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Osteopenia in transgenic mice with osteoblast-targeted expression of the inducible cAMP early repressor

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ABSTRACT

ICER is a member of the CREM family of basic leucine zipper transcription factors that acts as a dominant negative regulator of gene transcription. Four different isoforms of ICER (I, Iγ, II and IIγ) are transcribed from the P2 promoter of the *Crem* gene. We previously found that each of the ICER isoforms is induced by parathyroid hormone in osteoblasts. The goal of the present study was to assess the function of ICER in bone by overexpressing ICER in osteoblasts of transgenic mice. ICER I and ICER II cDNAs, each containing an N-terminal FLAG epitope tag, were cloned downstream of a fragment containing 3.6 kb of the rat Col1a1 promoter and most of the rat Col1a1 first intron to produce pOBCol3.6-ICER I and pOBCol3.6-ICER II transgenes, respectively. Multiple lines of mice were generated bearing the ICER I and ICER II transgenes. At 8 weeks of age, ICER I and ICER II transgenic mice had lower body weights and decreased bone mineral density of femurs and vertebrae. Further studies were done with ICER I transgenic mice, which had greatly reduced trabecular bone volume and a markedly decreased bone formation rate in femurs. Osteoblast differentiation and osteocalcin expression were reduced in ex vivo bone marrow cultures from ICER I transgenic mice. ICER I antagonized the activity of ATF4 at its consensus DNA binding site in the osteocalcin promoter in vitro. Thus, transgenic mice with osteoblast-targeted overexpression of ICER exhibited osteopenia caused primarily by reduced bone formation. We speculate that ICER regulates the activity and/or expression of ATF/CREB factors required for normal bone formation.

Introduction

The cAMP responsive element modulator (CREM) is a member of the CREB/ATF family of basic leucine zipper (bZIP) transcription factors [1]. *Crem* encodes multiple isoforms that give rise to both activators and inhibitors of gene expression. *Crem* expression is regulated at multiple levels, including transcription from four different promoters [1–4], mRNA splicing [4,5] and the use of alternative polyadenylation [4] and translational initiation sites [6,7].

The inducible cAMP early repressor (ICER) is transcribed from the P2 promoter of the *Crem* gene [8,9]. The P2 promoter is located within the 10 kb intron between exons G and γ. The P2 promoter contains two contiguous pairs of cAMP response element (CRE) sites termed cAMP autoregulatory response elements (CAREs) that are strongly inducible by cAMP [10]. Four ICER isoforms (I, Iγ, II, and IIγ) can be generated by alternative splicing of the γ domain and DNA binding domain I. The transcripts for ICER I and Iγ contain the contiguous DNA binding domains I and II sequences. However, a stop codon at the end of the DNA binding domain I prevents translation of DNA binding domain II. Due to alternative splicing, the transcripts for ICER II and IIγ contain only DNA binding domain II. DNA binding domains I and II are very similar and thus, all ICER proteins, which consist almost exclusively of the bZip domain of CREM, are thought to have similar activity as transcriptional repressors [1].

ICER was first discovered in pineal gland and plays a role in the regulation of circadian rhythms [11]. ICER was subsequently shown to regulate a variety of other cellular functions including interleukin-2 [12,13] and interleukin-4 [14] production in T cells, cyclin A expression and cell proliferation in AtT20 cells [15] and Fas ligand expression in T

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and natural killer lymphocytes [16]. Rat and human prostate tumor cells engineered to overexpress ICER are unable to form tumors in nude mice [17,18]. The sustained induction of ICER leads to cardiac myocyte apoptosis [19]. An important aspect of ICER biology is its ability to repress its own production. ICER homodimers inhibit transactivation of the P2 promoter by binding to the CAREs [1,20]. This represents an autoregulatory feedback loop that allows the resetting of ICER-inhibited gene expression. Thus, ICER may contribute to shaping the transient induction of gene expression in response to cAMP.

We previously reported that each of the four ICER isoforms is rapidly and transiently induced by PTH in osteoblasts via the cAMP-PKA signaling pathway [21,22]. Moreover, we showed that induction of a transfected *Pghs2* promoter-reporter construct with forskolin, a direct adenylase cyclase stimulator, is inhibited by transfection of an ICER IIγ expression construct in MC3T3-E1 cells. This led us to speculate that ICER induction in osteoblasts might represent a mechanism for regulating gene expression in response to PTH and other agonists that increase cAMP levels. To gain insight into the actions of ICER in vivo, we developed transgenic mice that overexpress ICER I and ICER II broadly in cells of the osteoblast lineage. Osteoblast-targeted ICER transgenic mice showed reduced body size, trabecular bone volume and bone formation. Bone marrow cultures from ICER transgenic mice displayed reduced osteoblast differentiation.

