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ADAR1 ablation decreases bone mass by impairing osteoblast function in mice

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Abstract

Bone mass is controlled through a delicate balance between osteoblast-mediated bone formation and osteoclast-mediated bone resorption. We show here that RNA editing enzyme adenosine deaminase acting on RNA 1 (ADAR1) is critical for proper control of bone mass. Postnatal conditional knockout of *Adar1* (the gene encoding ADAR1) resulted in a severe osteopenic phenotype. Ablation of the *Adar1* gene significantly suppressed osteoblast differentiation without affecting osteoclast differentiation in bone. In vitro deletion of the *Adar1* gene decreased expression of osteoblast-specific *osteocalcin* and *bone sialoprotein* genes, alkaline phosphatase activity, and mineralization, suggesting a direct intrinsic role of ADAR1 in osteoblasts. ADAR1 regulates osteoblast differentiation by, at least in part, modulation of osterix expression, which is essential for bone formation. Further, ablation of the *Adar1* gene decreased the proliferation and survival of bone marrow stromal cells and inhibited the differentiation of mesenchymal stem cells towards osteoblast lineage. Finally, shRNA knockdown of the *Adar1* gene in MC-4 pre-osteoblasts reduced cyclin D1 and cyclin A1 expression and cell growth. Our results identify ADAR1 as a new key regulator of bone mass and suggest that ADAR1 functions in this process mainly through modulation of the intrinsic properties of osteoblasts (i.e., proliferation, survival and differentiation).

Keywords

ADAR1; cyclin D1; osterix; osteoblast; proliferation; differentiation; bone

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1. Introduction

Adenosine (A) to inosine (I) RNA editing, which is carried out by adenosine deaminase acting on RNA (ADAR)(Bass, 2002), is a posttranscriptional process that regulates gene functions through distinct molecular mechanisms (Nishikura, 2006), such as changes of the mRNA coding sequences (Burns et al., 1997; Sommer et al., 1991), generation or elimination of splicing sites (Rueter et al., 1999), and modification of microRNA precursor sequences which impacts the efficacies of corresponding microRNAs (Heale et al., 2009; Yang et al., 2005). ADAR1, the first member of ADAR family, has been demonstrated essential for embryonic development (Shtrichman et al., 2012; Wang et al., 2004) and hematopoietic stem cell differentiation (XuFeng et al., 2009). ADAR1 KO resulted in a massive cell death in the embryos at the early stage of organogenesis (Wang et al., 2004) and the homozygous embryos were not able to survive beyond 11.5–12.0 dpc (Hartner et al., 2004; Wang et al., 2004). Genetically, mutations in ADAR1 is linked to dyschromatosis symmetrica hereditaria (DSH)(Hayashi and Suzuki, 2012). A more recent study showed that mutations in ADAR1 cause the autoimmune disorder Aicardi-Goutieres syndrome (AGS) (Rice et al., 2012). ADAR1 modulates the host response triggered by measles virus infection by suppressing the induction of interferon by measles virus (Li et al., 2012). Our recent study showed that deletion of the *Aadar1* gene causes regression of chronic myelogenous leukemia in mice (Steinman et al., 2012). These studies demonstrate that ADAR1 plays critical roles in regulation of embryonic organogenesis, pathogenesis of human genetic diseases and tumors, and host response to virus infection. However, the postnatal role of ADAR1 in specific tissues and cell types (e.g., bone and osteoblasts) is largely unknown.

Skeletal integrity requires a delicate balance between bone-forming osteoblasts and bone-resorbing osteoclasts. Imbalance between bone formation and bone resorption results in metabolic bone diseases such as osteoporosis. Mesenchymal cells (MSCs) proliferate and differentiate into osteoblasts, which synthesize and deposit the mineralizing extracellular matrix of bone (Stein, 1996). However, molecular mechanisms controlling osteoblast differentiation from MSCs are not completely understood. At the molecular level, osteoblast differentiation is controlled by key transcription factors. One is osterix (Osx), a zinc-finger transcription factor that is specifically expressed in osteoblast lineage cells. Osx is required for embryonic osteoblast differentiation and bone formation (Nakashima et al., 2002). In Osx null mice, the mesenchymal cells do not deposit bone matrix and thereby no bone formation occurs (Nakashima et al., 2002). Because Osx is not expressed in Runx2 null mice (Nakashima et al., 2002), it acts downstream of Runx2, a master regulator of osteoblast differentiation and bone formation (Banerjee et al., 1997; Ducy et al., 1997; Komori et al., 1997; Mundlos et al., 1997; Otto et al., 1997). Conditional deletion of Osx in mice revealed that Osx is also essential for postnatal osteoblast differentiation and bone formation (Zhou et al., 2010). These studies established that Osx is critical for osteoblast differentiation and bone formation during skeletal development and throughout life. However, molecular mechanisms that control Osx expression are poorly understood. As will be shown, in this study we demonstrate that ADAR1 plays a critical role in regulation of Osx expression during osteoblast differentiation.

