Hypoxia inhibits osteogenesis through direct regulation of RUNX2 by TWIST

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ABSTRACT INTRODUCTION

Bone loss induced by hypoxia is associated with various pathophysiological conditions such as ischemia, vascular diseases, and osteolytic bone metastases; however, little is known about the mechanism of hypoxia-regulated osteogenesis and bone formation. RUNX2 (also known as CBFA1) is a master regulator of skeletogenesis and its expression is required for osteoblast differentiation and maturation. However, the role of bone marrow microenvironment which survived under hypoxia, we discovered TWIST, a downstream target of HIF-1α, as a transcription repressor of RUNX2 through binding to the E-box located on the promoter of Type 1 RUNX2. Suppression of RUNX2 inhibited the expression of BMP2, Type 2 RUNX2 and downstream targets of RUNX2 such as alkaline phosphatase and osteocalcin in MSCs. Further, knockdown of twist1a and twist1b in zebrafish induced an increase in runx2b (one orthologue of mammalian Runx2) expression, which was associated with a ventralized embryo and an increased in bone formation. Our finding pointed out an important role of hypoxia-mediated signalling in osteogenic differentiation and bone formation through direct regulation of RUNX2 by TWIST.

METHODS:

Primary human mesenchymal stem cells (MSCs) induced in osteogenic induction medium (OIM) under normoxia or hypoxia were exposed to hypoxia or DFX. We plan to investigate makers of osteogenesis under hypoxia which induced HIF-1α-TWIST pathway to inhibit osteogenesis by real-time PCR analysis and western blot analysis. To examine whether overexpression of TWIST can suppress T1, T2 RUNX2 and osteogenesis, we used pFLAG-CMV1 transfected MSCs in normoxia or pSuper-TWIST-si or pSuper-Scr in hypoxia. To determine an E-box, a novel role of TWIST binding domain of T1 RUNX2 in bone formation through direct interaction under hypoxia, we used RUNX2 promoter assay, truncation bHLH of TWIST (pFLAG-tbTWIST) promoter assay, site-directed mutagenesis E-boxs of T1 RUNX2 promoter assay, EMSA and ChIP to prove TWIST binding T1 RUNX2 through E-box. In vivo, zebrafish used to confirm bone loss of hypoxia or DFX by Alizarin Red S & Alcian blue stain. Further, runx2b expression was analyzed by runx2b whole-mount in situ hybridization and real-time PCR and HE stain under embryos were microinjected twist1a or twist1b atgMOs.

RESULTS:

Because MSCs isolated from bone marrow, which is hypoxic in nature (1-7% O₂) survive under hypoxia, we induced bone marrow MSCs from three individual donors in osteogenic induction medium (OIM) under normoxia (21% O₂) and hypoxia (1% O₂) to understand the mechanism of hypoxia-regulated osteogenesis and bone formation by hypoxia or HIF-1α-TWIST pathway to inhibit osteogenesis by real-time PCR analysis and western blot analysis. To examine whether overexpression of TWIST can suppress T1, T2 RUNX2 and osteogenesis, we used pFLAG-CMV1 transfected MSCs in normoxia or pSuper-TWIST-si or pSuper-Scr in hypoxia. To determine an E-box, a novel role of TWIST binding domain of T1 RUNX2 in bone formation through direct interaction under hypoxia, we used RUNX2 promoter assay, truncation bHLH of TWIST (pFLAG-tbTWIST) promoter assay, site-directed mutagenesis E-boxs of T1 RUNX2 promoter assay, EMSA and ChIP to prove TWIST binding T1 RUNX2 through E-box. In vivo, zebrafish used to confirm bone loss of hypoxia or DFX by Alizarin Red S & Alcian blue stain. Further, runx2b expression was analyzed by runx2b whole-mount in situ hybridization and real-time PCR and HE stain under embryos were microinjected twist1a or twist1b atgMOs.

DISCUSSION:

This is the first time T1 RUNX2 has been demonstrated as an important target for controlling osteogenesis and bone formation by hypoxia or HIF-1α-TWIST, an important environment encountered by a lot of pathophysiological conditions associated with normal development and regeneration, or acquired and genetic diseases. Therefore, signaling pathways or molecules that control the transcription of RUNX2 may be applied as tools in vivo to control osteogenesis and bone formation. We further demonstrated the direct downregulation of RUNX2 by TWIST through binding to E-box of P2 promoter. In addition, we proved knockdown of twist1a and twist1b in zebrafish enhanced runx2b transcription, induced ventralized patterning, and promoted bone formation both under normoxia and hypoxic conditions (DFX treatment).

To test whether twist downregulates runx2a/runx2b and inhibits bone formation in vivo; we used the zebrafish embryonic model, in which the distribution of both runx2a and twist1a, b and c are well clarified. Interestingly, microinjection of twist1a and twist1b atgMOs but not MO-Scr (scramble), twist2 and twist3 atgMOs dose-dependently induced an increase in class 3 and 4 ventilared embryos, suggesting knockdown of twist1a/T2 and/or twist1b increased the expression of runx2b. Consistently, quantitative RT-PCR revealed twist1a and twist1b atgMOs increased in T1 and T2 runx2b expressions compared to wild type/ MO-SC embryos at 8, 14 and 48 hpf. Whole-mount in situ hybridization at 8, 14 and 48 hpf further demonstrated the expression of runx2b was induced by twist1a and 1b atgMO. Finally, we confirmed twist1a and twist1b atgMO injection promoted functional mineralization both under normoxia and hypoxia. The embryos survived and had normal morphology with up to an 8 dpf increase in bone formation as shown by an apparent increase of ARS staining at the Ot in whole embryos studies, and as well as an increase in cell condensation and mineralization in the cranial and pharyngeal region in thin tissue sections stained with H&E. Staining with or without DFX treatment. These data taken together suggest knockdown of twist1a or twist1b in zebrafish enhanced runx2b transcription, induced ventralized patterning, and promoted bone formation both under normoxia and hypoxic conditions (DFX treatment).

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