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Negative regulation of *Odd-skipped related 2* by TGF-beta achieves the induction of cellular migration and the arrest of cell cycle

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ABSTRACT

The transcription factor *Odd-skipped related 2* (*Osr2*) functions in craniofacial and limb developments in mammals. We previously found that *Osr2* gene expression is regulated by intracellular transcription factors such as *Runx2*, and *C/EBP*, whereas it remains unclear if extracellular factors would functionally regulate the *Osr2* expression. In this study, we showed that TGF-β down-regulated the *Osr2* expression, which is involved in regulation of cellular migration and cell cycle. Furthermore, the down-regulation was found to be mediated by Smad3/Smad4 and p38/ATF2 signaling molecules. The *Osr2* promoter was shown to possess Smad3/4 binding element and ATF2 binding element between −647 and −64 of promoter. TGF-β induced cellular migration in C3H10T1/2 cells and arrested cell cycle at G1 phase in NMuMG-Fucci cells. In contrast, the *Osr2* reduced the migration and also stimulated the cell-cycle progression. These results suggest that *Osr2* is involved in TGF-β regulating cell migration and cell cycle via a Smad3-ATF2 transcriptional complex mediating pathway.

1. Introduction

Odd-skipped related 2 (*Osr2*) which contains DNA-binding C2H2-type zinc finger domains in the C-terminal half functions as a transcription factor in craniofacial and limb developments [1,2]. We previously showed that *Osr2* gene promoter is regulated by other transcription factors such as *Runx2*, *C/EBPα*, *C/EBPβ*, and *C/EBPδ* [3], whereas it remains open question if extracellular factors also regulate *Osr2* expression, which may be involved in cellular physiological actions. We recently showed that cellular quiescence by serum starvation induces *Osr2* expression through epigenetic regulation [4], suggesting that extracellular factors in serum can regulate the *Osr2*. It is known that several serum factors differentially regulate cellular fundamental operations such as proliferation, differentiation, and migration [5]. Among them, transforming growth factor-β (TGF-β) is abundant with higher level (approximately 10–20 ng/mL) than others in fetal serum [6,7].

TGF-β regulates multiple biological processes including early development and maintenance of homeostasis in adult [8], which is mediated by several transcriptional factors including Smad2/Smad3/Smad4 [9], p38 MAP kinase, and ATF2 [10]. In cancer, TGF-β has dual roles to act as a tumor suppressor in early tumor development and to promote cellular migration in later tumor stages [11,12]. TGF-β inhibits cell cycle progression in a wide variety of cell types by blocking the late G1 activation and preventing S phase entry [13]. Biological roles of *Osr2* remain largely unknown, thus its novel function would be clarified if *Osr2* is involved in the regulatory cascades mediated by the multifunctional factor.

In this study, we examined the involvement of *Osr2* in the signaling cascade regulated by TGF-β, and identified the cascade-associated molecules. Furthermore, the role of *Osr2* was examined in the fundamental cell operations regulated by TGF-β, i.e., migration and cell cycle. Consequently, we here suggest that *Osr2* is involved in TGF-β regulating cell migration and cell cycle.

2. Materials and methods

2.1. Cell culture

The mouse fibroblastic mesenchymal cell line C3H10T1/2 (RCB0247) and a mouse epithelial-like cell line expressing Fucci (fluorescent ubiquitination-based cell cycle indicator), NMuMG-Fucci (RCB2831), were purchased from Riken Bioresource Center (Tsukuba, Ibaragi, Japan). C3H10T1/2 cells were maintained in α-modified Eagle's medium (α-MEM; Wako Pure Chemical Industries, Osaka, Japan) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA). NMuMG-Fucci cells were

Abbreviations: TGF-β, transforming growth factor-beta; Osr2, Odd-skipped related 2; CRE, cAMP response element; EMT, epithelial-mesenchymal transition.

