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Odd-skipped related 1 gene expression is regulated by *Runx2* and *Ikzf1* transcription factors

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ABSTRACT

Odd-skipped related 1 (*Osr1*) gene encodes a zinc-finger transcription factor that plays important roles in embryonic, heart, and urogenital development, however, it is unknown how its expression is regulated. In this study, we analyzed the promoter region of *Osr1* to elucidate its regulation mechanism. The mouse *Osr1* promoter region was cloned and characterized, and found to have two repressor elements in the −4504/−2766 and −1616/−109 regions, and two enhancer elements in the −2766/−1616 and −109/+199 regions. Several Runx2 and Ikzf1 binding sites were found in both mouse and human *Osr1* promoters. *Osr1* promoter activity was suppressed by cotransfection with *Runx2*- and *Ikzf1*-expressing vectors in a dose-dependent manner. Electrophoresis mobility shift assays showed that purified Runx2 bound to proximal (−611/−606) Runx2 binding motifs and that Ikzf1 bound to proximal (−1652/−1644) *Ikzf1* binding motifs. Chrosmatin immunoprecipitation demonstrated that Runx2 bound to both the distal (−3047/−3042) and proximal regions, and that Ikzf1 bound to both the far-distal (−3036/−3028) and proximal elements. These findings indicate that *Osr1* expression is regulated by Runx2 and Ikzf1, which are known as master-gene of osteogenesis and hematopoiesis, respectively.

1. Introduction

The *odd-skipped* (*odd*) gene, first identified as a *Drosophila* pair-rule transcription factor, contains four DNA-binding C2H2-type zinc fingers in the C-terminal half of the molecule (Coulter et al., 1990). The *Drosophila odd* gene is generally classified as a secondary pair-rule gene (Ingham et al., 1988), and reported to be primarily expressed in stripes that spread in even-numbered segments, and then later in narrow stripes in the center of both even- and odd-numbered segments of narrow strips (Coulter et al., 1990). Mutations in the *odd* gene cause pattern defects in the anterior regions of the odd-numbered segments and partial substitution by mirror-image duplications in adjacent regions (Coulter and Wieschaus, 1988). These defects are closely associated with altered expression levels of other segmentation genes, such as *fushi tarazu*, *engrailed*, and *wingless* (DiNardo and O'Farrell, 1987). Expression of the *odd* gene is governed by the so-called primary pair-rule genes (*eve*, *h,* and *runt*) (Ingham et al., 1988) and it was recently reported that the *odd* gene functioned in a manner similar to those primary pair-rule genes (Berman et al., 2004; Schroeder et al., 2004).

Homologues for *odd* genes have been found in *Caenorhabditis elegans*, *Saccharomyces cerevisiae,* and *Xenopus laevis* (Brohl et al., 1994; Buckley et al., 2004; Klein et al., 2002), and 2 mammalian homologues, *odd-skipped related 1* (*Osr1*) and *odd-skipped related 2* (*Osr2*), were subsequently cloned from both mice and humans (Katoh 2002; Lan et al., 2001; So and Danielian, 1999). These *Osr1* and *2* genes share a high degree of homology (83% and 78%, respectively) with the *odd* gene throughout its zinc finger domains, though no significant homology is displayed outside of those domains. Mouse *Osr1* has been shown to have a 65% homology with *Osr2* (Lan et al., 2001), however, the tissue distribution of those mRNA/proteins appear to differ. For example, mouse *Osr1* is expressed in the intermediate mesoderm, limb, and branchial arch of fetuses on embryonic day 9.5–12.5 (So and Danielian, 1999). Further, human *OSR1* is detected in fetal lungs, as well as adult colon, small intestine, prostate, and testis tissues (Katoh, 2002). In contrast, *Osr2* is preferentially expressed in sites where epithelial–mesenchymal interactions occur during limb, tooth, and kidney development.

