Osteopenia and male-specific sudden cardiac death in mice lacking a zinc transporter gene, \textit{Znt5}

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We isolated a mammalian gene whose expression transiently increased in response to intimal denudation of rabbit aorta. It was identical to a gene encoding a zinc transporter, \textit{ZNT5}, reported very recently by others. Mice deficient for this gene showed poor growth and a decrease in bone density due to impairment of osteoblast maturation to osteocyte. More than 60% of male null mice died suddenly because of the bradyarrhythmias. Analysis of gene-expression profiles in murine hearts by means of an oligonucleotide microarray disclosed that a subset of genes encoding immediate-early response factors (IEGs) and heat shock proteins (HSPs) were down-regulated in \textit{Znt5}-null mice. These results indicate that Znt5 protein plays an important role in maturation of osteoblasts and in maintenance of the cells involved in the cardiac conduction system, partly owing to dysregulated expression of IEGs and HSPs.

Bone remodeling, i.e., formation and absorption of bone, is under precise regulation; osteoblasts deposit calcified bone matrix and osteoclasts absorb it. Deregulation of this process leads to a variety of metabolic bone diseases, one of them being osteoporosis, a condition in which an increase of bone catabolism over anabolism increases the risk of bone fracture. Osteoporotic fracture is one of the most common and the most serious complications affecting elderly people, with the number estimated to increase three-fold by the middle of this century, from 1.7 million in 1990 to 6.3 million by 2050 (http://www.who.int/inf-pr-1999/en/pr99-58.html). However, the molecular mechanisms involved in its pathogenesis are not well understood, partly because of its genetic heterogeneity as well as a number of environmental factors in relation to its pathology in humans. Hence, a mammalian model will be of great help for better molecular understanding of this disease.

The heart has a specialized pacemaker and a conduction system to maintain rhythmical contraction. Disturbance of either can cause bradyarrhythmias such as sick sinus syndrome or atrioventricular (AV) block, conditions that often result in syncope and/or sudden cardiac death (SCD). Several candidate molecules related to SCD due to an impaired conduction system have been reported, including homeobox transcription factor NKX2-5, responsible for AV conduction abnormalities in humans (1). Mice deficient for HF-1b, a transcriptional factor preferentially expressed in the cardiac conduction system and ventricular myocytes, showed impaired formation of the conduction system, and suffered from malignant lethal arrhythmia (2). However, mutations in the NKX2-5 gene were accompanied by various forms of cardiac malformation, and HF-1b deficiency eventually resulted in tachyarrhythmia leading to death. Neither molecule may represent a basis for understanding the pathogenesis of bradyarrhythmia.

Here we report the isolation and characterization of a mammalian gene whose expression increased during the time course following intimal denudation of rabbit aorta. It was identical to a gene encoding a zinc transporter, \textit{Znt5}, reported very recently by Kambe \textit{et al}. (3). \textit{In vivo} analyses revealed that mice deficient for this cation transporter not only showed severe osteopenia, but were also subject to male-specific sudden death from bradyarrhythmia during their reproductive period.

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RESULTS

Isolation and characterization of a mammalian gene up-regulated in balloon-injured aorta of rabbit

By means of a differential-display method, we isolated a novel gene whose expression had transiently increased in response to balloon catheter injury in rabbit aortas (4), a model of arterial intimal hyperplasia. We excised, cloned and sequenced a DNA fragment corresponding to one of the bands that showed stronger intensity in aortas of rabbits with balloon injury than in control rabbits. Using the rabbit cDNA as a probe, we screened cDNA libraries to isolate human and mouse homologs. Subsequent 5’ and 3’ RACE experiments revealed two transcripts, representing variants at the 5’ and 3’ ends of the cDNA respectively (Fig. 1A). The human gene, spanning a 37 kb genomic region on chromosome 5q13, encodes 765 or 692 amino acid peptides, designated as α and β forms, respectively. The protein is highly conserved among species, with the human homolog showing respectively 97% and 95% identity in amino acids to the rabbit and mouse protein. The predicted protein appeared to contain a cation-efflux domain as well as a histidine-rich loop between membrane-spanning domains, a potential metal-binding motif (Fig. 1B and C). A recent homology search revealed that the gene was identical to the recently reported one encoding a zinc transporter, ZNT5 (3).