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maintained in Dulbecco's modified Eagle's medium (DMEM; Wako) supplemented with 10% FBS. Recombinant proteins of human TGF-β1, human BMP4, human TNF-α, and rat Jagged 1 were purchased from R&D Systems Inc. (Minneapolis, MN).

2.2. Real Time quantitative Reverse Transcription PCR (Real-Time qRT-PCR)

Real-Time qRT-PCR was performed as described [14]. Total RNA from the cells was prepared using TRIsure reagent (Bioline, London, UK) and reverse-transcribed with an iScript cDNA Synthesis kit (Bio-Rad Laboratories Inc., Hercules, CA). A qPCR assay was performed using a StepOnePlus system (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. The reaction was carried out with SYBR Green PCR Master Mix (Applied Biosystems) in a 10-μL volume containing 0.5 μM of *Osr2* primers. The primer set for mouse *Osr2* mRNA was 5′-GTCCTTCCAGCCCTACACAAG-3′ (forward) and 5′-CTTTCTGTCCGGGTTCAGTTTAC-3′ (reverse). The primer set for mouse GAPDH mRNA was 5′-ACCCAGAAGACTGTGGA TGG-3′ (forward) and 5′-CACATTGGGGGTAGGAACAC-3′ (reverse). The expression levels of mRNA are indicated as the relative cycle number normalized by the cycle number of GAPDH. Each procedure was repeated at least 3 times to assess reproducibility.

2.3. Cellular migration

C3H10T1/2 cells (1.0×10^5 cells per six-well culture dish) were cultured until confluent. The cell layers were scratched using a plastic tip and washed three times with serum-free medium to remove debris, as described previously [15]. C3H10T1/2 cells were transduced with an adenovirus *Osr2B* vector [16]. The cells were further incubated in αMEM containing 10% FBS at 37 °C for 12 h. The numbers of migrated cells to in the scratched areas were microscopically determined, and the closure rate of each scratched area was measured using NIH Image analysis, as described previously [15]. At least 10 fields were analyzed and all assays were performed in triplicate on three separate occasions.

3. Results

3.1. TGF-β inhibits Osr2 expression

To elucidate *Osr2* regulation by extracellular growth factors, the effects of Jagged1, TNF-α, TGF-β, and BMP4 were evaluated on *Osr2* expression in C3H10T1/2 cells. Jagged is a ligand of Notch receptor which is involved in joint formation at Drosophila leg upstream of *Osr* [17]. TNF-α is an important extracellular molecule in apoptosis which Osr1 also may be involved in [18]. BMPs and TGF-β are important extracellular molecules in bone formation where *Osr2* also plays an important role [1]. BMP regulates odd-skipped gene expression in chicken [19]. TGF-β significantly suppressed *Osr2* expression in a dose dependent manner, followed by BMP4 and TNF-α (Fig. 1). In contrast, Jagged 1 showed negligible effect. No factors were found to up-regulate *Osr2* expression.

