Journal of Bone and Mineral Metabolism (2018) 36:640–647
https://doi.org/10.1007/s00774-017-0885-9

ORIGINAL ARTICLE

Zinc-finger transcription factor *Odd-skipped related 1* regulates cranial bone formation

Shinji Kawai[1] · Masashi Yamauchi[2] · Atsuo Amano[3]

Received: 25 March 2016 / Accepted: 15 November 2017 / Published online: 12 December 2017

Abstract

Knowledge of the molecular mechanisms of bone formation has been advanced by novel findings related to genetic control. *Odd-skipped related 1* (*Osr1*) is known to play important roles in embryonic, heart, and urogenital development. To elucidate the in vivo function of *Osr1* in bone formation, we generated transgenic mice overexpressing full-length *Osr1* under control of its 2.8-kb promoter, which were smaller than their wild-type littermates. Notably, abnormalities in the skull of *Osr1* transgenic mice were revealed by analysis of X-ray, skeletal preparation, and morphological findings, including round skull and cranial dysraphism. Furthermore, primary calvarial cells obtained from these mice showed increased proliferation and expression of chondrocyte markers, while expression of osteoblast markers was decreased. BMP2 reduced *Osr1* expression and *Osr1* knockdown by siRNA-induced alkaline phosphatase and osteocalcin expression in mesenchymal and osteoblastic cells. Together, our results suggest that *Osr1* plays a coordinating role in appropriate skull closure and cranial bone formation by negative regulation.

Keywords Osteoblast · Bone formation · Transcription factor · Cranium · Transgenic mice

Introduction

The *odd-skipped* gene, initially identified as a *Drosophila* segmentation gene, contains four DNA-binding C2H2-type zinc fingers in the C-terminal half of the molecule as a pair-rule transcription factor [1]. The mammalian homologs *Odd-skipped related 1* (*Osr1*) and *Osr2* have been cloned from both mice and humans [2, 3]. Mouse *Osr1* has a 65% homology with *Osr2*, whereas their tissue distribution differs. Human *Osr1* has been detected in fetal lung, adult colon, small intestine, prostate, and testis tissues [3], whereas *Osr2* is specifically expressed in limb, tooth, and kidney tissues [4].

Osr1 was shown to function in heart morphogenesis and urogenital development by targeted null mutation [5]. In that study, null mutant embryos did not succeed in forming an atrial septum, and exhibited dilated atria with hypoplastic venous valves and blood backflow from the heart into systemic veins. Expression of *Osr1* is restricted to the central dorsal domain of the atrial myocardium during normal heart development. Furthermore, *Osr1* null mutant embryos exhibit defects of adrenal glands, metanephric kidneys, gonads, and pericardium formation, while the key regulators of early intermediate mesoderm development are down-regulated. In addition, it was shown that nephrogenic mesenchyme underwent massive apoptosis, which caused a disruption of nephric duct elongation and failure of metanephric induction in *Osr1* null mutant embryos [5].

We previously reported that *Osr2* regulates osteoblast function, as dominant-negative *Osr2* transgenic mice exhibited decreased osteoblast proliferation, and delayed mineralization in calvarial and tibial bone tissues [6]. By cloning the 5′-flanking 4.8-kb DNA region of the mouse *Osr1* gene, our findings also revealed the regulation mechanism, and showed that *Runx2* and *Ikzf1* suppress *Osr1* promoter

Shinji Kawai and Masashi Yamauchi contributed equally to this work.

✉ Shinji Kawai
skawai@dent.osaka-u.ac.jp

[1] Challenge to Intractable Oral Diseases, Center for Frontier Oral Science, Osaka University Graduate School of Dentistry, 1-8 Yamadaoka, Suita, Osaka 565-0871, Japan

[2] Department of Pediatric Dentistry, Osaka University Graduate School of Dentistry, Osaka, Japan

[3] Department of Preventive Dentistry, Osaka University Graduate School of Dentistry, Osaka, Japan

activity [7]. *Drosophila runt*, *hedgehog*, and *wingless* belong to the group of segmentation genes, and their mammalian counterparts *Runx2*, *Hh*, and *Wnt* function in bone formation [8]. The *Drosophila odd-skipped* gene is involved in segmentation and it is expected that mammalian *Osr1* functions in bone formation, though direct proof of the latter remains to be elucidated.

In the present study, we generated transgenic mice with full-length *Osr1* under control of its own promoter. Round skull and cranial dysraphism were observed in these transgenic mice. Our findings are the first to show that *Osr1* functions in adequate skull and cranial bone formation.

Materials and methods

Cell cultures and reagents

The mesenchymal cell line C3H10T1/2 (RCB0247), myoblastic cell line C2C12 (RCB0987), and osteoblastic cell line MC3T3-E1 (RCB1126) were purchased from Riken Bioresource Center (Tsukuba, Ibaragi, Japan). The HEK293A cell line was obtained from Life Technologies (Carlsbad, CA, USA). C3H10T1/2 and MC3T3-E1 cells were maintained in α-modified Eagle's medium (α-MEM; Sigma-Aldrich, Saint Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; Life Technologies). HEK293A cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich) supplemented with 10% FBS. Mouse *Osr1* was amplified by polymerase chain reaction (PCR) using the mouse embryonic cDNA library and cloned into a pcDNA3 plasmid vector (Life Technologies). The sequences of these inserts were confirmed by DNA sequence analysis. Alkaline phosphatase (ALP) promoter-luciferase (ALP-Luc) and osteocalcin promoter (OG2)-luciferase (OCN-Luc) vectors were used as described in our previous report [6].