Expression and subcellular localization of ZNT5

Northern blot analysis using the human cDNA as a probe detected two different transcripts of 4.2 kb and 3.4 kb that were expressed in all tissues examined; levels were the highest in prostate, testis and ovary (Fig. 1D). The longer transcript seemed to correspond to a longer 3’ non-coding region. RT–PCR analysis revealed that this gene was also expressed in osteoblasts, adipo cytes and fibroblasts. Computer analysis predicted 15 possible transmembrane domains (5) (Fig. 1B). To examine the subcellular location of the protein, we constructed a plasmid clone, pEGDNA3.1C-HA-aZNT5, that was designed to express the longer transcript of human ZNT5 and carried a hemagglutinin (HA) tag at the C-terminal end. Plasmid DNA was transfected into H1299 cells and protein was detected by immunofluorescent staining. The signal of αhZnt5 protein exactly coincided with that of the Golgi-apparatus membrane protein (Fig. 1E).

Ectopic expression using pEGFP, or transfection into COS-7 cells, resulted in the same localization (data not shown).

Generation of Znt5-null mice

To examine the physiologic role of ZNT5 in vivo, we generated knockout mice by constructing a targeting vector with a neo-cassette inserted into exon 9 of Znt5. The transcribed products were truncated and lacked the cation-efflux domain (Fig. 2A and B). Heterozygous mice were phenotypically normal, and crosses between heterozygotes produced homozygous mutant mice according to Mendelian expectations. Znt5-null mice were viable and fertile, but grew poorly; both male and female mice were significantly smaller and leaner than heterozygous or wild-type mice (Fig. 3A and B). The total weights of reproductive, inguinal, mesenteric and retroperitoneal fat pads of 20-week-old Znt5-null mice were significantly lower than those of wild-type mice (null versus wild-type; 0.63 g versus 2.06 g, n = 6, P < 0.001 by Student’s t-test). However, serum levels of cholesterol, triglyceride, albumin and cholinesterase were normal in Znt5-null mice, indicating that their poor growth and lean phenotype were not due to malnutrition (data not shown).

Null mice showed obvious muscle weakness. To evaluate muscle strength, we measured the time that 20-week-old mice could hang on with all four limbs to iron bars 2 mm in diameter. Though all of the 14 wild-type mice could hang on for more than 5 min, 14 Znt5-null mice fell off in a significantly shorter time (null versus wild-type: 13.4 s versus >300 s, P < 0.001 by Student’s t-test). Detailed histopathologic analyses of skeletal muscles and peripheral nerves of Znt5-null mice revealed no abnormality such as dystrophy, atrophy, hypertrophy or necrosis (data not shown).

Impaired function of osteoblasts in Znt5-null mice

With advancing age, Znt5-null mice developed hunched backs. X-ray analysis showed a systemic decrease in bone density compared with wild-type mice (Fig. 3C). The long bones of Znt5-null mice were shorter and more radiolucent (Fig. 3D), and three-dimensional computed tomographic analyses showed obvious decreases in cortical bone thickness, number and diameter of trabeculae, and bone volume (Fig. 3E). In addition, histologic analysis revealed that the epiphysial growth plate was also thinner in null mice (Fig. 3F). However, heterozygous mutant mice did not show any bone abnormalities (data not shown).

