Expression of Krüppel-Like Factor 6, KLF6, in Rat Pituitary Stem/Progenitor Cells and Its Regulation of the PRRX2 Gene

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Abstract. Paired-related transcription factors, PRRX1 and PRRX2, which are present in mesenchymal tissues and participate in mesenchymal cell differentiation, were recently found in the stem/progenitor cells of the pituitary gland of ectodermal origin. To clarify the role of PRRX1 and PRRX2 in the pituitary gland, the present study first aimed to identify transcription factors that regulate Prrx1 and Prrx2 expression. A promoter assay for the upstream regions of both genes was performed by co-transfection of the expression vector of several transcription factors, many of which are frequently found in the pituitary stem/progenitor cells. The results for the promoter activity of both genes showed expression in a cell type-dependent manner. Comprehensive comparison of transcriptional activity of several transcription factors was performed with CHO cells, which do not show Prrx1 and Prrx2 expression, and the results revealed the presence of common and distinct factors for both genes. Among them, KLF6 showed specific and remarkable stimulation of Prrx2 expression. In vitro experiments using an electrophoretic mobility shift assay and siRNA interference revealed a potential ability for regulation of Prrx2 expression by KLF6. Finally, immunohistochemistry confirmed the presence of KLF6 in the SOX2/PRRX2 double-positive stem/progenitor cells of the postnatal pituitary gland. Thus, the finding of KLF6 might provide a novel clue to clarify the maintenance of stem/progenitor cells of the postnatal pituitary gland.

Key words: Differentiation, KLF6, Pituitary, PRRX2, Stem cell

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and PRRX2 are likely involved in ontogenesis in a similar and/or distinct manner, but much less is known about their roles in the pituitary originating from the embryonic ectoderm.

In this study, we first examined whether both Prrx1 and Prrx2 are regulated similarly or distinctively by many pituitary transcription factors and found that Krüppel-like factor 6 (KLF6), which plays a role in the regulation of cell proliferation, differentiation and development, is characteristically responsible for only Prrx2 expression. Then, we demonstrated that KLF6 plays a role in the regulation of Prrx2 by binding to the proximal 5' upstream region of Prrx2. Immunohistochemistry ultimately revealed that KLF6 is certainly present in PRRX2-positive pituitary stem/progenitor cells.

Materials and Methods

Animals and immunohistochemistry

Wistar-Imamichi and S100β-GFP transgenic Wistar-crlj strain (S100β-GFP) [20] rats were housed individually in a temperature-controlled room under a 12 h light/12 h dark cycle. The present study was approved by the Institutional Animal Care and Use Committee, Meiji University, based on the NIH Guidelines for the Care and Use of Laboratory Animals.

Frozen sections of rat pituitaries on embryonic day (E) 16.5 and postnatal day (P) 20–30 were prepared as described in a previous paper [10]. Immunohistochemistry was performed by reaction with antibodies for human SOX2, rat PRRX1 and rat PRRX2 as described previously [10] and with rat KLF6 (dilution 1:100 of sc-365633, Santa Cruz Biotechnology, Santa Cruz, CA, USA), followed by incubation with the corresponding fluorescence (Cy3, Cy5 and fluorescent isothiocyanate) conjugated secondary antibodies [10] and embedding in mounting medium containing 4', 6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA, USA). Immunofluorescence was observed with a BZ-9000 fluorescence microscope (Keyence, Osaka, Japan).

Transcription reporter assays

Upstream regions of the mouse Prrx1 (NC_000067.6) and Prrx2 (NC_000068.7) genes were amplified using primers (Table 1) and were ligated to the secreted alkaline phosphatase (SEAP) plasmid vector pSEAP2-Basic (BD Biosciences Clontech, Palo Alto, CA, USA). This resulted in reporter vectors: Prrx1 (−2297/+103), Prrx1 (−1400/+103), Prrx1 (−450/+103), Prrx2 (−5060/+21), Prrx2 (−3567/+21), Prrx2 (−1752/+21), Prrx2 (−1091/+21) and Prrx2 (−372/+21). Mouse Klf6 full-length open reading frame (NM_011803.2) was ligated in frame into the mammalian expression vector pcDNA3.1Zeo+ (Invitrogen, Carlsbad, CA, USA). In addition, the expression vectors of cDNAs coding several pituitary transcription factors, which were involved in differentiation, were also constructed in frame in the pcDNA3.1 vector (Table 2).

The culture and transfection conditions for CHO cells generated from Chinese hamster ovary and NIH3T3 cells (mouse fibroblast cell line), which were obtained from RIKEN Cell Bank, Ibaraki, Japan, and for TtT/GF cells (TtT/GF, kindly provided by Dr K Inoue) have been described previously [21, 22]. The TtT/GF cell line, which was generated from a mouse pituitary tumor and is known to express S100, a Ca2+-binding protein and marker for non-endocrine pituitary cells such as folliculo-stellate cells. After incubation, each 5 µl of cultured medium was assayed for secreted alkaline phosphatase activity as described previously [21].