Generation of transgenic mice

Osr1 transgenic mice have a fusion gene composed of a 2.8-kb fragment of the mouse *Osr1* promoter [7] fused to the first intron of the rabbit beta-globin gene, *Osr1* full-length cDNA, and 3′ untranslated and polyA signals of SV40. Transgenic founders were generated by pronuclear injection into mouse strain BDF1 (DBA2/C57BL6) using standard techniques [9]. The genotype of the transgenic animals was determined by PCR. The primer set for the mouse *Osr1* gene was 5′-CCTGGACGTGACCAAGCTAT-3′ (forward) and 5′-TGTAGCGTCTTGTGGACAGC-3′ (reverse), and was not amplified *Osr2* mRNA. All animal experiments were performed using protocols approved by the Osaka University Graduate School of Dentistry animal care and use committee (Permit number: Doushi-19-020-0).

Skeletal analysis

For skeletal preparations, newborn mice were dissected and fixed overnight in 95% ethanol. Staining with Alcian blue 8GX and Alizarin red S (Sigma-Aldrich) was performed using standard protocols [10]. For histological analysis, the mice were killed as newborns and calvaria were then obtained and fixed in 10% formalin and embedded in paraffin. Thereafter, 5-μm-thick sections were stained with hematoxylin and eosin (H&E, Sigma-Aldrich) using procedures described previously [11]. Gross appearance was imaged using 3-week-old male mice. Skeletons were analyzed with X-ray apparatus (Softex, Kanagawa, Japan) using 3-week-old male mice.

In vitro differentiation of osteoblasts

Primary cells were isolated from the calvarial bones of 1- to 3-day-old newborn mice, as previously described [12], then cultured in α-MEM with 10% FBS or mineralization medium (α-MEM with 10% FBS containing 50 μg/ml ascorbic acid, 10 mM β-glycerophosphate, and 100 ng/ml rhBMP2; R&D Systems, Minneapolis, MN, USA). Cells were maintained for 7 days to determine ALP and for 21 days to detect mineralized matrix (Alizarin red S), with standard protocols utilized [6]. Cell proliferation was determined using a CellTiter 96 AQueous One Solution Cell Proliferation Assay kit (Promega, Madison, WI, USA), according to the manufacturer's instructions.

PCR assays

Total RNA from cells was prepared using TRIsure reagent (Bioline, London, UK) and reverse-transcribed with a PrimeScript RT reagent Kit (Takara Bio Inc., Shiga, Japan). PCR assays were performed using Taq PCR Master Mix (QIAGEN, Valencia, CA, USA). The primer set for the mouse *Osr1* gene was 5′-CCTGGACGTGACCAAGCTAT-3′ (forward) and 5′-TGTAGCGTCTTGTGGACAGC-3′ (reverse), and for mouse GAPDH mRNA was 5′-CCGTAGACAAAATGGTGAAGGT-3′ (forward) and 5′-GTGAGTGGAGTCATACTGGAACAT-3′ (reverse). A quantitative real-time reverse transcription-PCR (qRT-PCR) assay was performed using the StepOnePlus System (Life Technologies), according to the manufacturer's instructions, with the reaction carried out with KAPA SYBR FAST qRT-PCR Kit Master Mix (KAPA Biosystems, Woburn, MA, USA). The expression levels of mRNA are indicated as the relative expression normalized

by GAPDH. Each procedure was performed in triplicate and repeated at least twice to assess reproducibility.

Transfection and luciferase activity assays

One day before transfection, the cells were plated in 24-well culture plates (Iwaki Glass, Chiba, Japan) at a density of 4×10^4 per well. An *Osr1* expression vector and promoter firefly luciferase reporter vector, each 0.2 μg, were transiently transfected using FuGENE6 transfection reagent (1.2 μL/well; Roche Diagnostics, Indianapolis, IN, USA). A pRL-CMV vector (20 ng; Promega), in which the expression of Renilla luciferase was driven by the CMV promoter, was concomitantly transfected as an internal control. Approximately 48 h after transfection, the cells were lysed with passive-lysis buffer and the luciferase activities of both types were measured using a dual luciferase reporter assay system (Promega). Promoter firefly luciferase activity was normalized by Renilla luciferase activity. The experiments were performed in triplicate and repeated at least twice.

Statistical analysis

Values are presented as the mean and standard deviation (SD) of results of separate experiments and compared using unpaired two-tailed Student's *t* test. Values of $P < 0.05$ were considered to indicate a significant difference (*).

Results

Construction of *Osr1* transgenic mice

To determine whether *Osr1* affects bone formation in vivo, we generated transgenic mice expressing *Osr1* under the control of the authentic 2.8-kb *Osr1* promoter [7]. The transgene consisted of the *Osr1* full-length coding sequence (Fig. 1a). Since we failed to measure the copy number by Southern blot technique, the expression level of transgene was estimated by qPCR. Among the transgenic founders, several lines, Tg12, Tg20, Tg25, Tg24, and Tg28, had a higher expression of the *Osr1* transgene in qRT-PCR analysis of tail total RNA, and those showed a similar skeletal phenotype (Fig. 1b). The transgenic founder Tg28 showed the highest expression. Next, we performed qRT-PCR

