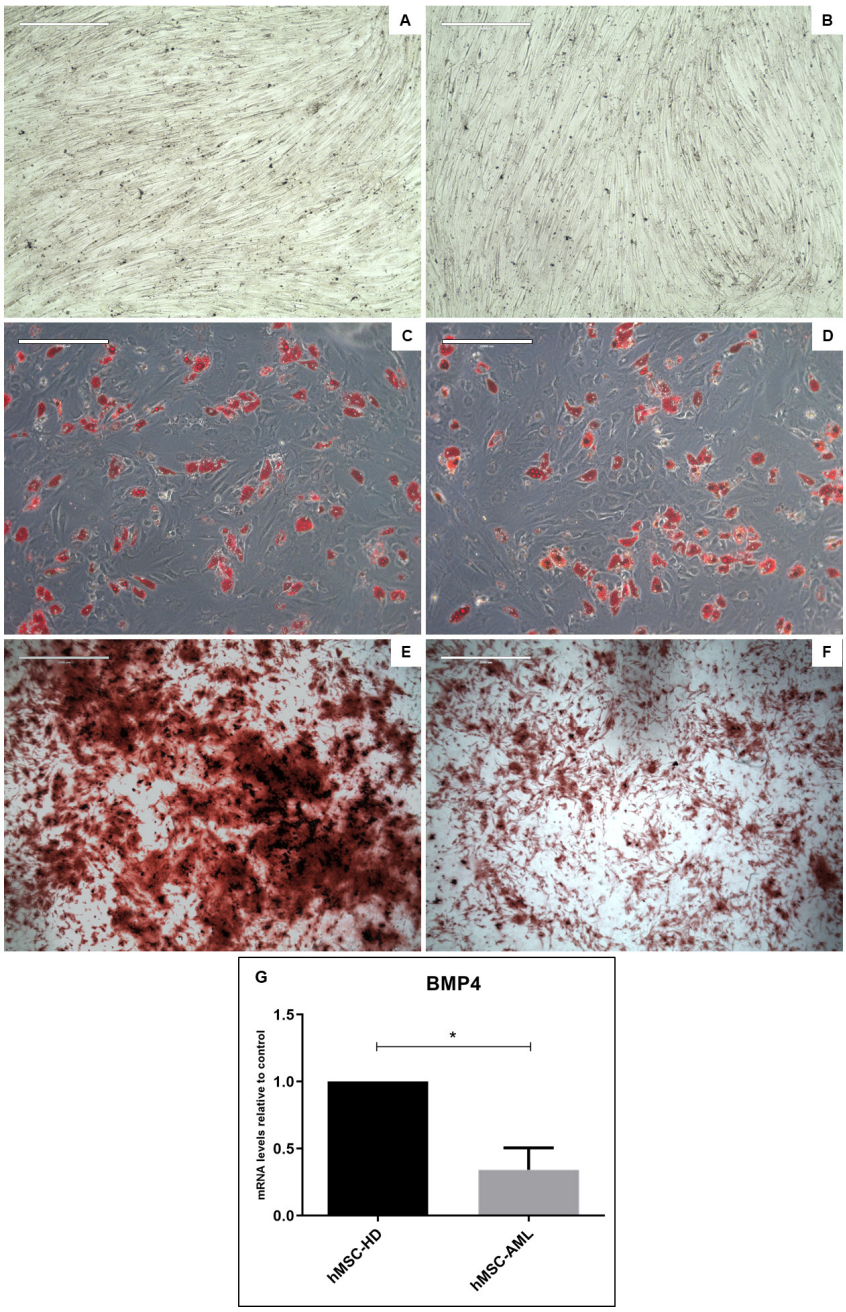

Fig. 1. hMSC multipotency capacity *in vitro*. (A, B) Undifferentiated hMSCs-HD and hMSCs-AML (100× magnification). (C, D) Adipogenic differentiation of hMSCs-HD and hMSCs-AML. The accumulation of neutral lipid vacuoles stained with Oil Red O indicates cell differentiation (20× magnification). (E, F) Osteogenic differentiation of hMSC-HDs and hMSCs-AML. Calcium deposition stained with Alizarin Red indicates cell differentiation (20× magnification). (G) *BMP4* is downregulated in hMSCs-AML after 21 days of osteogenic induction. To verify *BMP4* expression, we used RT-qPCR to determine changes in the mRNA expression obtained from hMSC-AML and hMSC-HD cultures. Data normalization was performed using the endogenous genes *B2M* and *GAPDH*. The bars indicate the mean mRNA expression levels (±standard deviation). *$p<0.01$. hMSCs-HD: mesenchymal stromal cells derived from healthy donors; hMSCs-AML: mesenchymal stromal cells derived from AML patients.

In micro-CT-based 3D reconstruction, bone neoformation was identified in the hMSC-HD (Fig. 2C) and hMSC-AML implants (Fig. 2D); moreover, we observed that the quality (mineral density) of the newly formed tissue was similar under both conditions (Fig. 2H). However, quantitative reductions in the bone volume formed, its thickness, and the relationship between bone volume formed and the volume of the tissue analysed were observed in the implants formed from hMSCs-AML compared to hMSCs-HD (Fig. 2E∼G). Thus, these results showed that hMSCs-AML maintained their *in vivo* bone differentiation capacity, but this capacity was reduced when compared to that of hMSCs-HD.