3.2. Smad3 and p38-ATF2 signaling pathway mediates inhibition of Osr2 expression by TGF-β

Osr2 is suggested to be downstream regulated by TGF-β which is mediated by Smad2/Smad3 with Smad4 [9], and p38 MAP kinase and transcription factor ATF2 [10]. Therefore, we examine whether these transcriptional factors are also involved in *Osr2* expression in order. Smad3 and Smad4 expression vectors were co-transfected with the *Osr2* promoter-luciferase expression construct in C3H10T1/2 cells, and then the cells were treated with TGF-β to phosphorylate and activate Smad3. Smad3 and Smad4 complex phosphorylated by TGF-β was found to decrease the *Osr2* promoter activity, whereas Smad7 increased the activity (Fig. 2A). Next, we examined the involvement of TGF-β-MAPK pathway using MAPK inhibitors and siRNA. C3H10T1/2 cells were treated by PD098059 (MEK inhibitor), SB203580 (p38 MAPK inhibitor) or SP600125 (JNK inhibitor) for two days and *Osr2* mRNA was quantified by qRT-PCR. p38 MAPK inhibitor, SB203580 significantly induced *Osr2* expression in a dose dependent manner, and JNK inhibitor, SP600125, showed slightly positive effects. However, the effect of MEK inhibitor, PD098059, was negligible (Fig. 2B). For siRNA knock-down, p38 siRNA (sip38) was transfected into C3H10T1/2 cells and *Osr2* mRNA was measured by qRT-PCR. The sip38 expectedly induced *Osr2* expression (Fig. 2C), indicating that p38 negatively regulates *Osr2* expression. Furthermore, ATF2, downstream molecule of p38 signaling, were overexpressed with *Osr2* promoter-luciferase reporter. ATF2 also dose-dependently suppressed *Osr2* expression (Fig. 2D). The *Osr2* was also down-regulated by Fos, but not by Jun (Supplementary Fig. S1). These results indicated that both Smad3/4 and p38/ATF2 directly regulate *Osr2* mRNA expression, showing that *Osr2* is regulated by TGF-β.

3.3. TGF-β suppressed Osr2 promoter activity

TGF-β was found to prevent *Osr2* expression through the Smad3/4 and p38/ATF2 cascades. Thus, we further examined if *Osr2* gene has direct binding sites on promoter region for Smad3 and ATF2 transcription factors, using 6 luciferase reporter vectors carrying various lengths of the *Osr2* promoter (Fig. 3A left). These constructs were transiently transfected into C3H10T1/2 cells, and then luciferase activity was measured. As shown in Fig. 3A, the deletion constructs from −4909 to −647 of the *Osr2* promoter exhibited inhibition of luciferase activity by TGF-β. Removal of the sequence corresponding to nucleotides −4909 to −647 from the parental construct increased suppression in luciferase activity by TGF-β. Sequential deletion of the regions corresponding to nucleotides −647/−64 caused an abolishment of decrease in promoter activity by TGF-β, implicating the presence of a suppressor element within a region spanning nucleotides −647 to −64. Sequence analysis of the *Osr2* promoter revealed the presence of multiple potential transcription factor binding sites (Fig. 3B). In the region (nucleotides −647 to −64), there were ATF-binding sequence, activator protein 1 (*AP-1*) binding site, Smad-binding sequence, and Ikzf1 sequence. These findings suggest that the region corresponding to nucleotides −647/−64 contains an inhibitory element for *Osr2* expression and that this region has inhibitory transcription factor binding sites of TGF-β.

3.4. Osr2 inhibits cellular migration, but enhanced cell cycle

Finally, we examined the involvement of *Osr2* in cell migration and cell cycle which are the fundamental cell operations regulated by TGF-β. Because *Osr2* which is involved in cellular proliferation [1,14] regulates many genes classified to 'cell communication' including the gene groups in cellular migration [14]. To determine the involvement of *Osr2* in cellular migration of C3H10T1/2 cells, we analyzed the effect of *Osr2* on cell scratch assay with *Osr2* adenovirus infection (Supplementary Fig. S2). TGF-β promoted the migration, whereas the *Osr2* overexpression clearly inhibited the effect of TGF-β (Fig. 4A). NMuMG cells are frequently used as a model of TGF-β-mediated migration and inducible epithelial-mesenchymal transition (EMT) [20]. Fucci is a cell cycle indicator that labels G1 phase nuclei and S/G2/M phase nuclei with red and green fluorescence, respectively [21]. As shown in Fig. 4B and Supplementary Fig. S3, the *Osr2* overexpression in NMuMG-Fucci cells