2. Materials and Methods

2.1. Reagents

Tissue culture media and fetal bovine serum were obtained from HyClone (Logan, UT). Other reagents were obtained from the following sources: Antibodies against Runx2, ATF4, cyclin A1, cyclin D1, p21, p27, cathepsin K, and horseradish peroxidase-conjugated goat anti-rabbit IgG from Santa Cruz (Santa Cruz, CA), antibody against Osx from Abcam Inc (Cambridge, MA), mouse monoclonal antibody against β -actin, alizarin red, cetylpyridinium

chloride, L-ascorbic acid, and β -glycerophosphate from Sigma (St Louis, MO), BrdU immunostaining kit (Zymed Laboratories Inc., San Francisco, California) from Invitrogen, and ApopTag Peroxidase In Situ Apoptosis Detection Kit from Chemicon (Temecula, CA). All other chemicals were of analytical grade.

2.2. Generation of the inducible conditional *Adar1* knockout mice (iKO) and tamoxifen (TM) treatment

Floxed *Adar1* mouse was described previously (Wang et al., 2004) and was crossed with Cre transgenic mouse in which Cre recombinase was globally expressed as a fusion protein tagged with estrogen receptor ligand binding domain as described previously (Hayashi and McMahon, 2002). All the progenies were subjected to PCR genotyping as described previously (Wang et al., 2004). Mice carrying the transgene and the floxed *Adar1* alleles at homozygous status (i.e., inducible *Adar1* knockout mice, hereafter referred as to iKO) were selected for experiments. Mice from the same litters carrying the wild-type *Adar1* alleles were used as controls (hereafter referred as to Ctrl). Tamoxifen (TM, Sigma T5648) was administrated into 4-week-old male mice of both Ctrl and iKO groups through peritoneal injection at the dosage 5 mg/20 g body weight, once a week for 4 weeks. This TM regimen essentially abolished ADAR1 protein expression in bone (Figure 1A) and in primary BMSCs isolated from the TM-treated iKO mice (Figure 2G). All research protocols were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh.

2.3. Micro-computed tomography (μ CT)

One week after the last administration of TM, Ctrl and iKO mice were sacrificed and femurs were isolated. Fixed non-demineralized femurs were used for μ CT analysis using a VIVACT40 (SCANCO Medical AG) following the standards of techniques and terminology recommended by American Society for Bone and Mineral Research (Parfitt et al., 1987) as we described (Cao et al., 2010; Yu et al., 2009). For trabecular bone parameters, transverse CT slices were obtained in the region of interest in the axial direction from the trabecular bone 0.1 mm below the growth plate (bottom of the primary spongiosa) to the mid-femur. Contours were defined and drawn close to the cortical bone. The trabecular bone was then removed and analyzed separately. 3D analysis was then performed on trabecular bone slices. A 3-mm section was used to obtain mid-femoral cortical bone thickness. The analysis of the specimens involves the following bone measurements: bone volume fraction (BV/TV, %), trabecular number (Tb. N), trabecular thickness (Tb. Th), and cortical thickness (Cort.Th).

2.4. Mouse BMSC cultures

Isolation of mouse BMSCs was described previously (Xiao et al., 2002). Briefly, mice were sacrificed and tibiae and femurs were isolated and the epiphyses were cut. Marrow was flushed with DMEM containing 20% FBS and 1% penicillin/streptomycin into a 60-mm dish and the cell suspension was aspirated up and down with a 20-gauge needle in order to break clumps of marrow. The cell suspension (marrow from 2 mice/flask) was then cultured in a T75 flask in the same media. After 10d, cells reached confluency and were ready for experiments.

2.5. RNA isolation, reverse transcription (RT), and quantitative RT-PCR

RNA isolation, RT, and quantitative real-time-PCR (qPCR) were performed to measure the relative mRNA levels using SYBR Green kit (Bio-Rad Laboratories Inc.) as previously described (Yu et al., 2008a). Samples were normalized to *Gapdh* expression. The DNA sequences of mouse primers used in this study are summarized in Table 1.

2.6. Western blot analysis

Western blot analysis was performed as previously described (Yu et al., 2008b). Tibias were frozen in liquid nitrogen and ground into powder using a mortar and pestle. Whole cells or bone tissue powder were extracted by RIPA buffer on ice as described (Zhang et al., 2008) and total proteins were separated by electrophoresis on 8% SDS polyacrylamide gels. The proteins were transferred electrophoretically to nitrocellulose membranes and staining with Ponceau Red to ensure that comparable amounts of proteins were loaded and the transfer was efficient. The membranes were blocked with 5% nonfat milk in TBST for 1 h at room temperature and immunoblotted with Osx, Runx2, ATF4, cyclin A1, cyclin D1, p21, and p27 antibodies. β -actin immunoblot was used as internal control.