To investigate whether the lower bone mass was due to altered bone remodeling, we performed static and dynamic histomorphologic analysis on the tibial metaphyses of 27-week-old knockout and wild-type mice. Bone volume/total tissue volume (BV/TV), a histomorphologic index of trabecular bone mass, of Znt5-null mice was only 39% of that in wild-type mice. Osteoblast-covered bone surface (Ob.S/Bs) and osteoid thickness (O.Th) indicating osteoid volume were lower in knockout mice than in wild-type littermates (Fig. 4A–C), although eroded surface (ES/BS), which represents the function of osteoclasts, the numbers of osteoclasts (N.Oc/B Pm) and osteoclast-covered bone surface (Oc.S/BS) were essentially equivalent to those in wild-type littermates (Fig. 4D–F). Furthermore, a double-labeling analysis of calcein, a marker of newly formed bone, revealed that calcification activities such as mineral apposition ratio (MAR), mineralized surface (MS/BS) and bone formation rate (BFR/BS), significantly decreased in Znt5-null mice, supporting the idea that deceleration of calcification was the cause of osteopenia (Fig. 4G–I). These results indicated that decreased bone mass in knockout mice was due to reduced activity of osteoblasts, rather than to enhancement of the activity of osteoclasts in increasing bone absorption.

To investigate further the consequences of genetic ablation of Znt5, we isolated primary osteoblasts from calvaria of newborn mice and differentiated them ex vivo in the presence of recombinant human bone morphologic protein 2 (rhBMP2),
Figure 1. Structure and expression of hZnt5. (A) Genomic structure. There were two types of transcript, one involving the 5' end and the other the 3' end of the cDNA. Shaded portions represent coding regions. The arrowhead indicates the first start codon. (B) Amino acid sequence of hZnt5 protein. The arrowhead indicates the first methionine of hZnt5 protein. Fifteen computer-predicted transmembrane domains are underlined, the histidine-rich loop is shaded and the cation efflux domain is boxed. (C) Amino acid alignment of cation efflux domains and transmembrane domains of Znt5, Zrc1, Cot1 and Znt1. Transmembrane domains predicted by PSORT (5) are boxed and the histidine-rich loop is underlined. (D) Expression of hZnt5 in various organs. Transcripts of 4.2 kb and 3.4 kb were expressed in all tissues examined, and the longer transcript seems to be the one reflecting a longer 3' non-coding region. β-Actin served as the RNA-loading control. Sk, skeletal; S., small. (E) Subcellular localization of ectopically expressed hZnt5 protein in H1299 cells. Cells were double stained with anti-HA antibody (FITC) and anti-human Golgi-apparatus membrane protein antibody (rhodamine).
ascorbic acid and β-glycerophosphate. The proliferation curve of calvaria-derived osteoblasts of knockout mice was equivalent to that of wild-type mice (data not shown). RT–PCR analysis revealed that expression of Znt5 was highest on the 10th day (data not shown). After 10 and 21 days of incubation, the osteoblasts were stained for alkaline phosphatase (ALP), a marker of bone differentiation that precedes the onset of mineralization, and by alizarin red, which monitors areas of mineralized extracellular matrix. The stained areas were remarkably smaller in osteoblasts of knockout mice, suggesting that impairment of osteoblast activity was the primary consequence of the absence of Znt5 (Fig. 4J–K). We also performed colony-formation assays of bone-marrow cells, but the numbers of colonies observed were equivalent between the null and normal groups [null versus wild-type; 34 ± 15 versus 39 ± 16 colonies/1.0 × 10⁶ bone marrow cells (not significant)] (Fig. 4L). These results suggest that the dysfunction of osteoblasts occurred not at the stages of proliferation or differentiation from bone-marrow precursors to osteoblasts, but at the point of maturation to osteocytes.

**Male-specific sudden death of Znt5-null mice**

When male Znt5-null mice reached reproductive age at about 15 weeks, they began to die suddenly in spite of normal appearance and activity on the previous day. Female Znt5-null mice, however, lived as long as wild-type mice (Fig. 5A). Kaplan–Meier analysis revealed that the average lifespan of male Znt5-null mice was significantly shorter than that of others \( P = 0.0001; \chi² = 17.7 \) by log-rank test). Sudden death in the male null mice generally occurred between the ages of 15 and 40 weeks. However, male null mice that escaped death during that period tended to survive as long as heterozygotes or wild-type mice.