These results corroborate those of Alice Pievani and colleagues, who observed significant alterations in mature bone formation from hMSCs derived from paediatric AML patients (14), and Frisch and coworkers, where a reduction in mineralized bone tissue formation was described after *in vivo* assays using an immunocompetent murine model of AML (15). Thus, these changes could be

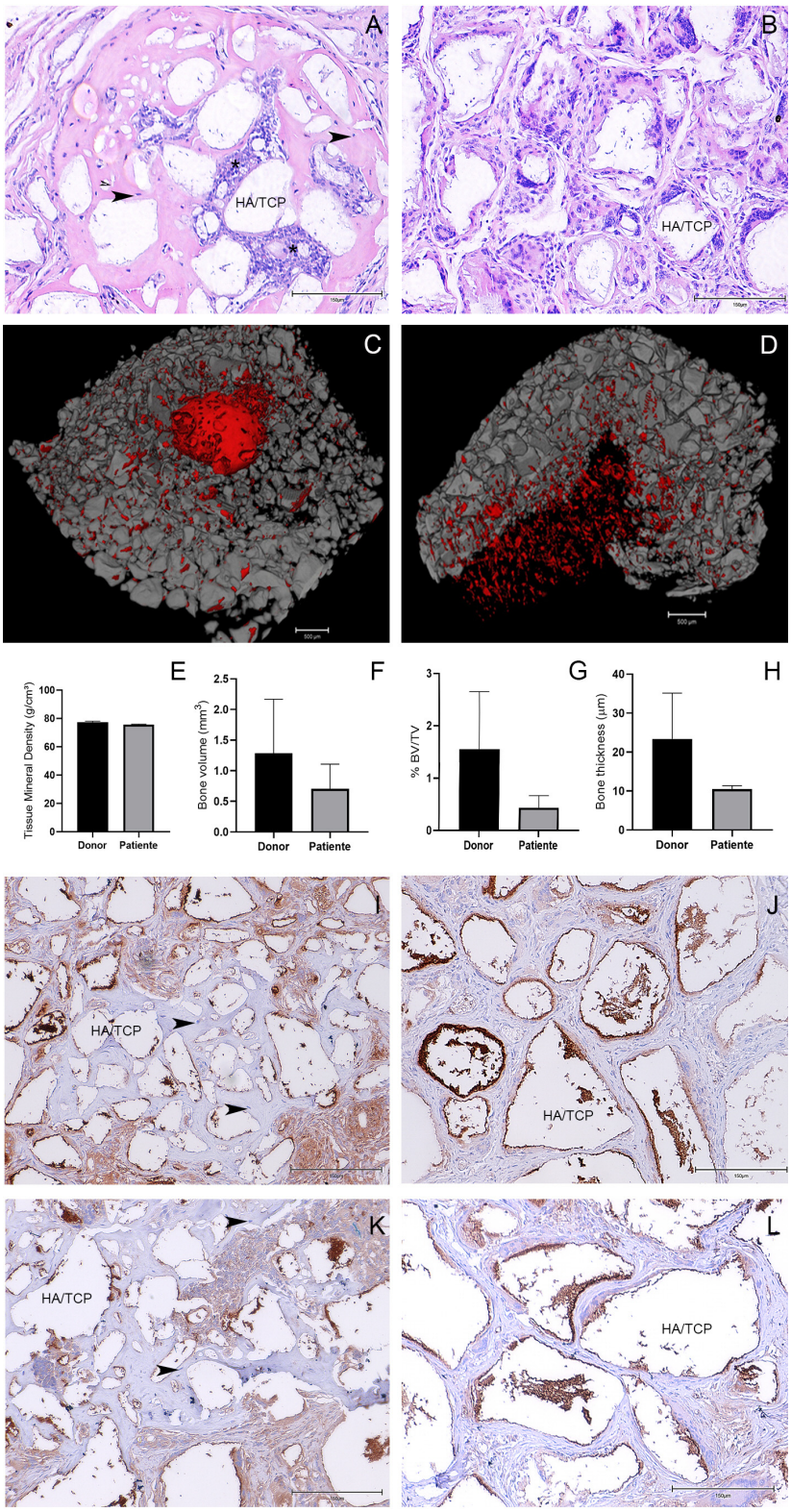

Fig. 2. *In vivo* osteogenic potential of BMSCs. *In vivo* transplantation assays were performed by combining hMSCs with HA/TCP followed by subcutaneous transplantation into immunocompromised mice. (A, B) H&E staining. (A) Implants from hMSC-HD cultures and (B) hMSC-AML cultures. hMSCs-HD formed ectopic ossicles that were sometimes populated by host haematopoietic marrow (asterisk). The arrowheads indicate osteocytes. (C∼H) Micro-CT analysis. (C) Bone tissue formed from hMSCs-HD and (D) hMSCs-AML from the 3D reconstruction of implants. For better visualization of the bone tissue formed (red), part of the HA/TCP (grey) was removed. (H) Tissue mineral density of implants formed from hMSCs-HD and hMSCs-AML. (E) Analysis of bone volume, (F) the relationship between bone volume and tissue volume, (G) and bone tissue thickness in implants formed from hMSCs-HD and hMSCs-AML. (I∼L) Human origin of the woven bone by immunohistochemical analysis. Expression of *BMP4* within the woven bone from (I) hMSCs-HD and (J) hMSCs-AML. Expression of Osterix within the woven bone from (K) hMSCs-HD and (L) hMSCs-AML. HA/TCP=hydroxyapatite/tricalcium phosphate.