2.7. Histological evaluation, bone histomorphometry and immunohistochemistry (IHC)

Tibiae were fixed in 10% formalin at 4 °C for 24h, decalcified in 10% EDTA (pH 7.4) for 10–14d, and embedded in paraffin. Sections of tibiae were used for TRAP staining as described previously (Liu et al., 2003). Bone histomorphometry such as osteoclast surface/bone surface (Oc.S/BS) and osteoclast number/bone perimeter (Oc.Nb/BPm) in both primary and secondary spongiosa of tibiae was measured using an Image Pro Plus 7.0 software (Media Cybernetics, Inc) as previously described (Cao et al., 2010). Five- μ m sections of tibiae were immunohistochemically stained with Osx antibody, cathepsin K antibody, or control IgG using the EnVision+System-HRP (DAB) kit (Dako North America, Inc) as described previously (Yu et al., 2009). Approximately 6–8 sections were obtained from each sample (4–6 samples per group) and analyzed.

2.8. Generation of adenovirus for *Adar1* shRNA and adenoviral infection

We have generated an adenovirus expressing an shRNA targeting the mouse *Adar1* mRNA (gcccaagaactacttcaagaaa) through a commercial company (Welgen, Inc. Worcester, MA). An adenovirus expressing a control shRNA was purchased from the same company. MC-4 cells were infected with adenovirus as described previously (Yang et al., 2011). Briefly, equal amounts of both adenovirus were added to cells in 1% FBS and incubated for 1 h at 37 °C. Dishes were rotated every 5 min for the first 15 min to ensure that all of the cells were exposed to virus. After 3 h, media were aspirated, and cultures were rinsed twice with serum-free medium, and then fresh media supplemented with 10% FBS were added to the dishes. 48 h later, cells were harvested for gene expression studies. Western blotting showed that the level of ADAR1 protein was dramatically decreased by *Adar1* shRNA in MC-4 cells (Figure 6A).

2.9. Colony forming unit-fibroblast (CFU-F) assay and colony forming unit-osteoblast (CFU-OB) assay

The CFU-F assay was performed using the Mesencult Proliferation Kit (Mouse) (Stemcell Technologies) for the expansion and enumeration of the mesenchymal stem cells (MSCs) as previously described (Xiao et al., 2012). Briefly, 1×10^6 bone marrow nucleated cells per 35-mm dish were seeded and cultured at 37°C in 5% CO₂ for 10d, followed by Giemsa staining. The numbers of CFU-Fs were counted under a microscope. CFU-OB assay was performed as previously described (Xiao et al., 2012). Briefly, 1×10^6 bone marrow nucleated cells per 60-mm dish were seeded and cultured for 21d in differentiation medium (α -MEM containing 10% FBS, 1% penicillin/streptomycin, 50 μ g/ml L-ascorbic acid and 2.0 mM β -glycerophosphate). Media were changed every 2d. Alizarin red staining was used to identify and enumerate the colonies containing mineralized bone matrix, which were designated as CFU-OB colonies.

2.10. ALP assay

ALP assay was performed as previously described (Xiao et al., 2012). Briefly, BMSCs were differentiated for 7d and harvested in 1× Passive Buffer (Promega, Madison, WI). Lysates were clarified by centrifugation (20 min, 13,000 × g, 4°C). Five µl of cell extracts were added to each well (96-well plate) containing 150 µl p-nitrophenyl phosphate at 37 °C for 10–60 min depending on the ALP activity in the extracts. ALP activity was determined by absorbance measurement at 405 nm on a 96-well plate reader. ALP activity was normalized to total protein.

2.11. MTS assay, BrdU staining, and TUNEL staining

MTS assay was used to measure the growth of BMSCs as previously described (Zhang et al., 2008). Briefly, cells were planted in a 96-well plate (1×10^4 cells/well) in 100 µl proliferation medium (α-MEM containing 10% FBS and 1% penicillin/streptomycin). Cells were incubated at 37 °C for 24h to allow attachment. The media were changed every 48h. At different time points, 20 µl of CellTitre96AQ solution reagent (Promega, Madison, WI) was added into each well and incubated for 2h. The absorbance was recorded at 490 nm using a 96-well plate reader. BrdU staining was used to measure cell proliferation as previously described (Cao et al., 2010; Yu et al., 2009; Zhang et al., 2008). Cell survival was evaluated using the ApopTag Peroxidase In Situ Apoptosis Detection Kit according to the manufacturer's instruction (Millipore).

2.12. Statistical analysis

Data was analyzed with a GraphPad Prism software (4.0). A one-way ANOVA analysis was used followed by the Tukey test. Students' t test was used to test for differences between two groups of data as needed. Results were expressed as means ± standard deviation (S.D.). Differences with a $p < 0.05$ was considered as statistically significant.