To investigate the cause of male-specific sudden death, we performed autopsies but found neither vascular lesions such as atherosclerosis, myocardial infarction or cerebral infarction, nor any signs of congestive heart failure. In spite of detailed pathohistologic examination of most of the organs, including liver, kidney, spleen, pancreas, submandibular gland, tongue, esophagus, stomach, small intestine, large intestine, trachea, lung, heart, aorta, thyroid, parathyroid, adrenal gland, pituitary gland, lachrymal gland, thymus, mesenteric lymph node, urinary bladder, testis, brain and spinal cord, skeletal muscle and peripheral nerve, we found no abnormalities related to death. Biochemical analyses also failed to find dysfunction of liver or kidney, or disturbances in mineral balance such as hyperkalemia. Furthermore, the serum concentrations of zinc, calcium and phosphate in male mutant and wild-type mice were similar (mutant versus wild-type; 72 ± 27 µg/dl versus 69 ± 13 µg/dl, 7.2 ± 0.4 mg/dl versus 7.5 ± 0.1 mg/dl, 7.9 ± 0.5 mg/dl versus 8.1 ± 0.4 mg/dl, respectively, not significant by Student’s t-test). Mutant mice did not show abnormal serum glucose concentrations (mutant versus wild-type; 102 ± 29 mg/dl versus 115 ± 20 mg/dl, not significant by Student’s t-test).

At this point, suspecting that cardiac arrhythmia was the cause of sudden death, we examined bipolar-lead resting electrocardiograms (ECGs) of anesthetized mice but found no significant differences in heart rate, PR interval or QRS duration between wild-type and mutant ones (data not shown). We surgically implanted microtransmitters into eight male and two female null mice, and into nine wild-type mice as controls, to record ECGs under conscious, freely moving and unanesthetized conditions. We observed sudden death in 9 of the 10 null mice, including both females. Examination of continuous ECG recordings revealed rhythm disturbance as a possible cause of death; all of the null mice that died suddenly showed complete AV block and sinus bradycardia just before and at the time of death (Fig. 5B–D), whereas all wild-type mice survived and showed significantly fewer bradyarrhythmias (Fig. 5E). The single surviving male null mouse also had a complete AV block and sinus pause even though its behavior and activity level were normal in spite of such arrhythmias (Fig. 5D). It is true that, in humans, bradyarrhythmias are often observed in patients with end-stage heart failure, but since we had found no pathologic evidence of cardiac hypertrophy, congestive heart failure or cardiomyopathy among our test animals, the arrhythmias in the null mice appeared to be the primary cause of death rather than a secondary effect related to heart failure.

Since male-specific sudden death occurred during the reproductive period, there was a possibility that sex hormones contributed to the pathology in relation to its male specificity. We investigated survival rates of castrated or 17β-estradiol (E2) injected male knockout mice up to 50 weeks of age. No significant differences in the average lifespan were observed by
Kaplan–Meier analyses between castrated or E2-injected mice and control groups (NS; $\chi^2 = 1.0$ and $\chi^2 = 0.01$, respectively, by log-rank test).

Expression analysis of heart tissue from Znt5-null mice

To search for candidate molecules for involvement in the observed pathologic phenotype of Znt5-null mice, we performed oligonucleotide-microarray analysis. Poly(A)$^+$ RNAs were isolated from whole hearts of 20-week-old male mice of both genotypes, and we compared the expression profiles. Expression of 12 genes, including two expressed sequence tags (ESTs), was altered between null and wild-type mice; eight known genes were decreased, and two known genes and two ESTs were increased (Table 1). Six of the eight downregulated elements represented immediate-early genes (IEGs) or encoded heat shock proteins (HSPs), molecules that increase in response to various external stimuli. The microarray results were confirmed by semi-quantitative RT–PCR (data not shown).