associated with the leukaemic transformation process and the development of AML.

This potential of hMSCs to differentiate into osteoblasts in BM is an essential process for normal haematopoiesis and for the maintenance of HSCs. The process occurs from commitment towards the osteogenic lineage, osteo-

progenitor cell proliferation, osteoblast maturation and bone matrix mineralization, driven by a complex network of cytokines, hormones, and growth factors (16, 17). Changes in the regulation of this process, associated with lower production of osteoblasts, may result in altered bone deposition and alter the maintenance of HSCs. It is believed that reduced bone deposition can promote the exit of quiescent HSCs from the endosteal niche, associated with an increase in the number of circulating blasts in BM (18).

Interestingly, we also observed that hMSCs-HD were able to develop a supportive haematopoietic stroma and reconstruct an *in vivo* bone marrow-like microenvironment in mice with haematopoietic cells (Fig. 2A), confirming that hMSCs can organize and are important components of the haematopoietic microenvironment (13, 19). This finding was not observed in the implants obtained from hMSCs-AML, evidence that corroborates the report by Priya Chandran and colleagues, who found that hMSCs-AML have altered capacity to expand differentiated haematopoietic progenitors *in vitro* (20).

BMP4, a member of the transforming growth factor β (*TGF-*β) superfamily of secreted signalling molecules, was initially identified for its ability to induce bone formation (21) and plays an important role in osteogenic differentiation. It induces the commitment of hMSCs towards the osteogenic lineage and enhances the activity of mature osteoblasts (16). BMP receptor activation in osteogenesis, involves both *Smad1/5/8* and *MAPK* downstream signaling activation, and works in conjunction with *Osterix* via both *Runx2* dependent and independent pathways (22). In mice, has already been evidenced that overexpressing *BMP4* had enlarged bones containing thick trabeculae (23), and the loss of *BMP4* resulted in severe impairment of osteogenesis (24). In this work, the *BMP4* expression of hMSCs-AML after 21 days of osteogenic induction *in vitro* was also decreased (Fig. 1G), similar to undifferentiated hMSCs-AML (4), indicating that it could be associated with reduced osteogenic potential. In fact, it was not possible to observe the expression of *BMP4* in implants obtained *in vivo* from hMSCs-AML (Fig. 2J) when comparing intense marking in the matrix adjacent to new bone formed from the hMSCs-HD (Fig. 2I).

In addition, *BMP4* is a critical component produced by haematopoietic microenvironment that regulates both HSCs number and function (7, 25). Thus, a decreased of *BMP4* expression in implants obtained *in vivo* from hMSCs-AML, could be associated with the changes in HSCs, contribute to the suppressing normal hematopoiesis, the leukaemic transformation and culminate in the AML.

Finally, osteoblast differentiation is a multistep process in which hMSCs differentiate into osteoblast lineage cells, including osteocytes. Osterix (OSX) is an osteoblast-specific transcription factor that activates a repertoire of genes during the differentiation of preosteoblasts into mature osteoblasts and osteocytes (16). Similar to *BMP4*, we observed OSX expression in hMSC-HD implants only (Fig. 2K), confirming that there was alteration in osteogenic differentiation and formation of mature osteoblasts in hMSCs-AML.

In conclusion, the current study showed a reduction in osteogenic differentiation potential *in vivo* and an absence of osteocytes and haematopoietic support when hMSCs-AML were used, in contrast to what we found using hMSCs-HD. These findings correlate with the decreased expression of key osteogenic markers such as *BMP4* and *OSX*. These alterations could influence the BM microenvironment, promoting the release of HSCs from the quiescent niche and indirectly being important factors in the leukaemic transformation process and leukaemia progression.

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Potential Conflict of Interest

The authors have no conflicting financial interest.

Author Contributions

PLA performed all experiments, drafted the manuscript, participated in the study design and contributed intellectual content; RBD performed xenotransplantation, histology and immunohistochemistry assays; LPN performed X-ray microtomography (micro-CT) and morphometric quantification; SM, RBigni and JSRA provided and classified all patient samples used in this study; EA participated in the study design, provided financial support and contributed intellectual content and RB conceived the study and its design and coordination, provided financial support, drafted the manuscript and contributed intellectual content. All authors read and approved the final manuscript.

Supplementary Materials

Supplementary data including one table can be found with this article online at https://doi.org/10.15283/ijsc21138.

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