3. Results

3.1. Postnatal conditional knockout of the *Adar1* gene decreases bone mass, resulting in an osteopenic phenotype in mice

To investigate the postnatal role of ADAR1 in bone, we deleted the gene encoding ADAR1 (i.e., *Adar1*) by treating the Ctrl and iKO mice with tamoxifen (TM) as described in Materials and Methods. One week after the last administration of TM, animals were sacrificed and tibiae and femurs were isolated. IHC staining using an anti-ADAR1 antibody displayed a strong signal in osteoblasts on trabecular surfaces of Ctrl tibiae, which was undetectable by IHC in trabecular osteoblasts of iKO tibiae (Figure 1A). We next examined if ablation of the *Adar1* gene affected bone mass by performing quantitative µCT analysis of histomorphometric parameters using fixed non-demineralized femurs from Ctrl and iKO mice. Results showed that, while trabecular number (Tb.N) was not altered by ADAR1 ablation (Figure 1D), ablation of the *Adar1* gene resulted in significant decreases in bone volume/tissue volume (BV/TV) (Figure 1E), trabecular thickness (Tb. Th) (Figure 1F), and cortical thickness (Cort.Th) (Figure 1G). These results demonstrate that ADAR1 is critical for control of bone mass postnatally.

3.2. Ablation of the *Adar1* gene compromises osteoblast differentiation in bone

We investigated if ablation of the *Adar1* gene impairs osteoblast differentiation, which is critical for bone formation. Tibiae from Ctrl and iKO mice were used for isolation of total RNAs and protein extracts, followed by qPCR analysis and Western blotting. Results showed that ablation of the *Adar1* gene significantly decreased the mRNA levels of *osteocalcin* (*Ocn*) and *bone sialoprotein* (*Bsp*) in bone (Figure 2A), both osteoblast

differentiation markers. Ablation of the *Adar1* gene significantly decreased the level of *osterix* (*Osx*) mRNA (Figure 2A), which is an upstream transcriptional activator of *Ocn* and *Bsp* and is essential for bone formation (Nakashima et al., 2002). In contrast, although Runx2 is essential for *Osx* expression and osteoblast differentiation (Nakashima et al., 2002), its expression was not reduced by *Adar1* ablation. Likewise, the level of *Atf4* mRNA, which is also required for *Ocn* and *Bsp* expression and osteoblast differentiation (Yang et al., 2004; Yu et al., 2009; Yu et al., 2008a), was not altered by *Adar1* ablation. Western blotting revealed that the level of *Osx*, but not Runx2 or ATF4, protein in bone was dramatically reduced by *Adar1* ablation (Figure 2B). Strikingly, IHC staining revealed that *Osx*-positive osteoblasts, which were identified on trabecular surfaces throughout the Ctrl tibia, were completely lost on all trabecular bone surfaces of the iKO tibiae (Figure 2C), suggesting that ADAR1 is critical for osteoblast differentiation in bone. In vivo deletion of the *Adar1* gene greatly impaired *Ocn* and *Osx* expression and ALP activity in BMSCs in vitro (Figure 2, D–F). PCR analysis confirmed that the *Adar1* gene was essentially ablated in primary BMSCs from iKO mice treated with TM but not vehicle (veh) (Figure 2G).

3.3. Ablation of the *Adar1* gene does not alter osteoclast differentiation in vivo

Bone mass is controlled by not only bone-forming osteoblasts but also bone-resorbing osteoclasts. Thus, to better understand the function of ADAR1 in control of bone mass, we have analyzed the role of ADAR1 in osteoclast differentiation. The tibiae of Ctrl and iKO mice were fixed, decalcified, and paraffin-embedded, and histological sections were stained for the osteoclast enzyme tartrate-resistant acid phosphatase (TRAP). The results showed that TRAP activity was not significantly different in tibiae of the two genotypes (Figure 3A). Bone histomorphometry showed that osteoclast surface/bone surface (Oc.S/BS) and osteoclast number/bone perimeter (Oc.Nb/BPm) were not altered in both primary and secondary spongiosa of tibiae by *Adar1* ablation (Figure 3, B–F). IHC staining of tibial sections using specific antibody for cathepsin K (Cat K), a marker for terminal osteoclast differentiation, or normal IgG as a control showed that ADAR1 ablation did not significantly affect the level of cathepsin K (Cat K) protein (Figure 3G). Finally, qPCR analysis showed that the expression levels of osteoclast differentiation marker genes including those encoding Cat K, nuclear factor of activated T cells c1 (Nfatc1), receptor activator of NF-kappaB (Rank), and matrix metalloproteinase-9 (Mmp-9) were not significantly altered by *Adar1* ablation (Figure 3H). Thus, *Adar1* ablation did not impact osteoclast differentiation in bone.

3.4. Ablation of the *Adar1* gene decreases the formation of osteoblast progenitors in bone marrows

To study the mechanism whereby ADAR1 impacts osteoblast differentiation, we investigated the effect of *Adar1* ablation on the development of mesenchymal stem cells (MSCs) by performing the CFU-F assays using bone marrow cells from Ctrl and iKO mice treated with TM as described in Materials and Methods. Results showed that *Adar1* ablation did not alter the number of CFU-Fs from bone marrow (Figure 4, A and B), suggesting that ADAR1 is not required for the expansion and formation of MSCs, which can develop into osteoblast progenitors and other cell types under distinct conditions. We next determined if *Adar1* ablation impacts the formation of osteoblast progenitors by performing CFU-OB assays using bone marrow cells from Ctrl and iKO mice. The results revealed that the number of CFU-OBs was significantly reduced in bone marrow cultures from iKO mice compared to that from Ctrl mice (Figure 4, C and D). Thus, ADAR1 is required for MSC differentiation towards osteoblasts.