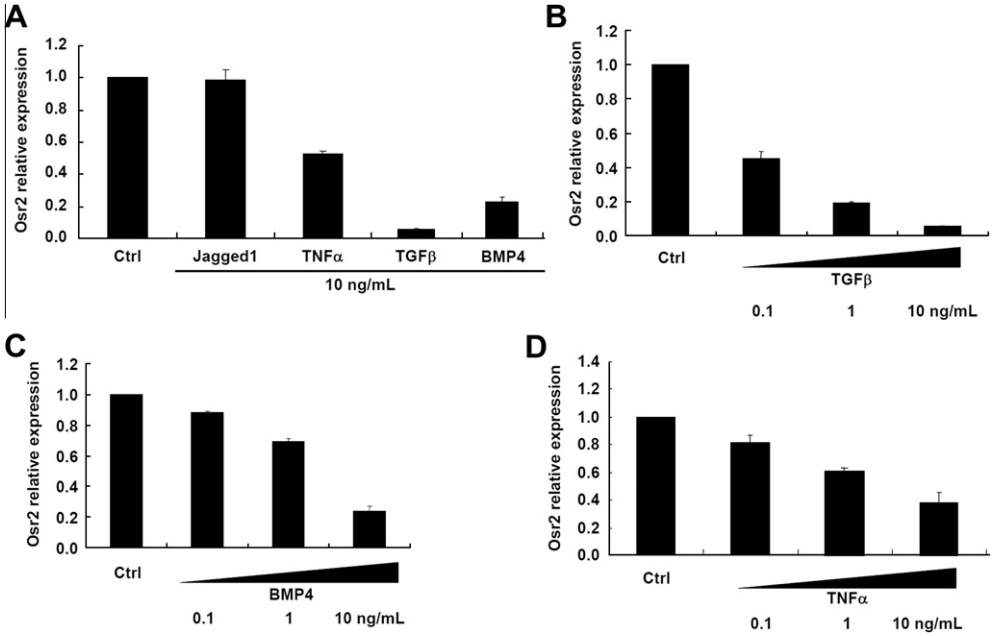

Fig. 1. Regulation of *Osr2* expression by several growth factors. (A) Fibroblastic C3H10T1/2 cells were treated with various growth factors (10 ng/mL). Total RNA was extracted by the AGPC method after 48 h, and *Osr2* expression was quantified by ABI StepOnePlus real-time PCR. The value was standardized with GAPDH ($N = 3$). The experiment was repeated at least twice. TGF-β remarkably down-regulated the expression of *Osr2*. TGF-β (B), BMP4 (C), and TNF-α (D) was observed to control the expression of *Osr2* dose-dependently. BMP4 was observed to dose-dependently regulate the expression of *Osr2*.

