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ESET histone methyltransferase regulates osteoblastic differentiation of mesenchymal stem cells during postnatal bone development

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ABSTRACT

To investigate the effects of histone methyltransferase ESET (also known as SETDB1) on bone metabolism, we analyzed osteoblasts and osteoclasts in ESET knockout animals, and performed osteogenesis assays using ESET-null mesenchymal stem cells. We found that ESET deletion severely impairs osteoblast differentiation but has no effect on osteoclastogenesis, that co-transfection of ESET represses Runx2-mediated luciferase reporter while siRNA knockdown of ESET activates the luciferase reporter in mesenchymal cells, and that ESET is required for postnatal expression of Indian hedgehog protein in the growth plate. As the bone phenotype in ESET-null mice is 100% penetrant, these results support ESET as a critical regulator of osteoblast differentiation during bone development.

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1. Introduction

The evolutionarily conserved SET (suppressor of variegation, enhancer of zest and trithorax) domain is present in a group of proteins that function as histone methyltransferases [1]. Among these histone methyltransferases, the ESET (an ERG-associated protein with a SET domain, also known as SETDB1 protein) was found to methylate histone H3 specifically at lysine 9 (H3-K9) [2]. It has been reported that the ESET gene is widely expressed in a variety of cells and tissues, suggesting that the ESET protein may have multiple cellular functions [3].

The ESET protein contains two major functional domains: the N-terminal tudor domain is responsible for interaction with other chromatin modification enzymes, while the C-terminal SET domain catalyzes methylation of H3-K9. Ubiquitous deletion of the ESET gene was found to cause peri-implantation lethality during embryogenesis [4], however, it was recently shown possible to generate viable animals with tissue-specific deletion of the SET domain encoded by exons 15–22 of the ESET gene [5,6].

To investigate the effects of histone methyltransferases on the differentiation of mesenchymal stem cells into osteoblasts and postnatal bone development, we analyzed the bone phenotype in mice harboring mesenchymal-specific deletion of the SET domain from the ESET protein (therefore inactivates its H3-K9 methyltransferase activity). Our results demonstrate that specific knockout of ESET in mesenchymal cells severely impairs osteoblast differentiation in mutant mice, and is associated with deregulation of Runx2 and Indian hedgehog (Ihh) that are well known for their critical roles in the differentiation of mesenchymal stem cells into both chondrocytes and osteoblasts.

2. Materials and methods

2.1. Generation of mesenchymal-specific ESET knockout, staining for osteoblasts and osteoclasts in bone sections

All experiments were reviewed and approved by the Institutional Animal Care and Use Committee at the VA Puget Sound Health Care System. Generation of the (exon 15&16)$^{Flox/Flox}$; Prx1-Cre mutants was described previously [5]. To identify alkaline phosphatase (ALP)-positive osteoblasts in bone sections, we followed a previously published protocol using an ALP staining kit (Sigma cat #86R-1KT) [7].

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Osteoclasts in the bone are characterized by expression of tartrate-resistant acid phosphatase (TRAP). To visualize osteoclasts in bone sections, fixed tissue sections were stained using a TRAP staining kit (Sigma cat #386A-1KT) following the manufacturer's instructions. Areas with the most osteoclasts were counted in multiple light fields and shown as the average number ± S.D. per light field.

2.2. Osteogenic differentiation of mesenchymal stem cells in vitro

Mesenchymal stem cells were isolated from mouse bone marrow as previously described [8]. Cells were then plated in fibronectin/collagen coated 48-well plates at a density of 40×10^3 cells/well. After reaching confluence, cells were switched to osteogenic differentiation medium (DMEM with 15% FBS, 0.1 μM dexamethasone, 10 mM β-glycerophosphate, and 0.05 mM L-ascorbic acid-2-phosphate). Differentiation medium was changed twice a week. Staining for ALP was done 2 weeks later, and Alizarin red staining for mineralized matrix was carried out after 3 weeks of culture in osteogenic medium.

2.3. Inducible siRNA knockdown of ESET expression in ATDC5 cells

Doxycycline induced siRNA knockdown of ESET was achieved using the pSLIK (single lentiviral vector for inducible knockdown) platform [9]. Oligonucleotides 5′-AGCGACCCGAGGCTTTGCTCTTAAATTAGTGAAGCCACAGATGTAATTTAAGAGCAAAGCCTCGGGC-3′ and 5′-GGCAGCCCGAGGCTTTGCTCTTAAATTACATCTGTGGCTTCACTAATTTAAGAGCAAAGCCTCGGGT-3′ were annealed for insertion into the lentiviral vector to generate a hybrid transcript that fuses green fluorescence protein (GFP) with a microRNA-like short hairpin against nucleotide 3639–3660 of mouse ESET mRNA. A control microRNA-like short hairpin that does not affect ESET expression was similarly generated through annealing of 5′-AGCGAGTTGATCGGCTGTTTGATGATTAGTGAAGCCACAGATGTAATCATCAAACAGCCGATCAACC-3′ and 5′-GGCAGGTGGATCGGCTGTTTGATGATTACATCTGTGGCTTCACTAATCATCAAACAGCCGATCAACT-3′ and subsequent cloning. After co-transfection into 293FT cells with the ViraPower lentiviral packaging DNA mix (Invitrogen), supernatants containing the lentivirus were used to infect ATDC5 cells. Hygromycin (150 μg/ml)-resistant colonies were expanded, treated in maintenance medium plus or minus doxycycline (1 μg/ml), checked for GFP induction in live cells, and lysed at different days post-doxycycline to confirm ESET knockdown.