3.5. Ablation of the *Adar1* gene decreases cell proliferation and increases apoptosis in primary BMSC cultures

The decrease in CFU-OBs in iKO bone marrow cultures observed above could be explained by a cell-autonomous defect in proliferation and/or survival of BMSCs due to *Adar1* ablation. Results from MTS assays showed that the growth of iKO BMSCs was significantly slower than that of Ctrl BMSCs at both d6 and d8 cultures (Figure 5A). BrdU staining revealed that the rate of proliferation of iKO BMSCs was significantly decreased compared to that of Ctrl BMSCs (Figure 5, B and C). TUNEL staining showed that the percentage of apoptotic cells was low (~1%) in Ctrl BMSC cultures, but was significantly increased in iKO BMSC cultures (Figure 5, D and E).

3.6. shRNA knockdown of ADAR1 decreases cyclin D1 and cyclin A1 expression and reduces cell growth in MC-4 pre-osteoblastic cells

We examined the effects of *Adar1* shRNA on expression levels of major regulators of cell cycle progression. Western blot analysis showed that the expression of ADAR1 protein was essentially abolished in MC-4 pre-osteoblastic cells infected with adenovirus expressing *Adar1* shRNA (Figure 6A). In addition, *Adar1* shRNA dramatically decreased the levels of cyclin D1 and cyclin A1 proteins in MC-4 cells (Figure 6A). In contrast, the levels of p21, and p27 proteins were not altered by *Adar1* shRNA in MC-4 cells (Figure 6A). qPCR analysis shows that the levels of *cyclin D1* and *cyclin A1* mRNAs were decreased by *Adar1* shRNA (Figure 6B). Finally, *Adar1* shRNA significantly reduced the growth of MC-4 cells (Figure 6C).

3.7. In vitro ablation of the *Adar1* gene directly impairs osteoblast differentiation in primary BMSCs

The defective osteoblast differentiation observed in primary BMSCs and bones from the iKO mice treated with TM could be secondary to an impaired bone microenvironment due to the *Adar1* ablation. To test this possibility, primary BMSCs from Ctrl and iKO mice that were not previously treated with TM were cultured in proliferation media until confluency and then treated with and without indicated concentrations of 4-hydroxytamoxifen (4OHT) (i.e., to delete the *Adar1* gene in vitro) for 24h and switched to differentiation media for 7d. Results showed that short time exposure of the BMSCs to 4OHT dose-dependently and dramatically inhibited the expression levels of *Ocn*, *Bsp*, and *Osx* mRNAs in primary iKO BMSCs but not Ctrl cells (Figure 7, A–D). Note: 4OHT slightly reduced *Bsp* mRNA expression in the Ctrl BMSCs in a dose-dependent manner (Figure 7B). 4OHT treatment reduced *Osx*, but not *Runx2* and *ATF4*, protein expression in iKO BMSCs but not Ctrl cells (Figure 7E). 4OHT treatment similarly inhibited ALP activity (Figure 7F) and osteoblast mineralization (Figure 7, G and H) in iKO BMSCs from iKO cells.

4. Discussion

In this study we examined the role of ADAR1, a RNA-editing enzyme, in regulation of osteoblast function and bone mass by ablating the *Adar1* gene in mice using an inducible Cre/loxP system in mice. Since global KO of the *Adar1* gene is embryonic lethal (Hartner et al., 2004; Wang et al., 2004), this inducible conditional knockout *Adar1* mouse model provides an invaluable tool to evaluate the postnatal role of ADAR1 in different cell types and tissues. By using this model system, we demonstrate that ADAR1 is essential for osteoblast function and the control of bone mass.

Results from this study demonstrate that ADAR1 plays a critical role in regulation of osteoblast differentiation. Ablation of the *Adar1* gene reduced all parameters for osteoblast differentiation in vitro and in bone. In vitro ablation of the *Adar1* gene also blocked

osteoblast differentiation in primary BMSC cultures, which suggests that the defective osteoblast differentiation caused by the loss of ADAR1 is not secondary to an impaired bone microenvironment. Because loss of ADAR1 did not impact osteoclast differentiation *in vivo*, the reduced bone mass is primarily due to impaired osteoblast function. ADAR1 regulates osteoblast differentiation by modulating, at least in part, expression of *Osx*, a key transcription factor for osteoblast differentiation and bone formation. First, postnatal ablation of the *Adar1* gene in mice dramatically reduced *Osx* expression in bone and strikingly reduced the number of *Osx*-positive osteoblasts located on trabecular bone surfaces. Second, *Osx* expression was significantly reduced in primary BMSC cultures from mice in which the *Adar1* gene is ablated. Third, *in vitro* ablation of the *Adar1* gene in primary BMSC cultures decreased *Osx* expression. Finally, the regulation of *Osx* by ADAR1 is specific because the expression of *Runx2* (an upstream activator of the *Osx* gene), and ATF4, two key osteoblast transcription factors, was not altered by the *Adar1* ablation *in vitro* and *in bone*.