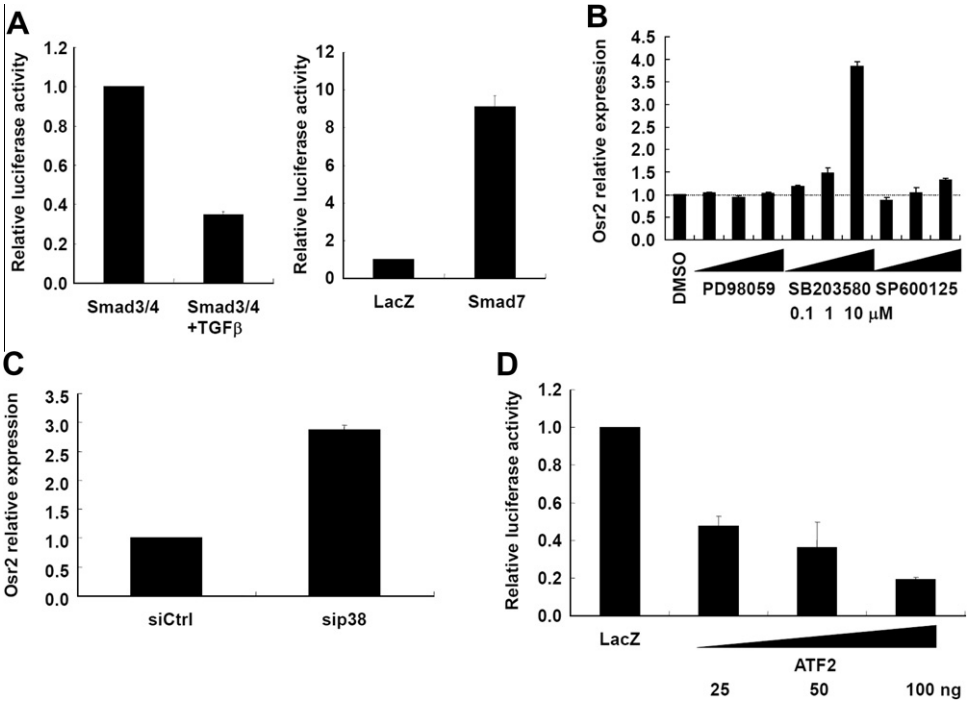

Fig. 2. Regulation of *Osr2* expression by Smads, p38, and ATF2. (A) Regulation of *Osr2* promoter by Smads. *Osr2* promoter-firefly luciferase and Smad were co-transfected in fibroblastic C3H10T1/2 cells. After 24 h of transfection, TGF-β was added. After a further 24 h, luciferase activity was measured. It was observed that Smad3/4 down-regulated the *Osr2* promoter and Smad7 up-regulated the *Osr2* promoter activity. (B) Regulation of *Osr2* expression by MAPK. Cells were treated with various concentrations of MAPK inhibitors. *Osr2* expression was measured by real-time PCR after 48 h. p38 inhibitor (SB203580) and JNK inhibitor (SP600125) dose-dependently up-regulated the expression of *Osr2*. MEK1 inhibitor (PD98059) did not influence the expression of *Osr2*. (C) Regulation of *Osr2* expression by p38. Cells were transfected by p38 siRNA (sip38), and *Osr2* expression after 48 h of transfection was measured by real-time PCR. From the increase of *Osr2* expression by sip38, p38 inhibited expression of *Osr2*. (D) Regulation of *Osr2* promoter by ATF2. *Osr2* promoter-luciferase and ATF2 expression vector were transfected in cells. Firefly and renilla luciferase activities were measured after 48 h. ATF2 was observed to control the *Osr2* promoter.

increased a number of green proliferative cells, whereas TGF-β treatment increased red arrested cells. Collectively, these results suggest that *Osr2* inhibits cellular migration and is able to evoke cell proliferation.