DISCUSSION

We have reported here evidence that knockout of Znt5 in mice causes poor growth, lean phenotype, muscle weakness, osteopenia and male-specific SCD because of bradyarrhythmia, suggesting that these mice have qualitative abnormalities in adipocytes, skeletal myocytes, osteoblasts and cardiomyocytes of conduction systems. Since all of these cell types may be derived from mesenchymal stem cells (6–9), Znt5 may play an important role in the development or maintenance of mesenchyme-related cells.

Recently, this protein has been shown to transport zinc into Golgi-enriched vesicles (3). In combination with the result of our experiment localizing the gene product at the Golgi apparatus (Fig. 1E), it is reasonable that we found no differences between wild-type and null mice as regards cellular tolerance to those cations, and serum concentrations were equivalent. Trace elements often form stable complexes that are integrated into components of enzymes or act as second messengers. It is conceivable that failure of subcellular compartmentalization of cations such as zinc leads to undetectable imbalances with respect to cations inside the Golgi apparatus, disturbing

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**Figure 3.** Poor growth and osteopenia of Znt5-null mice. (A) Body weights of male null mice; females showed the same results. The null mice grew more poorly than wild-type mice, although all were fed with the same chow diet. Open, closed, and shaded symbols indicate wild-type, heterozygous and null mice, respectively. *P < 0.005. (B) Wild-type and null mice photographed at 27 weeks. (C) Plain X-ray analysis of wild-type and null mice, showing a systemic decrease in bone density of null mice compared with wild-type mice. The null mice also showed hunched backs. (D) X-ray photos of femora and tibiae. (E) Three-dimensional CT images of distal femora. (F) Plastic sections of tibiae from 30-week-old wild-type and Znt5-null mice stained with toluidine blue. High-power views are shown, and scale bars in the panels indicate 0.1 mm.
functions, e.g. glycosylation, processing and sorting. Failure of those activities would result in dysfunctional proteins and cells. Osteoblast growth and differentiation can be divided into three stages: proliferation, maturation and mineralization (10). The \(Znt5\)-null mice showed significantly poor growth and a high degree of osteopenia due to the reduced bone formation resulting from impaired activity of osteoblasts. Colony-formation assays of bone-marrow cells and calvaria-derived osteoblasts showed no significant differences between genotypes, an indication that loss of \(Znt5\) caused no obvious impairment in proliferation of pre-osteoblasts or osteoblasts. However, when differentiation was induced by rhBMP, alkaline phosphatase (ALP) activity and the regions of calcification were decreased in osteoblasts derived from knockout mice. Since the impairment of osteoblasts was observed ex vivo, its dysfunction was primary; that is, osteopenia in the knockout mice did not reflect secondary effects of other factors such as hormones, serum mineral concentration, or physical stress due to muscle weakness. The results also indicate that the cause of osteoblast dysfunction is not failure of proliferation or differentiation from bone-marrow precursor cells, but failure of osteoblasts to mature to osteocytes. The \(Znt5\) gene therefore has a critical role in osteoblast maturation, and its depletion results in impaired function of osteocytes, reduced bone formation, poor skeletal growth and osteoporosis.

To our knowledge, the \(Znt5\)-null mouse is the first animal model to exhibit male-specific sudden death during the reproductive stage of their lives. Telemetric ECG revealed severe degrees of bradyarrhythmia in the \(Znt5\)-null mice shortly before death. It is true that factors, related to surgery, such as infection, cannot be excluded as one of the causes of death. However, since wild-type mice with implanted ECG transmitters did not die, sudden death in knockout mouse cannot be explained only by these factors, different genetic backgrounds of wild-type and knockout mouse, \(Znt5\) deficiency, must contribute to their sudden death. In addition, wild-type mice showed only mild degrees of bradyarrhythmias temporarily that did not lead to death. In combination with the fact that Fig. 5B was recorded just before death, we think it is reasonable to indicate that a severe degree of bradyarrhythmia was the direct cause of this genotype-specific sudden death.