While our results clearly establish an important role of ADAR1 in regulation of *Osx* expression, how ADAR1 up-regulates *Osx* expression in osteoblasts, however, remains to be defined. It is well known that ADARs act on the mRNA substrates leading to the protein coding changes that alter the protein properties, such as the editing on the Q/R site in the pre-mRNA substrate coding for a subunit of the neuron receptor α -amino-3-hydroxy-5methyl-4-isoxazolepropionate (AMPA), the glutamate-activated cation channel subunit B (GluR-B). This editing changes the code for glutamine (Q) to arginine (R) in the second transmembrane region which considerably decreases the Ca^{++} permeability and ion conductance and thereby changes this channel's gating behavior (Higuchi et al., 1993; Kohler et al., 1993). Similarly, editing on the 5 sites of 5-hydroxytryptamine receptor 2C changes its G protein coupling capacity (Burns et al., 1997; Wang et al., 2000). To test if the *Osx* mRNA is an editing substrate of ADAR1, we have analyzed the available RNA databases and failed to identify any potential editing sites in the coding regions (unpublished data). Future studies will determine if potential editing sites exist in non-coding regions. It is known that some of the ADAR1 functions are not mediated through its RNA editing activity. Herbert and coworkers showed that induction of protein translation by ADAR1 is not dependent on RNA editing (Herbert et al., 2002). Further, ADAR1 interacts with NF90 through double-stranded RNA and regulates NF90-mediated gene expression independently of RNA editing (Nie et al., 2005). ADAR1 also interacts with the mRNA surveillance protein hUpf1 in the cell nucleus (Agranat et al., 2008). It is possible that ADAR1 regulates *Osx* expression through protein-protein interactions and/or modulation of expression of upstream activator(s) and suppressor(s) of *Osx* in osteoblasts. Future studies will determine these possibilities.

Our studies demonstrate that ADAR1 is critical for the growth and proliferation of primary BMSCs and MC-4 pre-osteoblastic cells. This regulation is achieved by modulating, at least in part, expression of cyclin D1 and cyclin A1, both key regulators of cell cycle progression and growth. Cyclin D1 is a key sensor and integrator of extracellular signals of cells and plays a critical role in cell cycle progression and proliferation (Stacey, 2003). The expression level of cyclin D1 has been shown to be rate-limiting in cell proliferation induced by a variety of stimuli (Zhao et al., 2001). The level of cyclin D1 protein was significantly reduced by *Adar1* shRNA in MC-4 pre-osteoblasts. Interestingly, *Adar1* shRNA also decreased expression of cyclin A1, another key regulator of cell cycle progression, which could additionally contribute to the defective cell proliferation and growth by the loss of ADAR1. Since *Adar1* shRNA did not impact the levels of both *cyclin D1* and *cyclin A1* mRNAs, this regulation must involve a post-transcriptional mechanism.

Finally, this study demonstrates that ADAR1 is critical for MSC differentiation towards osteoblastic lineage. *Adar1* ablation did not impact the formation of CFU-F in bone marrow cultures, which suggests that ADAR1 is not required for the expansion and formation of MSCs, which can differentiate into osteoblast, adipocyte, chondrocyte, and other cell types under distinct conditions. However, ablation of the *Adar1* gene dramatically decreased the formation of CFU-OBs in bone marrow cultures. Because each colony is derived from a single osteoblast progenitor, the number of CFU-OB colonies reflects the number of osteoblast progenitors present in the original bone marrow isolate that are capable of differentiating into osteoblast. These results suggest that ADAR1 plays an important role in regulation of early differentiation.

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Abbreviations

ADAR1	adenosine deaminase acting on RNA 1
Alp	alkaline phosphatase
AMPA	α -amino-3-hydroxy-5methyl-4-isoxazolepropionate
ANOVA	analysis of variance between groups
ATF4	activating transcription factor 4
BMSC	bone marrow stromal cells
BrdU	5-bromo-2'-deoxyuridine
Bsp	bone sialoprotein
Cat K	cathepsin K
CFU-F	colony-forming unit- fibroblasts
CFU-OB	colony-forming unit- osteoblasts
μCT	Micro-computed tomography
Gapdh	glyceraldehydes-3-phosphate dehydrogenase
GluR-B	glutamate-activated cation channel subunit B
IHC	immunohistochemistry
MSC	mesenchymal stem cells
Mmp-9	matrix metalloproteinase-9
Nfatc1	nuclear factor of activated T cells c1
Ocn	osteocalcin
Oc.Nb/BPm	osteoclast number/bone perimeter
Oc.S/BS	osteoclast surface/bone surface
4OHT	4-hydroxytamoxifen
Osx	osterix
qPCR	quantitative or real-time (RT-) polymerase chain reaction