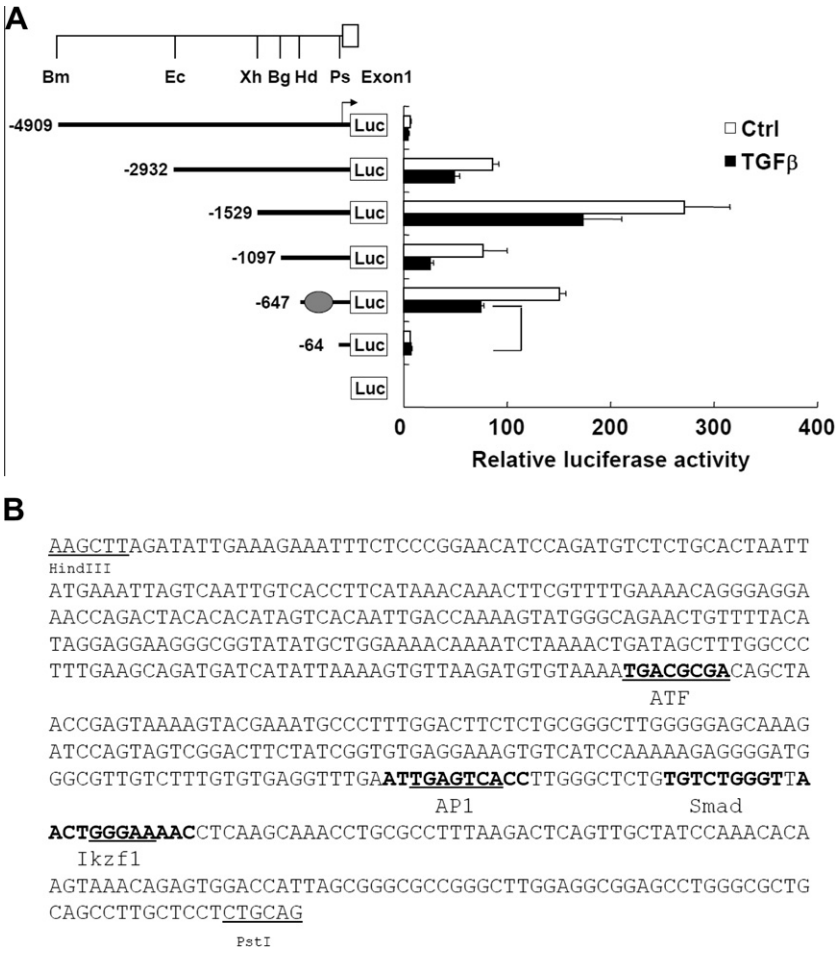

Fig. 3. Regulation of *Osr2* promoter by TGF-β. (A) The firefly luciferase that connected serial deleted *Osr2* promoter was transfected in fibroblastic C3H10T1/2 cells. After 24 h of transfection, TGF-β was added and treated for a further 24 h. Luciferase activity was measured and the value was standardized by the renilla luciferase ($N = 3$). TGF-β acted through the −647 and −64 regions of the *Osr2* promoter. (B) Sequence of −647 to −64 region of the *Osr2* promoter. The predicted binding sites for ATF2, AP1, Smad, and Ikzf1 were indicated.

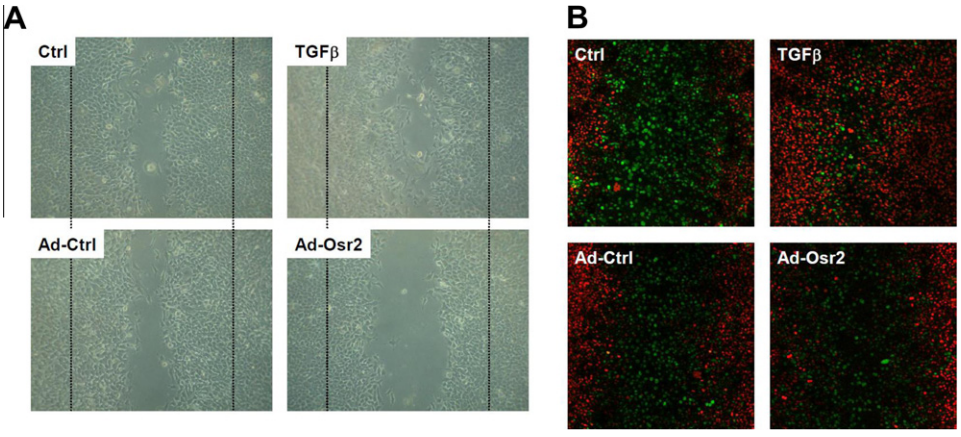

Fig. 4. Inhibition of cellular migration by *Osr2*. (A) The effect of migration by *Osr2* was analyzed on cell scratch assay in C3H10T1/2 cells. The *Osr2* adenovirus infected scratched C3H10T1/2 cells and cell migration was observed after 16 h by microscope. TGF-β enhanced cellular migration, but *Osr2* suppressed the migration. (B) The *Osr2* adenovirus infected scratched NMuMG-Fucci cells and cell migration was observed after 24 h by confocal microscope. TGF-β enhanced cellular migration and arrested cell cycle at G1, but *Osr2* suppressed the migration and accelerated cell cycle.