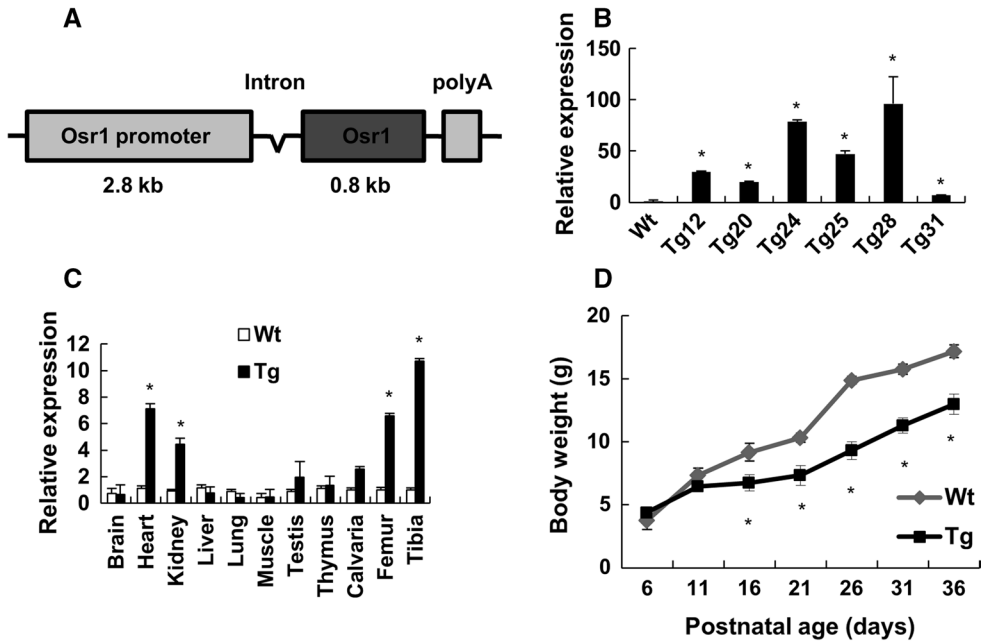

Fig. 1 Generation of *Osr1* transgenic mice. **a** Schematic representation of the *Osr1* transgene. The transgene contained full length *Osr1* under the control of a 2.8-kb *Osr1* promoter fragment, and possessed the first intron of the rabbit beta-globin gene and polyA signals of SV40. **b** Expression level of *Osr1* transgene in transgenic (Tg) founders. Total RNA samples were obtained from mouse tails and analyzed by qRT-PCR with transgene-specific primers. The transgenic founder Tg28 showed the highest expression. Data are shown as the mean and SD. Significant ($P < 0.05$) induction in comparison with the wild-type (Wt) is indicated by an asterisk (*). **c** The tissue distribution of *Osr1* in Wt and Tg28 mice was determined by qRT-PCR assays. Data are shown as the mean and SD. *$P < 0.05$ (*t* test) versus Wt. The experiments were performed in triplicate and repeated at least twice. **d** Body weights of Wt and Tg28 male littermates ($n = 3$ in each group)

analysis to confirm transgene expression in calvaria, femur, tibia, heart, and kidney tissues of Tg28. The expression level of the transgene in the tibia was approximately ten-fold higher than the level of endogenous expression, and about seven-fold higher in femur and kidney tissues (Fig. 1c). A difference in *Osr1* expression between Wt and Tg because of artificial 2.8-kb *Osr1* promoter construct was observed. The body weight of *Osr1* Tg28 mice was less and the weight difference became prominent after 11 days (Fig. 1d).

Osr1 transgenic mice exhibited shorter-bodied phenotype

We analyzed 3-week-old male wild-type and transgenic littermates. *Osr1* transgenic mice were smaller than the wild-type (Fig. 2a), which was confirmed by analysis of radiographic and skeletal preparation findings (Fig. 2b, c). The body length was 6.4 ± 0.84 and 5.8 ± 0.35 cm in Wt and Tg, respectively, meaning that Tg mice had an abnormality in the vertebrae. In contrast, long bone size was similar to that of the wild-type littermates (Fig. 2d, e). To determine whether *Osr1* has a physiologic function in tooth development, we characterized the tooth phenotype of the transgenic mice. In mandible preparations, *Osr1* transgenic mice displayed much shorter mandible and small condyle of mandible compared with their wild-type littermates (Fig. 2f). *Osr1* transgenic mice displayed a white incisor compared with their wild-type littermates, suggesting a malfunction of ameloblasts (Fig. 2g). Furthermore, the present *Osr1* transgenic mice did not demonstrate prenatal or postnatal lethality, while *Osr1*-deficient mice died between E11.5 and E12.5 [5]. There was no difference in the longevity and other tissues such as muscle between wild-type and *Osr1* transgenic mice. Although *Osr1* functions in heart morphogenesis and urogenital development [5], there was no abnormality in heart and kidney in Tg mice. Together, these results suggested that the small phenotype of *Osr1* transgenic mice is caused by impaired bone formation and teeth abnormality from impaired teeth formation.