Rank	receptor activator of NF-kappaB
RT	reverse transcription
Runx2	runt-related transcription factor 2
shRNA	short hairpin RNA
TM	tamoxifen
TRAP	tartrate-resistant acid phosphatase
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling

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Research Highlights

- We have ablated *Adar1* gene postnatally using an inducible Cre/loxP system in mice.
- *Adar1* ablation decreases bone mass, resulting in severe osteopenia.
- *Adar1* ablation suppresses *Osx* expression and osteoblast differentiation
- *Adar1* ablation decreases cyclins D1 and A1 expression and osteoblast proliferation.
- *Adar1* ablation inhibits MSC differentiation towards osteoblast progenitor.

Figure 1. Postnatal conditional knockout of the Adar1 gene significantly decreases both trabecular and cortical bone in mice

(A) IHC staining. Five- μ m tibial sections were immunohistochemically stained with an anti-ADAR1 antibody. Strong ADAR1 signal was detected in osteoblasts located on trabecular bone surfaces of Ctrl mice, which was lost in iKO osteoblasts. Original magnification: $\times 400$. (B–C) Representative images from 3D μ CT reconstruction of femur trabecular (B) and cortical (C) bones. (D–G) Quantitative analysis of trabecular number (Tb.N) (D), bone volume/tissue volume (BV/TV) (E), trabecular thickness (Tb.Th) (F), and cortical thickness (Cort.Th) (G) of femurs. $N=4-6$, $*p < 0.05$ (versus Ctrl).

Figure 2. In vivo ablation of the *Adar1* gene severely impairs osteoblast differentiation in bone and the ability of primary BMSCs from iKO mice to differentiate in vitro

(A) qPCR. Total RNA from Ctrl and iKO tibiae was used for qPCR using specific primers for *Osx*, *Bsp*, *Osx*, *Runx2*, and *Atf4* mRNAs, which were normalized to *Gapdh* mRNA. N=4–6, **p* < 0.05 (versus Ctrl). (B) Western blot analysis. Protein extracts from Ctrl and iKO tibiae were used for Western blotting using specific antibodies against for *Osx*, *Runx2*, and *ATF4* proteins. β -actin was used as a loading control. (C) IHC staining. Five- μ m tibial sections were immunohistochemically stained with anti-*Osx* antibody or control IgG as indicated in the figure. Arrows indicate the nuclei of *Osx*-positive osteoblasts located on trabecular bone surfaces that were stained brown. *Osx*-negative cells were stained blue.

Original magnification: $\times 100$ (top), $\times 400$ (bottom). **(D–F)** BMSCs isolated from Ctrl and iKO mice were differentiated in vitro for 7d, followed by qPCR for *Ocn*, *Osx*, and *Runx2* mRNAs (which was normalized to *Gapdh* mRNA) (**D**), or by Western blotting (**E**) for *Osx* and *Runx2* (β -actin was used as a loading control), or by ALP activity assay (**F**). Bars represent means $\pm$ S.D. from three independent experiments. * $p < 0.05$ (versus Ctrl). **(G)** Genomic DNAs were isolated from primary Ctrl and iKO BMSCs and used for PCR analysis using specific primers.

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Figure 3. Ablation of the *Adar1* gene does not alter osteoclast differentiation in bone (A–F) TRAP staining. Tibial sections of Ctrl and iKO mice were stained for TRAP activity. TRAP activity in metaphyseal regions of tibiae is shown (A). Arrows indicate TRAP-positive osteoclasts on trabecular surfaces (B). Oc.S/BS and Oc.Nb/BPm in primary (C and D) and secondary (E and F) spongiosa of tibiae were measured. N=4–6. (G) IHC staining. Five- μ m tibial sections of Ctrl and iKO mice were immunohistochemically stained with anti-Cat K antibody or control IgG as indicated in the figure. (H) qPCR. Tibiae from Ctrl and iKO mice were harvested for RNA isolation and qPCR analysis using specific primers

for *Cat K*, *Nfatc1*, *Rank*, and *Mmp9* mRNAs, which were normalized to *Gapdh* mRNA. N=4–6.

Figure 4. Ablation of the Adar1 gene dramatically decreases the formation of osteoblast progenitors in bone marrow cultures

(**A** and **B**) CFU-F assay. Bone marrow nucleated cells (1×10^6 cells) from Ctrl and iKO mice were seeded in 35-mm culture dishes and cultured using the Mesencult Proliferation Kit (Mouse) for d10, followed by Giemsa staining. The numbers of CFU-Fs were counted under a microscope. (**C** and **D**) CFU-OB assay. Bone marrow nucleated cells (1×10^6 cells) from Ctrl and iKO mice were seeded in 60-mm culture dishes in osteoblast differentiation medium (complete α -MEM containing 50 μ g/ml L-ascorbic acid and 2.0 mM β -glycerophosphate). Media were changed every 2d. At d21, alizarin red staining was used to identify and enumerate the colonies containing mineralized bone matrix, which were designated as CFU-osteoblast (CFU-OB) colonies. Bars represent means $\pm$ S.D. from three independent experiments. * $p < 0.05$ (versus Ctrl).