4. Discussion

A novel role of *Osr2* is shown to be regulation of cellular migration in downstream of TGF-β. The suppression of *Osr2* expression by TGF-β was mediated by Smad3/Smad4 and by p38/ATF2, and *Osr2* proximal promoter included Smad3/4 binding site and ATF2 binding site. It was further shown that *Osr2* suppressed cellular migration and accelerated cell cycle progression, whereas TGF-β

oppositely promoted migration and prevented cell cycle. One of functions of TGF-β is induction of migration/chemotaxis/chemokinesis [22]. We found that *Osr2* inhibited migration, suggesting that TGF-β elicits migration via repression of *Osr2* expression. The migration by TGF-β is indispensable to the process of invasion and metastasis of cancer [12]. This finding brings up a possibility that *Osr2* may have a function in invasion and metastasis of cancer. In agreement with this possibility, the expression of *Osr2* is dysregulated various types of tumors (Supplementary Fig. S4). TGF-β acts as a tumor suppressor in early tumor development and promotes cellular migration in later tumor stages [11]. *Osr2* may progress tumor proliferation in early stage, but may inhibit tumor invasion and metastasis in later stage.

TGF-β has bidirectional function for cellular proliferation, which depend on cell type and culture condition. We previously found that *Osr2* enhanced osteoblast proliferation [1] and regulated a group of genes in cell cycle as well as proliferation [14]. TGF-β arrested cell cycle at G1 in NMuMG cells, whereas *Osr2* progressed proliferation (Fig. 4B and Supplementary Fig. S3), suggesting that TGF-β provokes arrest of cell cycle via repression of *Osr2* expression. TGF-β inhibits cell cycle progression by preventing Rb1 (retinoblastoma 1) phosphorylation and S phase entry [13]. It suggests that *Osr2* associates with Rb1 or regulates phosphorylation state of Rb1 to activate E2F transcription factor. *Osr2* may interact with E2F to progress S phase entry.

Another function of TGF-β is induction of EMT. TGF-β decreased the expression of epithelial marker E-cadherin through Snails and Zebs transcription factors in epithelial cells and changed the phenotype of cells to mesenchyme (Supplementary Fig. S7). TGF-β down-regulated *Osr2* expression in both epithelial NMuMG cells (Supplementary Fig. S5) and mesenchymal C3H10T1/2 cells (Fig. 1B). The *Osr2* expression was higher in mesenchemal C3H10T1/2 cells than epithelial NMuMG cells (Supplementary Fig. S6) and *Osr2* preferentially expressed in mesenchyme in the embryonic development of mesonephros, mandible, maxilla, forelimb, hindlimb, nasal processes, eye, palatal shelves, tongue, esophagus, thyroid gland, and tooth buds [23]. Since TGF-β downstream Snails and Zebs were only slightly increased *Osr2* expression (Supplementary Fig. S7), Snails and Zebs seemed not to regulate *Osr2* expression. Smad3/4 and p38/ATF2 mainly regulate *Osr2* expression and Smad3/ATF2 probably form a transcriptional complex with other repressors such as Ski or Tgif1 [24] and represent a mechanism of gene repression. Although TGF-β decreased expression of *Osr2* and E-cadherin, their signal mediators were different. The relationship between *Osr2* and EMT is still unknown and should be further investigated.

We revealed that TGF-β was one of extracellular factors to regulate *Osr2* expression. The regulation of *Osr2* expression by TGF-β was mediated by Smad3/Smad4 and by p38/ATF2 signaling molecules. *Osr2* promoter (from −647 to −64) contained Smad3/4 binding site and ATF2 binding site of TGF-β. TGF-β induced cellular migration and arrested cell cycle, while *Osr2* diminished migration and stimulated cell cycle progression. We propose that TGF-β elicits migration and cell cycle arrest through repression of *Osr2* expression.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.04.064.

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