Skull formation abnormality in *Osr1* transgenic mice

Next, we performed detailed analysis of the skull phenotype and found a calvaria abnormality in the transgenic mice indicating that intramembranous ossification was affected by *Osr1* overexpression. The calvaria of the transgenic mice was shorter and more round than that of their wild-type littermates (Fig. 3a). The length of the anteroposterior axis of the cranium in Tg mice was shorter than in Wt mice (Fig. 3b). The facial bones, especially both the maxilla and mandible, were much shorter in Tg mice (Fig. 3c). Delayed cranial closure was also observed in the transgenic mice,

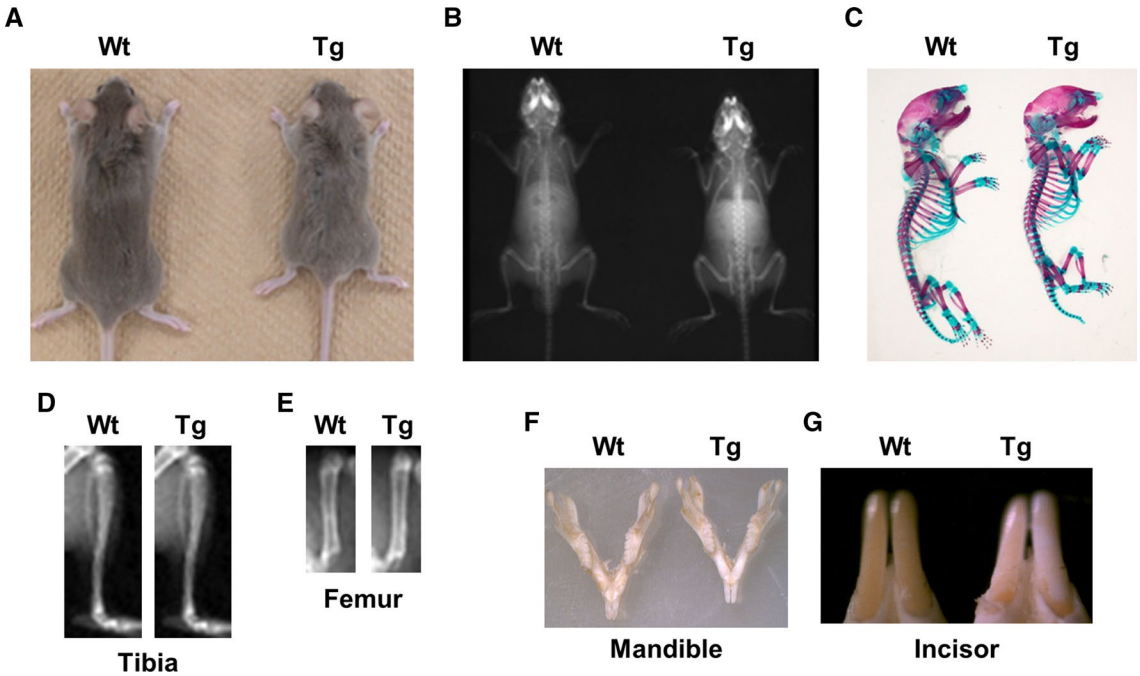

Fig. 2 Phenotype of *Osr1* transgenic mice. **a** Gross appearance of 3-week-old male wild-type (Wt) and *Osr1*-expressing (Tg) mice. **b** Radiographic analyses of skeletons from 3-week-old male Wt and Tg mice. **c** Skeletal preparations of newborn Wt and Tg mice. The skeletons were stained first with Alcian blue (cartilage), then Alizarin red (bone). **d**, **e** Radiographic images of tibia (**d**) and femur (**e**) specimens. **f** Surgical extraction of mandibular bone from 3-week-old male mice. **g** Enlarged mandibular image (incisor) from **f**. The experiments were performed in the Tg28 line ($n = 3$ in each group) (color figure online)