Figure 5. Ablation of the Adar1 gene decreases cell growth and increases cell apoptosis in primary BMSC cultures

(A) MTS assay. Primary Ctrl and iKO BMSCs were seeded at a density of 1×10^4 cells/well in 96-well plates in proliferation medium. MTS assays were performed on days 0, 2, 4, 6, and 8 as indicated. * $p < 0.05$ (vs. Ctrl). (B–E) Ctrl and iKO BMSCs cultured in 8-well chambers (5×10^5 cells/well) in proliferation medium for 72 h followed by BrdU staining (B and C) or TUNEL staining (D and E) as described in (Yu et al., 2009; Zhang et al., 2008). Arrows indicate BrdU-positive (proliferating) cells (B) or apoptotic cells (C). Magnification: 100X. Bars represent means $\pm$ S.D. from three independent experiments. * $p < 0.05$ (vs. Ctrl).

Figure 6. shRNA knockdown of the Adar1 gene significantly decreases expression of cyclin D1 and cyclin A1 as well as cell growth in MC-4 pre-osteoblast cultures

(A–D) Adenoviral shRNA knockdown of ADAR1 in MC-4 cells. Cells were infected with equal amount of adenoviral vectors for ADAR1 shRNA or control shRNA. 48 h later, cells were harvested for Western blotting for cyclin A1, cyclin D1, p21 and p27. β-actin was used as a loading control (A), qPCR analysis for *cyclin A1*, *cyclin D1*, and *cyclin D3* mRNAs, which were normalized to *Gapdh* mRNA (B), MTS assays (C), and direct cell count (D). Bars represent means ± S.D. from three independent experiments. * $p < 0.05$ (vs. Control siRNA).

Figure 7. In vitro ablation of the Adar1 gene greatly impairs osteoblast differentiation in primary BMSC cultures

(A–H) Osteoblast differentiation assays. Primary BMSCs isolated from Ctrl and iKO mice that were not previously treated with TM were treated with and without indicated concentrations of 4-hydroxytamoxifen (4OHT) for 24h in vitro and switched to osteoblast differentiation media for 7d (A–F), followed by RNA isolation for quantitative real-time PCR using specific primers for *Ocn*, *Bsp*, *Osx*, and *Atf4* mRNAs, which were normalized to *Gapdh* mRNA (A–D), or for isolation of whole cell extracts for Western blot analysis using specific antibodies against for Osx, Runx2, and ATF4 proteins (β -actin was used as a loading control) (E), or for ALP assay (F). Primary BMSCs were treated with 4OHT as in

(A–F) for 24h and switched to differentiation media for 14d, followed by alizarin red staining (G). Alizarin red was extracted with 10% cetylpyridinium chloride and OD562 was read (H). Bars represent means $\pm$ S.D. from three independent experiments. * $p < 0.05$ (versus Ctrl).

Table 1

real-time PCR primers

Gene name	5' primer	3' primer
<i>Atf4</i>	GAGCTTCCTGAACAGCGAAGTG	TGGCCACCTCCAGATAGTCATC
<i>Bsp</i>	AAGAGGAAGAAAATGAGAACGA	GCTTCTTCTCCGTTGTCTCC
<i>CatK</i>	AATACGTGCAGCAGAACGGAGGC	CTCGTTCCCCACAGGAATCTCTGTAC
<i>Cyclin A1</i>	ATTGTGCCTTGCCTGAGTGAGC	GCGGCTCCATGAGGGACA
<i>Cyclin D1</i>	GAGGAGGGGGAAGTGGAGGA	CCTCTTTGCGGGTGCCACTA
<i>Gapdh</i>	CAGTGCCAGCCTCGTCCCGTAGA	CTGCAAATGGCAGCCCTGGTGAC
<i>Mmp9</i>	TGCCCTGGAACTCACACGACATCTTC	TGCCCTGGAACTCACACGACATCTTC
<i>Nfatc1</i>	CCCCATCCGCCAGGCTACA	GGTTGTCTGCACTGAGCCAACTCC
<i>Ocn</i>	TAGTGAACAGACTCCGCGCTA	TGTAGGCGGTCTTCAAGCCAT
<i>Osx</i>	AGAGGTTCACTCGCTCTGACGA	TTGCTCAAGTGGTCGCTTCTG
<i>Rank</i>	AGAGGGGAGCCTCAGGGTCC	AAGTTCATCACCTGCCCCTAGTA
<i>Runx2</i>	TAAAGTGACAGTGGACGGTCCC	TGCGCCCTAAATCACTGAGG