Materials and methods

Animals

All animal care procedures were reviewed and approved by the University of Connecticut Health Center Animal Care Committee. To produce ICER transgenic mice, FLAG-ICER I and FLAG-ICER II cDNAs were amplified by PCR from pCR3.1-F-ICER with an Xba I-built-in 5′ primer and a 3′ primer corresponding to the 3′ end of ICER I. The PCR products were cloned directly to a pCR2.1 vector (Invitrogen Company, Carlsbad, CA). After verifying the orientation and the sequence of the inserts, the Xba I fragment was released and cloned into a ClaPa polylinker, which is flanked by Cla sites and contains a the bovine growth hormone polyadenylation (bGH poly A) sequence [23]. The FLAG-ICER-bGH poly A cassette was released by digestion with Cla1 and cloned into pBC-SK+, which contains the rat Col1a1 gene fragment from −3518 to +1594 bp including part of the first exon in which the AUG initiator has been mutated and most of the first intron [23]. This step produced the pOBCol3.6-ICER transgenes (Fig. 1). The downstream Xho I site of the pOBCol3.6-ICER transgenes were mutated to an Sst II site with a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Sst II fragments of pOBCol3.6-ICER I and II were gel purified and microinjected into CD-1 embryos to generate transgenic lines. The transgenes were detected as a 700 nt product by PCR of tail DNA using primers corresponding to the rat Col1a1 first intron (5′-ACCCTCCTCCATTTTAGCC) and the FLAG sequence (5′-CATCGTCG-TCCTTGTAGTC) with denaturation at 94 °C for 30 s, annealing at 65 °C for 30 s and extension at 72 °C for 2 min for 32 cycles [24]. To generate experimental mice, mating units typically consisted of wild-type females and hemizygous transgenic males.

In some experiments, pOBCol3.6-ICER I mice (line 284) were bred with mice containing a green fluorescent protein (GFP) transgene driven by a 2.3-kb fragment of the rat Col1a1 promoter and part of the first Col1a1 intron (pOBCol2.3-GFP) (kindly provided by Dr. David Rowe), which is a real time, fluorescent marker of mature osteoblasts [25]. Homozygous pOBCol2.3-GFP mice were bred with hemizygous pOBCol3.6-ICER I mice. This yielded progeny that were all hemizygous for the pOBCol2.3-GFP transgene and either wild type or hemizygous for the pOBCol3.6-ICER I transgene.

Immunostaining

Primary calvarial osteoblasts from 7-day-old wild-type and transgenic mice were prepared as previously described [26]. Immunostaining for the FLAG-tagged ICER I transgene was performed as previously described with some modifications [24]. Primary neonatal calvarial osteoblasts were plated in αMEM medium with 10% heat-inactivated fetal calf serum at 8600 cells/well in a 4-well chamber slide. Cells were grown to 60–80% confluence. The medium was removed and cells were washed, fixed and stained using the M2 monoclonal primary antibody that recognizes the FLAG epitope (Sigma-Aldrich, St. Louis, MO), a biotinylated secondary anti-mouse IgG (1:200) and horseradish peroxide streptavidin (1:100).

Transient transfection

MC3T3-E1 and HEK293 cells were transfected with the expression constructs pCR3.1-ICER I and pCMV-ATF4 and a promoter-reporter luciferase construct: one containing 4 tandem OSE1 sites driving luciferase (p4OSE1-Luc) and one containing 6 tandem OSE2 sites driving luciferase (p6OSE2-luc) [27]. Transfections were performed using the Lipofectamine PLUS™ kit (Invitrogen). The amount of DNA in each transfection

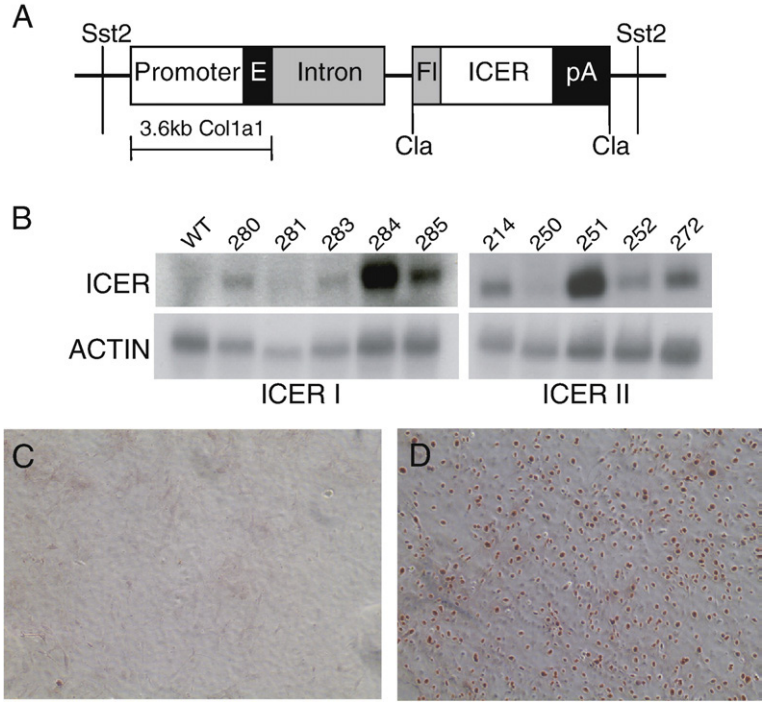

Fig. 1. Generation of ICER transgenic mice. (A) The pOBCol3.6-ICER transgenes consist of the ICER I or II cDNA, a 3.6-kb fragment of the rat Col1a1 gene including 3.5 kb of promoter and 115 bp of first exon, 1.6 kb of the rat Col1a1 first intron and the bovine growth hormone polyadenylation site. (B) Northern blot analysis of ICER mRNA in long bones expressing either the ICER I or ICER II transgenes. (C, D) Immunostaining of primary calvarial osteoblast cultures established from wild-type (WT) and ICER I (line 284) transgenic (TG) mice. Primary cultures were prepared from neonatal calvaria as described in Materials and methods. Cultures were grown almost to confluence and stained with the M2 antibody that recognizes the FLAG epitope in the ICER I transgene.