Under natural conditions, only male null mice died suddenly. However, female null mice also exhibited SCD after surgical implantation of telemetric devices. This result raised the
interesting implication that the cardiac conduction system is also impaired in female mutant mice, but that the threshold of physical stress for triggering sudden death is higher than it is in males; dysfunction becomes overt only under conditions of severe stress such as surgery.

The reason why only the male null mice suffered from SCD under natural conditions is unknown, but some gender differences do exist in the electrophysiology of the heart (11), and the fact that most of the male null mice died during the reproductive stage of their lives may suggest that sex hormones contribute to the difference in the prevalence of SCD between the sexes. Recent studies have revealed that female hormones, including 17β-estradiol, have cardioprotective effects that include lowering of cholesterol, antioxidant activity, epithelial cell-dependent vasodilation, and antiplatelet effects (12). However, we found no differences in survival curves between E2-injected and castrated male knockout mice and controls. This may indicate the possibility that gender-specific proteins other than estrogen or androgens contribute to the pathogenesis of sudden death.

Analysis of gene-expression profiles by means of an oligonucleotide microarray revealed that expression of some stress proteins was down-regulated in the hearts of Znt5-mutant mice. Immediate-early response factors (IEGs) and HSPs can be induced in response to a variety of stimuli such as growth hormones, cytokines, heat, ischemia, heavy metals, or...
mechanical and emotional stress (13–18), and they play important roles in cell maintenance, proliferation and differentiation. Expression of IEGs and HSPs in mutant mice may respond poorly to some kinds of stimuli in vivo, with consequences including dysfunction of osteoblasts, adipocytes, myocytes and other cells involved in cardiac conduction systems. Indeed, inhibition of c-fos expression is known to impair differentiation of osteoblasts (19,20). The regulation of IEGs is complex, and differences in their expression may account for sex differences in cardiac phenotype. For example Cry61, whose expression was down-regulated in the hearts of male Znt5-null mice, is estrogen-inducible (21). These data suggest the importance of some IEGs and HSP genes for the phenotype, although the mechanism that decreases expression of these genes and the reason why down-regulation of these genes may result in the phenotype we observed here remain to be investigated.

Nguyen-Tran et al. reported that mice deficient for the transcriptional factor HF-1b exhibited normal cardiac structure and function but often suffered from bradyarrhythmias such as sinus bradycardia, sinus pause and AV block, and succumbed to SCD (2). Since the phenotype of our knockout mice was similar to that of HF-1b-null mice, we considered that HF-1b might be a transcriptional factor for Znt5. However, ectopic introduction of a plasmid designed to express HF-1b did not enhance transcription of hZNT5 in HEK293 cells (data not shown). Therefore, the molecular mechanisms of bradyarrhythmia in these two types of knockout mice are probably different.

The Znt5-null mouse should be a highly valuable model for studying arrhythmias, especially as regards sex differences in arrhythmogenesis. Furthermore, if mutations in the hZNT5 gene are found to be associated with cardiac-conduction defects in humans, mutational analyses could be undertaken to uncover genetic risks of SCD in individual patients. Since sudden cardiac death due to bradyarrhythmia can be avoided by implantation of a cardiac pacemaker, identification of people at high risk would have great medical significance.

**METHODS**

**Endothelial denudation and preparation of RNA**

We surgically inserted Fogarty balloon catheters into the thoracic aortas of 3-month-old male Japanese white rabbits through the femoral artery, inflated the balloons to allow dissection, and isolated total RNA from excised aorta tissue on a time course of 1, 2, 4, 7 and 14 days after balloon inflation using TRIZOL (Life Technologies, Houston, TX, USA) according to the supplier’s instructions. RNA was also isolated from the aorta of a control rabbit. The differential-display procedure was performed as described previously (4) to detect genes that were expressed differently between treated and untreated rabbits. One partial sequence was selected for investigation and later designated Znt5.