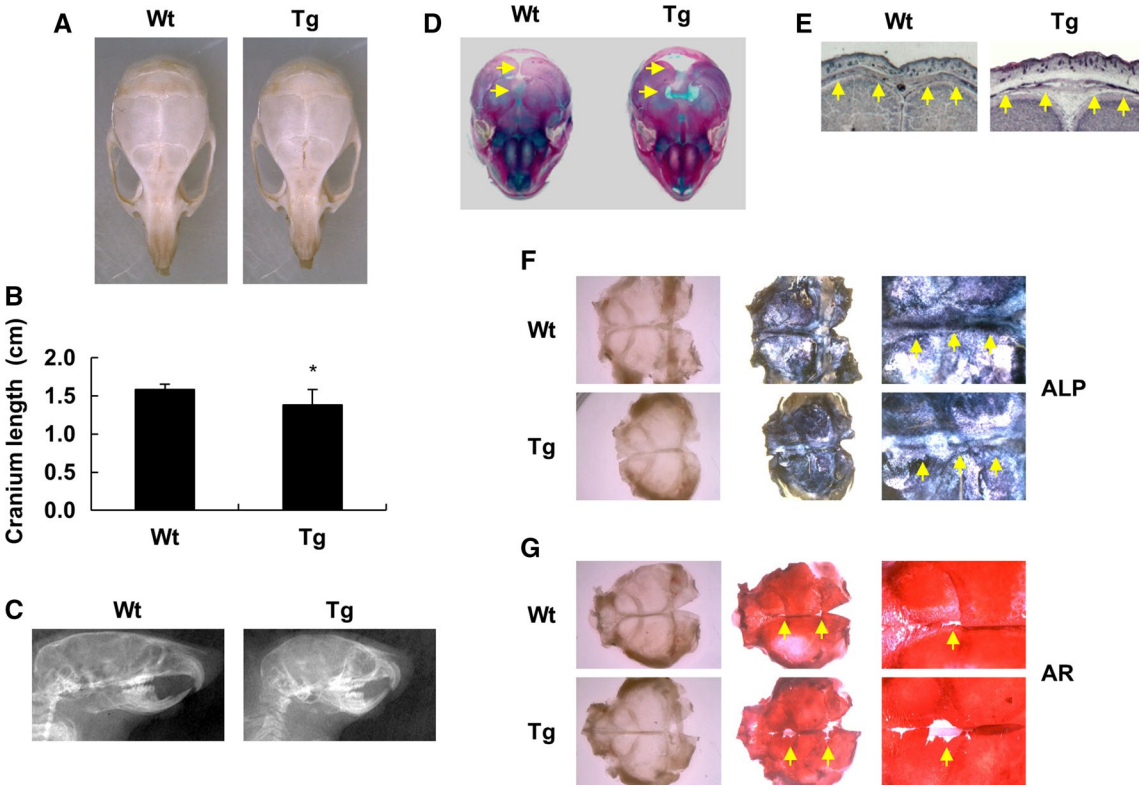

Fig. 3 Skull of *Osr1* transgenic mice. **a** Surgical extraction of skulls from 3-week-old male mice. **b** Quantification for cranium length. Data are shown as the mean and SD. $*P < 0.05$ (*t* test) versus Wt. **c** Radiographic analyses of skulls from 3-week-old male mice. **d** Skeletal preparations of newborn Wt and Tg mice with alcian blue (cartilage) and Alizarin red (bone). Arrows indicate delayed cranial closure. **e** Histological analysis of calvaria from newborn mice. Coronal sections of parietal bones were stained using H&E staining. Arrows indicate parietal bones. **f**, **g** Calvaria of newborn mice. The calvaria specimens were stained with ALP (**f**) and Alizarin red (AR, **g**). Arrows indicate delayed cranial closure. The experiments were performed in the Tg28 line ($n = 3$ in each group) (color figure online)

indicating that *Osr1* overexpression caused cranial dysraphism. Notably, the spaces of both the lambdoid and sagittal sutures were wider in the transgenic mice (Fig. 3d). No difference was observed in Alcian blue staining, meaning that there was no difference in chondrocalvarium (cartilage appeared transiently in calvaria of fetal and newborn mice, Fig. 3d). Histological analysis of calvaria specimens from *Osr1* transgenic mice showed an abnormality in cranial closure (Fig. 3e). We then performed ALP and Alizarin red staining of calvaria from wild-type and *Osr1* transgenic mice, and weaker staining in the *Osr1* transgenic calvaria was detected in the area of cranial closure (Fig. 3f, g). These observations indicated that *Osr1* is involved in cranial closure and skull formation.

Proliferation and differentiation of primary calvarial cells

We analyzed the activities of primary calvarial osteoblasts obtained from wild-type and *Osr1* transgenic mice to confirm that the phenotype of the latter was caused by an intrinsic defect of osteoblasts. Proliferation of osteoblasts was evaluated using an MTS assay and found to be increased in the transgenic mice (Fig. 4a). We considered that the enhanced osteoblastic proliferation in those might be caused by an aberrant function of osteoblasts. Thus, we performed RT-PCR expression analysis to compare osteoblast differentiation between the wild-type and transgenic mice. RNA was prepared from 7-day cultures of primary calvarial cells, in which we observed overexpression of *Osr2* as well as *Osr1*, whereas the expression of *Msx1* and *Msx2* was suppressed (Fig. 4b). The expression of osteoblastic genes was also affected, as osteocalcin and Osterix were decreased, ALP, type I α2 collagen, and *Runx2* were unchanged, and type I α1 collagen was increased in calvaria cells from the *Osr1* transgenic mice (Fig. 4c). Chondrogenic genes including type II α1 and type X α1 collagen, and Sox9 were increased, whereas aggrecan was decreased (Fig. 4d). Together, these findings suggest that *Osr1* negatively regulates osteoblast differentiation, while it positively regulates cell proliferation and chondrocyte differentiation.