An investigation of mice carrying a targeted null mutation of *Osr1* showed that the gene was essential for heart and intermediate mesoderm development (Wang et al., 2005). *Osr1* null mutant embryos did not succeed to form an atrial septum, and exhibited dilated atria with hypoplastic venous valves and blood backflow from the heart into systemic veins. Furthermore, expression of *Osr1* was

Abbreviations: Osr1, odd-skipped related 1; Odd, odd-skipped; TSS, transcription start site; RT-PCR, reverse transcription-polymerase chain reaction; qPCR, real-time PCR; RLM-RACE, RNA ligase-mediated and oligo-capping rapid amplification cDNA ends; EMSA, electrophoresis mobility shift assay; ChIP, chromatin immunoprecipitation; MBP, maltose binding protein; FITC, fluorescein isothiocyanate.

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found to be restricted to the central dorsal domain of the atrial myocardium during normal heart development. In addition, *Osr1* null mutant embryos exhibited complete defects of adrenal glands, metanephric kidneys, gonads, and pericardium formation, and the key regulators of early intermediate mesoderm development (*Lhx1*, *Pax2*, and *Wt1*) were all downregulated in the *Osr1* null mutant embryos. They also found that nephrogenic mesenchyme underwent massive apoptosis, which caused a disruption of nephric duct elongation and failure of metanephric induction in the *Osr1* null mutant embryos. These findings suggested that *Osr1* functions in heart morphogenesis and urogenital development. We previously reported that *Osr2* regulates osteoblast function, because dominant-negative *Osr2* transgenic mice exhibited decreased osteoblast proliferation and delayed mineralization in calvarial and tibial bone tissues (Kawai et al., 2007). Although there is a possibility that *Osr1* is also involved in bone formation, such novel functions of *Osr1* remain to be elucidated.

The critical roles of *Osr1* in embryos are receiving increasing attention for the reasons noted above, however, the mechanism involved with regulation of its expression remains unknown. In the present study, we performed molecular dissection of the regulation mechanism, by cloning the 5′-flanking DNA region (spanning 4703 bp) of the mouse *Osr1* gene, and functional analysis of that fragment revealed that *Runx2* (also known as *Cbfa1*, *PEBP2A1*, and *AML3*) and *Ikzf1* (also known as *Lyf1* and *Ikaros*) are regulators that suppress *Osr1* promoter activity. Herein, we report the first known direct evidence that *Runx2* and *Ikzf1* have an ability to regulate *Osr1* expression.

2. Materials and methods

2.1. Cloning of 5′-flanking region of mouse Osr1 gene

First, we performed a BLAST search for mouse *Osr1* cDNA (GenBank accession number NM_011859) and found that the mouse chromosome 12 genomic contig of strain C57BL/6J (GenBank Accession number NT_039548) contained the 5′-flanking region of the *Osr1* gene. The 5094-bp 5′-flanking region of the *Osr1* gene was amplified by PCR using mouse genomic DNA with LA Taq polymerase (Takara Bio Inc., Shiga, Japan). The primers used were 5′-GTTTGGAGCTGGTACTCTGATACCAGTTTA-3′ (forward) and 5′-GCGTCGACTTCTGTAGCTGCGGTGACTCCA-3′ (reverse; underlined bases indicate *Sal* I site used later for cloning). The amplified product was cloned into a pCR4Blunt-TOPO vector (Invitrogen Corp., Carlsbad, CA). DNA sequencing was performed using BigDye cycle sequencing and read on an ABI PRISM 310 genetic analyzer (Applied Biosystems, Foster City, CA). The sequence of the construct was verified by comparing with NT_039548.