**Cloning of full-length cDNA**

To isolate a full-length rabbit Znt5 gene, 5’ RACE was performed using the Marathon cDNA Amplification Kit (Clontech Laboratories, Palo Alto, CA, USA). Full-length cDNAs of human and mouse homologs were cloned by screening heart cDNA libraries of each species (Clontech).

**Multi-tissue Northern blot analysis**

Human multi-tissue northern blots (Clontech) were hybridized with a partial cDNA fragment of hZNT5 and labeled by a random oligonucleotide priming method. Pre-hybridization, hybridization and washing were performed according to the supplier’s recommendations.

**Immunocytochemistry**

Transiently transfected H1299 cells with pcDNA3.1/C-HA-hZnt5 were replaced on poly-D-lysine-coated multiwell chamber slides (Beckton Dickinson, Franklin Lakes, NJ, USA), fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS), permealized with 0.1% Triton X-100 in PBS for 5 min, and covered with blocking solution (3% bovine serum albumin (BSA) in PBS) for 60 min at room temperature. Then, cells were incubated with rat anti-HA antibody, 3F10 (diluted 1 : 1000 in blocking solution) and mouse anti-Golgi 58K protein antibody, 58K-9 (Sigma, St Louis, MO, USA, diluted 1 : 200 in blocking solution) for 2 h at room temperature. These antibodies were stained with a goat anti-rat secondary antibody conjugated to fluorescein isothiocyanate (FITC) and a goat anti-mouse secondary antibody conjugated to rhodamine (diluted 1 : 3000) for 30 min and viewed with an ECLIPSE E600 microscope (Nikon, Tokyo, Japan).

### Table 1. Comparison of RNA expression profiles of mouse heart

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Eight gene products were determined to be ‘decreased’ in Znt5-null mice, and six of those transcripts were stress-inducible proteins such as immediate-early response factors and heat shock proteins (underlined). AFC represents ‘average fold change’, which is an average of fold change between a sample of knockout and wild-type mice.
Generation of Znt5-null mice

We constructed a targeting vector containing a 12 kb DNA we isolated from a genomic phage library of mouse strain 129/Sv (Stratagene, La Jolla, CA, USA), using mZnt5 cDNA as a probe. The vector also contained a neo cassette (inserted into exon 9) for positive selection and a thymidine kinase cassette (TK) for negative selection. After this vector was electroporated into mouse embryonic stem (ES) cells, clones were selected in the presence of G418. Three G418-resistant clones of ES cells were identified as homologous recombinants based on Southern analysis using the PCR product of mouse genomic DNA as a probe. Positive clones were microinjected into murine blastocysts that were subsequently transferred to pseudo-pregnant females. Chimeric male offspring were crossed with strain C57BL6. About 4–5 weeks after birth, these mice were genotyped by PCR, using as primers mZnt5-F (5'-CACCCCAATAACAGGACACCTC-3') and mZnt5-R (5'-CACAAAATGAAGAAGACACGC-3').

X-ray analysis of bones and histomorphometry

Bone radiographs of the excised femora and tibiae were taken with soft X-ray apparatus (Type SRO-M50; Softron, Tokyo, Japan). Three-dimensional CT scans of thigh bones were taken using a composite X-ray analyzing system (NX-HCP, NS-ELEX Inc., Tokyo, Japan). Mice 27 weeks old were injected with calcein for in vivo fluorescent labeling (16 mg/kg body weight) 3 and 10 days before sacrifice. Specimens of the proximal right tibia were fixed with 70% ethanol and embedded in glycolmethacrylate after staining with toluidine blue O. We obtained 3 mm thick midfrontal sections of proximal tibiae using a microtome (model 2050 supercut; Reihert-Jung, Heidelberg, Germany). Histomorphometry of bone sections was performed using a semiautomated system of bone analysis (Osteoplan II, Carl Zeiss, Thornwood, NY, USA) at 200-fold magnification (22). Nomenclature recommended by the Nomenclature Committee of the American Society of Bone and Mineral Research (23) was used.