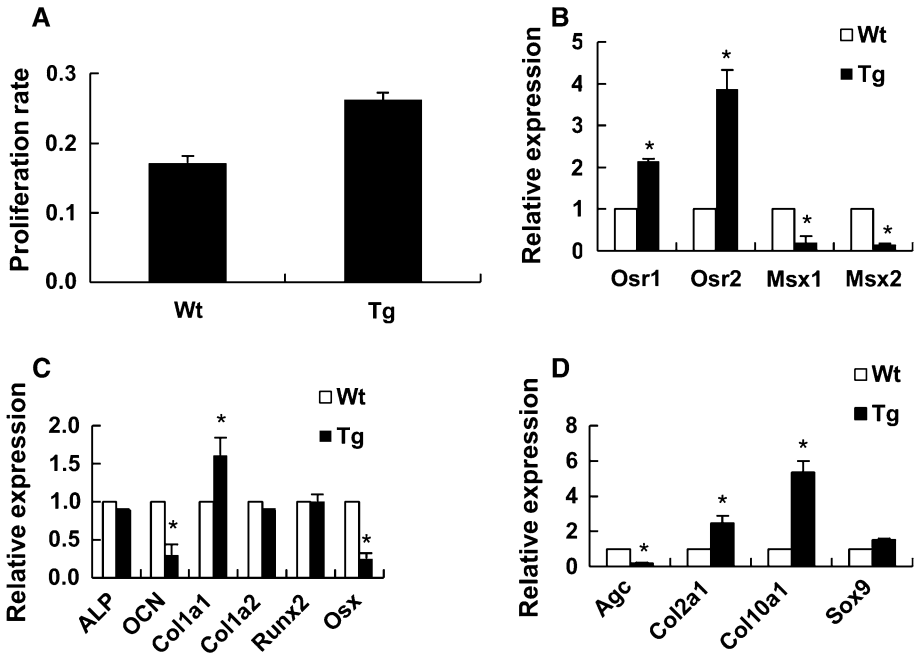

Fig. 4 Primary cultures of calvarial cells from newborn wild-type and transgenic mice. **a** Proliferation of primary calvarial cells was analyzed with an MTS assay. **b–d** RT-PCR analysis of expression in primary cultures of cells from newborn mice after 7 days of differentiation. Primary cultures prepared from two separate calvaria were analyzed. Abbreviations; OCN (osteocalcin), Osx (Osterix), Col1a1 (type I alpha 1 collagen), Col1a2 (type I alpha 2 collagen), Agc (aggrecan), Col2a1 (type II alpha 1 collagen), Col10a2 (type X alpha 1 collagen). The experiments were performed in triplicate and repeated at least twice. *Significant difference from Wt ($P < 0.05$)

BMP2 down-regulates Osr1 expression

BMP2 induces ALP production in osteoblastic cells [13]. Primary cells from calvaria were cultured for 7 days in BMP2-treated or mineralization medium and then subjected to ALP staining. The level of ALP staining of cells from the transgenic mice was reduced compared to those from the wild-type cultured in both types of media (Fig. 5a). The staining intensity of ALP of the transgenic mice was reduced compared to the wild-type cultured in mineralization medium (Fig. 5b). Primary cells from calvaria were then cultured for another 21 days and subjected to Alizarin red S staining for detecting calcium deposits. Staining intensity was weakly mineralized in *Osr1* calvaria cells cultured in mineralization medium, whereas primary cell cultures treated with BMP2 displayed less staining (Fig. 5a). Next, we analyzed *Osr1* expression after BMP2 treatment in mesenchymal C3H10T1/2 and osteoblastic MC3T3-E1 cells (Fig. 5c), and the results of those assays indicated that BMP2 negatively regulated *Osr1* expression. To further analyze whether *Osr1* affects osteoblast differentiation, *Osr1* was knocked down in the cell lines. siRNA for *Osr1* and the luciferase reporter were transfected into mesenchymal C3H10T1/2 or osteoblastic MC3T3-E1 cells, which were then cultured for 2 days, after which luciferase activity was measured. That activity was enhanced by *Osr1* knockdown. In addition, a reporter assay for the ALP promoter indicated that *Osr1* siRNA induced ALP promoter and osteocalcin promoter activities (Fig. 5d–g). Together, these results show that *Osr1* negatively regulates the expression of ALP and osteocalcin.

Discussion

In this study, the zinc-finger transcription factor *Osr1* was found to function in both skull and cranial bone formation. Our genetic analyses showed that transgenic mice overexpressing *Osr1* had round skulls and delayed skull closure in the calvaria. We also noted essential functions of *Osr1* in the processes of proliferation and differentiation of cranial osteoblasts along with alternation of expression of osteoblast marker genes. These results strongly suggest that *Osr1* plays a regulatory role in critical skull and cranial bone formation.

Osr1 transgenic mice display an abnormal cranial suture. Several genes, such as *Gsk3a* and *Gsk3b* [14], *Runx2* [15], *Msx2* [16], and *ameloblastin* [17], have also been demonstrated to play crucial roles in precise skull formation. For example, *Runx2* expression was found in cranial suture samples [18] and *Runx2* deficiency was seen in cleidocranial dysplasia [19], while we previously reported that *Runx2* decreased *Osr1* expression [7].

Regulation of *Osr1* expression by *Runx2* seems to be critical for skull formation. Nevertheless, the interaction between *Osr1* and *Runx2*, and modulation of *Runx2* expression by *Osr1* remain to be elucidated. *Msx2* was reported to be involved in premature suture closure [16]. In the present study, we found that *Osr1* overexpression resulted in delayed closure and *Msx2* expression was down-regulated in *Osr1* transgenic mice. These findings suggest that adequate adjustment of these genes is important for proper suture closure. Although the relationships between *Osr1* and *Gsk3a*, *Gsk3b*, and *ameloblastin* should be further analyzed, the present results demonstrated that *Osr1* is an important