2.4. Transfection and luciferase assays

Using the FUGENE 6 reagent, 500 ng of pOG2-Luc [10], 1000 ng of pCMV-HA-Runx2 and 1000 ng of pSG5-FL-ESET plus 50 ng of pRL-SV40 were transfected into 5×10^5 of C3H10T1/2 cells in one well of a 6-well plate. Similarly, 2500 ng of pOG2-Luc plus 50 ng of pRL-SV40 was transfected into 5×10^5 ATDC5 cells. After 48 h, the cells were washed once with PBS and lysed with 0.5 ml passive lysis buffer for measurement of luciferase activity using the Dual-Luciferase Reporter Assay System (Promega). *Firefly* luciferase reporter activities were normalized according to the *Renilla* luciferase controls. At least three independent transfections were performed to rule out experimental variations. A separate set of the transfection was collected in 0.1 ml lysis buffer per well for western blotting with a rabbit polyclonal anti-ESET (Santa Cruz Biotch, cat #SC-66884), a rabbit polyclonal anti-Runx2 (Santa Cruz Biotech, cat #SC-10758), or with HRP-conjugated mouse monoclonal anti-Flag (clone M2) and rat monoclonal anti-HA (clone 3F10).

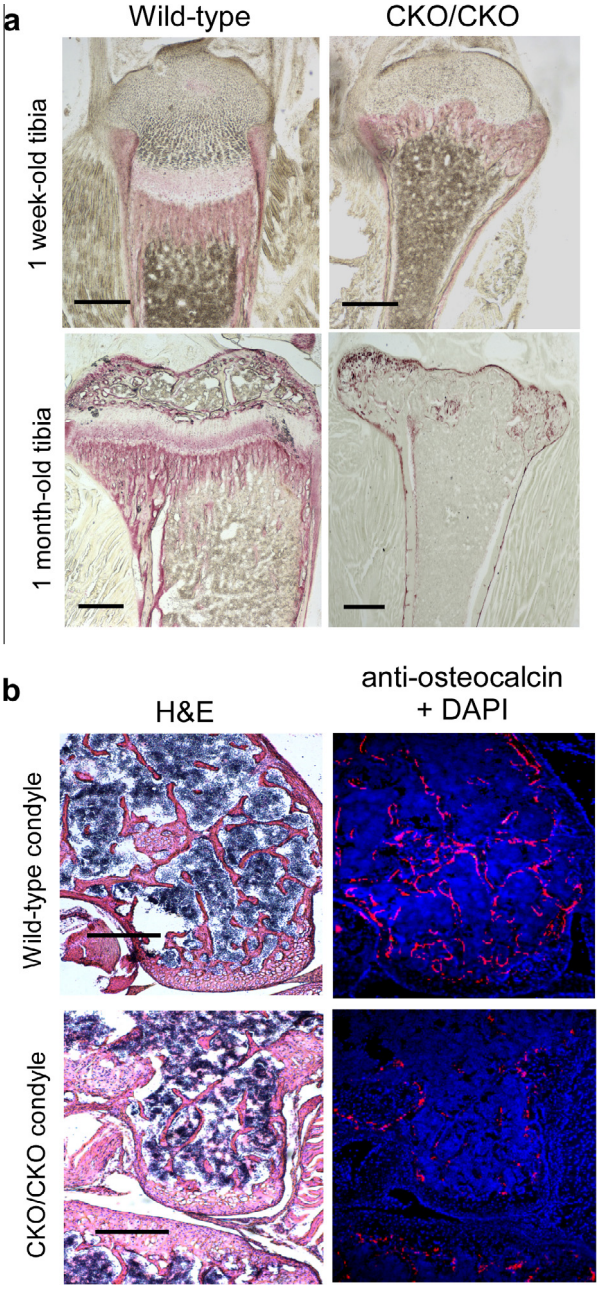

Fig. 1. Significant decrease of osteoblast activity in ESET-deficient mice. (a) Proximal tibias from wild-type and (exon 15&16)$^{CKO/CKO}$ littermate were stained for APL-positive osteoblasts (red) at one week (top panel) and one month (bottom panel) after birth. Note that in addition to decrease in ALP-positive osteoblasts, ESET-deficient tibia also lacked the epiphyseal plate. (b) Paraformaldehyde-fixed and decalcified sections of femoral condyles from one month-old mice were stained by H&E to show general cell morphology, and by anti-osteocalcin (red) to show differences in mature osteoblast activity. Scale bar: 400 μm.

2.5. Immunohistostaining

Paraformaldehyde fixing of tissue sections and antigen retrieval were described previously [5]. A rabbit polyclonal antibody against osteocalcin from Abcam (catlog #ab93876) was used at 1:200 dilution, a rabbit polyclonal antibody against Ihh (Cat #SC-13088, Santa Cruz biotechnology) was used at 1:50 dilution. After overnight incubation with the primary antibody and washes in PBS, the sections were incubated with a Cy3-conjugated goat anti-rabbit IgG from Jackson ImmunoResearch Laboratories at a 1:200 dilution