Isolation and culture of osteoblasts and bone-marrow cells

Primary osteoblasts were isolated from calvariae of newborn wild-type and mutant mice. Calvariae were dissected aseptically and digested sequentially for 10 min in α-MEM containing 0.1% collagenase and 0.2% dispase. Fractions 3–5 were cultured in α-MEM containing 10% fetal calf serum (FCS) and replanted at a subconfluent stage. To induce differentiation, osteoblasts were cultured in α-MEM containing 10% FBS, 100 ng/ml rhBMP2 (R&D systems, Minneapolis, MN, USA), 10 µM β-glycerophosphate, and 50 mg/ml ascorbic acid. Osteoblasts were stained with ALP (Sigma) and alizarin red on the 10th and 21st days, respectively. For colony-formation assays, we harvested bone-marrow cells from 8-week-old wild-type and knockout mice, and cultured them for 10 days before staining them with ALP.

Anesthesia, surface-ECG recording and telemetry monitoring

A surface six-lead ECG was obtained, by means of cutaneous clips at each of the four limbs after the mice were anesthetized by intraperitoneal injection of averitn (1.25%, 0.02 ml per gram body weight). We also undertook continuous ECG monitoring of eight male null mice, two female null mice and nine wild-type mice by implanted telemetry, to detect lethal arrhythmias that could explain the sudden deaths. A wireless ECG transducer (TA10EA-F20, Data Science International, St Paul, MN, USA) was implanted in the intraperitoneal space under anesthesia, with intraperitoneal injection of 10 mg/kg of ketamine and 15 mg/kg of xylazine. Each mouse was separately housed in a cage with a signal receiver at the bottom. The ECG data were stored on a computer hard disk using a data-acquisition system (Power Lab, AD Instrumentals Pty Ltd, Castle Hill, Australia). Observation was continued for 2 weeks or more.

Survival analysis of E2-injected and castrated knockout mice

For the E2-injected group (n = 10), 2 µg of estradiol (Sigma) dissolved in 0.1 ml of sesame oil with 10% ethanol was injected intraperitoneally every day for 50 weeks, and the same volume of solvent was injected into male null mice (control group, n = 10). Castration surgery of mice was performed at the age of 10 weeks (n = 12), and sham operation was also performed (control group, n = 12).

Analysis of gene-expression profiles by oligonucleotide microarray

Hybridization samples for GeneChip analysis (Affymetrix, Santa Clara, CA, USA) were prepared from total RNA obtained from whole hearts of 20-week-old male wild-type and mutant mice. Biotinylated cRNA was synthesized using the Bizarre High Yield kit (Enzo, Farmingdale, NY, USA) were prepared from total RNA isolated from a genomic phage library of mouse strain 129/Sv (Stratagene, La Jolla, CA, USA), using mZnt5-F (5'-CACCCCAATAACAGGACACCTC-3') and mZnt5-R (5'-CACAAAATGAAGAAGACACGC-3'). We constructed a targeting vector containing a 12 kb DNA we isolated from a genomic phage library of mouse strain 129/Sv (Stratagene, La Jolla, CA, USA), using mZnt5 cDNA as a probe. The vector also contained a neo cassette (inserted into exon 9) for positive selection and a thymidine kinase cassette (TK) for negative selection. After this vector was electroporated into mouse embryonic stem (ES) cells, clones were selected in the presence of G418. Three G418-resistant clones of ES cells were identified as homologous recombinants based on Southern analysis using the PCR product of mouse genomic DNA as a probe. Positive clones were microinjected into murine blastocysts that were subsequently transferred to pseudo-pregnant females. Chimeric male offspring were crossed with strain C57BL6. About 4–5 weeks after birth, these mice were genotyped by PCR, using as primers mZnt5-F (5'-CACCCCAATAACAGGACACCTC-3') and mZnt5-R (5'-CACAAAATGAAGAAGACACGC-3').

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REFERENCES