2.2. Reverse transcription-polymerase chain reaction (RT-PCR) and real-time PCR (qPCR)

Total RNA from cells was prepared using TRIsure reagent (Bioline, London, UK) and reverse-transcribed with an iScript cDNA Synthesis Kit (Bio-Rad Laboratories Inc., Hercules, CA). The primer sequences for *Osr1* were 5′-CCTGGACGTGACCAAGCTAT-3′ (forward) and 5′-TGTAGCGTCTTGTGGACAGC-3′ (reverse). The primer set for β-actin mRNA was also used, as described previously (Kawai et al., 2006). PCR assays were performed using Taq PCR Master Mix (QIAGEN Inc., Valencia, CA). The product (about 0.8 kb) was resolved by electrophoresis on a 1.5% agarose gel. qPCR assays were performed using a LightCycler system (Roche Diagnostics Corp., Indianapolis, IN), according to the manufacturer's instructions. The primer sequences for *Osr1* were 5′-GAGCGACCTTACACCTGTGA-3′ (forward) and 5′-GTCTTGTGGACAGCGAGAGT-3′ (reverse). The primer set for GAPDH mRNA was used for quantification, as described previously (Kawai et al., 2006). Each reaction was carried out with QuantiTect SYBR Green PCR Master Mix (QIAGEN). The expression levels of mRNA are shown as the relative expression normalized by GAPDH. Each procedure was repeated at least 3 times to assess reproducibility.

2.3. RNA ligase-mediated and oligo-capping rapid amplification cDNA ends (RLM-RACE)

To identify the transcription start site (TSS) of *Osr1* mRNA, an RLM-RACE reaction was performed using a commercial GeneRacer kit from Invitrogen, according to the manufacturer's protocol. The gene specific primers for *Osr1* were 5′-TTAGCATTTGATCTTGGAGGTTTTGAGCTC-3′ and 5′-TGTAGCGTCTTGTGGACAGC-3′. Following cloning of the PCR products into a pCR4Blunt-TOPO vector (Invitrogen), independent colonies were randomly chosen and subjected to sequencing analysis.

2.4. Identification of putative transcription factor binding sites

The putative transcription factor binding sites on the 5′-flanking region of the *Osr1* gene were identified by a TFSEARCH (http://www.cbrc.jp/research/db/TFSEARCHJ.html) based on the TRANSFAC database (Wingender et al., 1996). Matrix similarity scores were set at 90.0, except when another value was indicated.

2.5. Construction of promoter-reporter expression vectors

The 5′-flanking region of the *Osr1* gene in a pCR4Blunt-TOPO vector was isolated by digestion with Xba I and Sal I (4703 bp), then subcloned into the Nhe I and Xho I sites of a pGL3-basic firefly luciferase reporter vector (Promega Corp., Madison, WI) that carried neither the eukaryotic promoter nor its enhancer. The resulting plasmid was termed pXb. Serial deletions of the 5′-flanking region of the *Osr1* gene were carried out by self-ligation after enzymatic digestion of pXb. The restriction enzyme sites used for construction of the serially deleted promoter regions are shown in Fig. 3, with a total of 7 promoter-luciferase reporter expression vectors constructed.

2.6. Cell culture, transfection, luciferase activity assay, and adenovirus infection

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 (Ibaraki, Japan). C3H10T1/2 and MC3T3-E1 cells were maintained in α-modified Eagle's medium (α-MEM; Sigma-Aldrich Corp., Saint Louis, MO) supplemented with 10% fetal bovine serum (FBS; JRH Biosciences Inc. Lenexa, KS), 100 U/mL of penicillin and 50 mg/mL of streptomycin. C2C12 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich) supplemented with 10% FBS, 100 U/mL of penicillin, and 50 mg/mL of streptomycin.

One day before transfection, cells were plated in 24-well culture plates at a density of 5×10^4 per well. The promoter firefly luciferase reporter vector (0.4 μg) was transiently transfected using FuGENE6 transfection reagent (1.2 μL/well; Roche Diagnostics). A pRL-CMV vector (20 ng; Promega), in which expression of Renilla luciferase was driven by the CMV promoter, was concomitantly transfected as an internal control. About 48 h after transfection, the cells were lysed with a passive-lysis buffer and both types of luciferase activity were measured using a Dual Luciferase Reporter Assay System (Promega), after which promoter firefly luciferase activity was normalized by Renilla luciferase activity. The experiments were done in triplicate and repeated at least twice. A *Runx2* vector was provided by S. Roman-Roman (Prostrakan Pharmaceuticals, Romainville, France). The *Ikzf1* gene was isolated by RT-PCR and cloned into a pcDNA3 vector (Invitrogen).

Adenovirus expressing *Runx2* was provided by R. Nishimura (Departments of Biochemistry, Osaka University Graduate School of