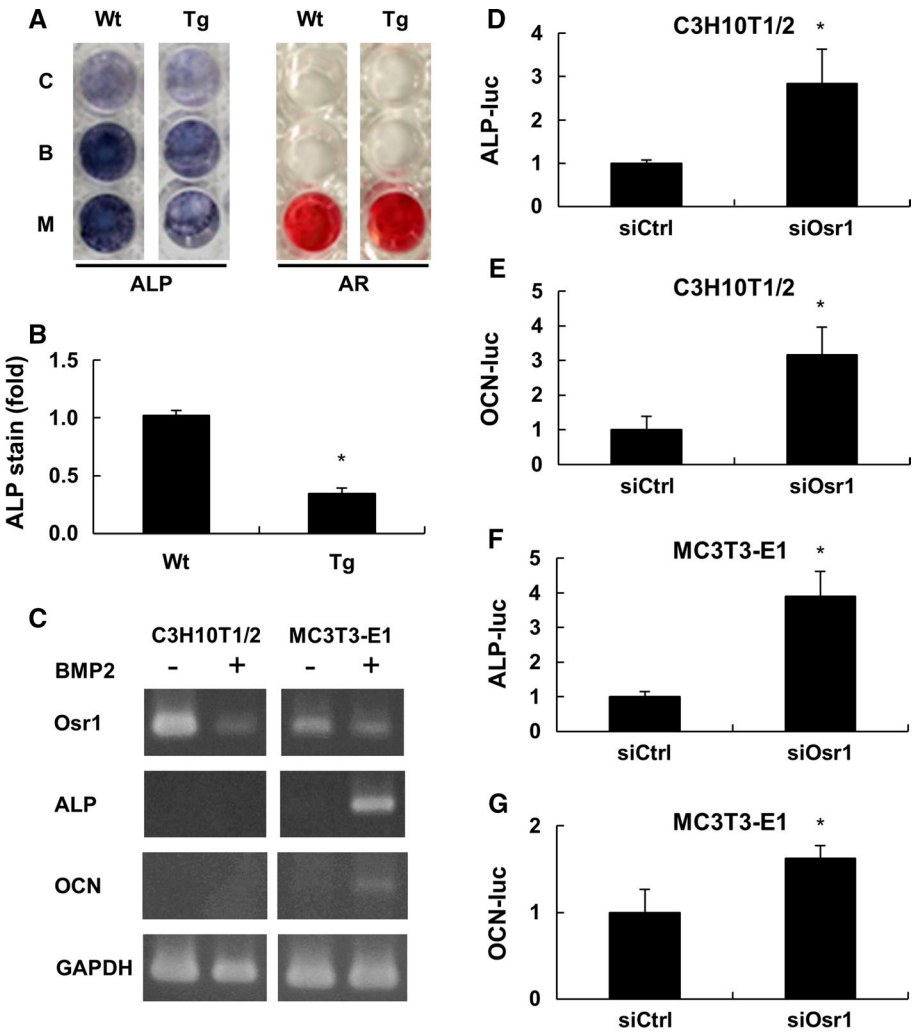

Fig. 5 Involvement of *Osr1* in osteogenic gene expression. **a** Primary cultures of calvarial cells from newborn mice were treated with BMP2 (lane B) or cultured in mineralization medium alone (lane M), followed by ALP staining after 7 days and Alizarin red staining (AR) after 21 days of culture. *C* control. **b** Quantification for ALP staining in mineralization medium (M). Data are shown as the mean and SD. **P* < 0.05 (*t* test) versus Wt. **c** Effect of *Osr1* expression by BMP2 in C3H10T1/2 and MC3T3-E1 cells. Cells were treated with 100 ng/mL of BMP2 for 2 days, and the expression levels of *Osr1*, ALP, and osteocalcin (OCN) were analyzed by qRT-PCR. (**d**–**g**) Reporter assay for ALP (ALP-Luc) and the osteocalcin promoter (OCN-Luc) in C3H10T1/2 and MC3T3-E1cells. Cells were transfected by siRNA for *Osr1* (si*Osr1*) and reporter genes for 2 days. The experiments were performed in triplicate and repeated at least twice. *Significant difference from siCtrl (*P* < 0.05) (color figure online)

transcriptional factor involved in skull shape formation and suture fusion.

It is also important to identify the molecular mechanisms regulating *Osr1* expression during bone formation. Recently, James et al. reported that a low concentration of BMP activated *Osr1* gene expression, whereas a high concentration repressed it [20], suggesting that the function of *Osr1* in bone formation is regulated by the BMP signaling pathway. In the present study, we analyzed *Osr1* expression by undifferentiated C3H10T1/2 and differentiated MC3T3-E1 cells cultured with a high dose of BMP2. Consistent with previously reported results, we found that *Osr1* expression was decreased by BMP2 treatment. This control of expression suggests that *Osr1* functions during osteoblast differentiation induced by BMP2. siRNA knockdown of *Osr1* induced ALP (an early osteogenic marker) and osteocalcin (a late marker), which also suggests that *Osr1* functions in commitment to osteoblast lineage. Based on our previous studies regarding the *Osr2* binding sequence [6], a possible explanation is regulation of the expression of osteoblast marker genes via the *Osr* binding sequence by *Osr1*. We also found induction of chondrocyte genes, suggesting a function of *Osr1* in chondrogenesis. These molecular activities in chondrogenesis require further elucidation in a future study, although our present results indicate a genetic function of *Osr1* in osteoblasts and chondrocytes during formation of skeletal tissue. The expression of *Osr1* in osteoclasts was 40 times lower than in osteoblasts (unpublished observation). As the relationship between *Osr1* and osteoclastogenesis remains unclear, further studies are needed to elucidate this association.

The kidneys regulate homeostasis of calcium and phosphate, which is related to bone metabolism, while 1,25-dihydroxy vitamin D, parathyroid hormone, and FGF23 regulate the serum levels of calcium and phosphate. James et al. reported that the *Osr1* gene functions in generation of kidney precursor cells and their differentiation into nephrons [21], and it was also reported that the *OSR1* rs12329305(T) allele in Caucasians alters the activity of the exon 2 splice enhancer, and reduces spliceosome-binding affinity and

stability of *OSR1* mRNA. Another study found that the *OSR1* rs12329305(T) allele had a relationship with small kidney volume and high cystatin C levels in cord blood [22]. An investigation of *Osr1* knockout mice also showed that *Osr1* functions in heart morphogenesis and urogenital development [5]. However, *Osr1* transgenic mice did not show obvious abnormalities in those tissues, even though high expression levels were found in the heart and kidneys. Elucidation of the detailed mechanisms of *Osr1* in heart and kidney tissues of *Osr1* transgenic mice requires additional investigation.

In summary, our findings are the first to show the underlying functions of *Osr1* in skull and bone formation. As *Osr1* transgenic mice exhibited decreased osteoblast activity as well as delayed skull closure in the calvaria, we speculate that *Osr1* is a regulator of osteoblast function. Our results also indicate the existence of a novel alternative cascade involved in skull formation. Thus, regulation of *Osr1* function by pharmacological compounds is a potential strategy for abnormal skull development.

Acknowledgements We thank all the members of the Challenge to Intractable Oral Diseases and Center for Frontier Oral Science for their assistance and encouragement, as well as Mark Benton for their comments regarding our manuscript. This work was supported by Grants-in-Aid for Scientific Research from the Japanese Society for the Promotion of Science (KAKENHI no. C21592356, C24592796), and by special funds for Challenge to Intractable Oral Diseases.

Conflict of interest All authors declare that they have no competing interests.

References

1. Coulter DE, Swaykus EA, Beran-Koehn MA, Goldberg D, Wieschaus E et al (1990) Molecular analysis of odd-skipped, a zinc finger encoding segmentation gene with a novel pair-rule expression pattern. EMBO J 9:3795–3804
2. So PL, Danielian PS (1999) Cloning and expression analysis of a mouse gene related to *Drosophila* odd-skipped. Mech Dev 84:157–160
3. Katoh M (2002) Molecular cloning and characterization of OSR1 on human chromosome 2p24. Int J Mol Med 10:221–225
4. Kawai S, Michikami I, Kitagaki J, Hashino E, Amano A (2013) Expression pattern of zinc-finger transcription factor Odd-skipped related 2 in murine development and neonatal stage. Gene Expr Patterns 13:372–376
5. Wang Q, Lan Y, Cho ES, Maltby KM, Jiang R (2005) Odd-skipped related 1 (Odd 1) is an essential regulator of heart and urogenital development. Dev Biol 288:582–594
6. Kawai S, Yamauchi M, Wakisaka S, Ooshima T, Amano A (2007) Zinc-finger transcription factor odd-skipped related 2 is one of the regulators in osteoblast proliferation and bone formation. J Bone Miner Res 22:1362–1372
7. Yamauchi M, Kawai S, Kato T, Ooshima T, Amano A (2008) Odd-skipped related 1 gene expression is regulated by Runx2 and Ikzf1 transcription factors. Gene 426:81–90
8. Deng ZL, Sharff KA, Tang N, Song WX, Luo J et al (2008) Regulation of osteogenic differentiation during skeletal development. Front Biosci 13:2001–2021
9. Gordon JW, Scangos GA, Plotkin DJ, Barbosa JA, Ruddle FH (1980) Genetic transformation of mouse embryos by microinjection of purified DNA. Proc Natl Acad Sci USA 77:7380–7384
10. McLeod MJ (1980) Differential staining of cartilage and bone in whole mouse fetuses by alcian blue and alizarin red S. Teratology 22:299–301
11. Lyle HM (1947) An improved tissue technique with hematoxylin-eosin stain. Am J Med Technol 13:178–181
12. Farley JR, Tarbaux NM, Hall SL, Linkhart TA, Baylink DJ (1988) The anti-bone-resorptive agent calcitonin also acts in vitro to directly increase bone formation and bone cell proliferation. Endocrinology 123:159–167
13. Rawadi G, Vayssiere B, Dunn F, Baron R, Roman-Roman S (2003) BMP-2 controls alkaline phosphatase expression and osteoblast mineralization by a Wnt autocrine loop. J Bone Miner Res 18:1842–1853
14. Barrell WB, Szabo-Rogers HL, Liu KJ (2012) Novel reporter alleles of GSK-3alpha and GSK-3beta. PLoS One 7:e50422
15. Komori T, Yagi H, Nomura S, Yamaguchi A, Sasaki K et al (1997) Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. Cell 89:755–764
16. Liu YH, Kundu R, Wu L, Luo W, Ignelzi MA Jr et al (1995) Premature suture closure and ectopic cranial bone in mice expressing Msx2 transgenes in the developing skull. Proc Natl Acad Sci USA 92:6137–6141
17. Atsawasuwan P, Lu X, Ito Y, Zhang Y, Evans CA et al (2013) Ameloblastin inhibits cranial suture closure by modulating MSX2 expression and proliferation. PLoS One 8:e52800
18. Park MH, Shin HI, Choi JY, Nam SH, Kim YJ et al (2001) Differential expression patterns of Runx2 isoforms in cranial suture morphogenesis. J Bone Miner Res 16:885–892
19. Otto F, Kanegane H, Mundlos S (2002) Mutations in the RUNX2 gene in patients with cleidocranial dysplasia. Hum Mutat 19:209–216
20. James RG, Schultheiss TM (2005) Bmp signaling promotes intermediate mesoderm gene expression in a dose-dependent, cell-autonomous and translation-dependent manner. Dev Biol 288:113–125
21. James RG, Kamei CN, Wang Q, Jiang R, Schultheiss TM (2006) Odd-skipped related 1 is required for development of the metanephric kidney and regulates formation and differentiation of kidney precursor cells. Development 133:2995–3004
22. Zhang Z, Iglesias D, Eliopoulos N, El Kares R, Chu L et al (2011) A variant OSR1 allele which disturbs OSR1 mRNA expression in renal progenitor cells is associated with reduction of newborn kidney size and function. Hum Mol Genet 20:4167–4174