Electrophoretic mobility shift assay (EMSA)

A FAM-labelled probe of the Prrx2 5’ upstream (−372/+21) region was made by PCR using a set of primers (FAM-labelled Prrx2-372 forward primer and Prrx2 reverse primer in Table 1) with the same conditions as described previously [23]. Bacterial recombinant KLF6 proteins (rec-KLF6) were expressed and purified as described previously [24]. The binding reaction was carried out in a mixture containing 100 fmol FAM-labelled double-stranded (ds) nucleotides, 500 ng rec-KLF6 and 1 µg ds-poly(dI:dc) in 10 µl of binding buffer (10 mM Heps buffer, pH 7.9, containing 0.4 mM MgCl2, 0.4 mM DTT, 50 mM NaCl, 0.1 mM ZnCl2, 0.1 mM spermidine and 4% (v/v) glycerol) with or without an unlabelled fragment (−372/+21) by incubation at 37°C for 30 min, followed by electrophoresis on a 4% polyacrylamide gel at 100 V for 60 min.

 Knockdown analysis of Klf6 mRNA and PCR analyses

Knockdown of Klf6-mRNAs was performed using siRNAs with the same conditions as described previously [11] using TtT/GF cells. siRNAs for Klf6 (MSS215606, MSS215607 and MSS215608) and control siRNA (12935-300) were purchased from Life Technologies (Carlsbad, CA, USA). Cell number was counted by staining of the nuclei with Diff-Quik (Sysmex, Kobe, Japan).

cDNAs were synthesized using total RNAs prepared from siRNA-treated TtT/GF cells, and quantitative real-time PCR was performed using a primer set for mouse Klf6 (NM_011803.2), 5’-ACCAGACACCTTCCGAAAGCA-3’ and 5’-TCTTAGCTGGAAGCTTCTTT-3’. The primer sets for mouse Prrx1, Prrx2 and TATA-box binding protein (Tbp) were the same as described previously [11]. Each datum measured by triplicate experiments was calculated by the comparative Ct method (DDCt method) to estimate the gene copy number relative to Tbp as an internal standard. The DNA sequence of the PCR product of each sample was confirmed by nucleotide sequence determination (data not shown).

Statistical analysis

Differences between two groups were subjected to the Student’s t-test, and differences among the above three groups were subjected to one-way ANOVA with Dunnett’s test. A P value less than 0.05 was considered statistically significant.

Results

Localization of PRRX1 and PRRX2 in the pituitary stem/progenitor cells

Immunohistochemistry of PRRX1 and PRRX2 was performed for embryonic (E16.5) and postnatal (P30) pituitaries. Double immunostaining with SOX2 and PRRX1 showed that PRRX1 was present in some of the SOX2-positive cells of both sections at E16.5 and P30, while cells positive for PRRX1 only were present in the periphery of the pituitary on E16.5 (Fig. 1A). On the other
hand, PRRX2 was not present in the embryonic pituitary but rather was present in the SOX2-positive cells of the anterior lobe of the postnatal pituitary on P30 (Fig. 1B).

Transcriptional activities of the 5’ upstream region of Prrx1 and Prrx2

Reporter vectors harboring the 5’ upstream regions of Prrx1 (–2297 b) and Prrx2 (–5060 b) together with their truncated regions were assayed for their transcriptional activity by transfection into three cell lines, CHO, NIH3T3 and TtT/GF. In the CHO cells, the promoter activities of the upstream regions of Prrx1 and Prrx2 decreased along with the increased upstream length (Fig. 2A and B). On the other hand, in the NIH3T3 and TtT/GF cells, the promoter activities were higher than those of the pSEAP-basic vector used as a control. The regions up to –450 b of Prrx1 and –372 b of Prrx2, respectively, showed significantly high promoter activity, indicating the presence of transcriptional enhancer elements in these regions.

| Table 1. List of primers used for construction of the 5’ upstream region of Prrx1 and Prrx2 |
|---------------------------------------------------------------|
| **Prrx1**                                                     |
| Forward primer                                               |
| –2297 5'-aatacgctTCTAGAACAATGGGGAG-3’                         |
| –1400 5'-aatacgctAGTGCTGGAGCGAGCCG-3’                         |
| –1140 5'-aatacgctTCTCATAGCTACAGGAGAG-3’                       |
| –450 5'-aatacgctTCTCCGCCAAAACAAGACGCT-3’                      |
| Reverse primer                                               |
| +103 5'-accacatgegaTAATAGGAGGCTGTA-3’                         |
|**Prrx2**                                                     |
| Forward primer                                               |
| –5060 5'-aatacgctAGAGAGATTTGTGAGCTCGT-3’                       |
| –3567 5'-aatacgctGGCATGCTGGAGCGAGCCG-3’                       |
| –1752 5'-aatacgctACACACCAAGAGGGCGCACT-3’                      |
| –1091 5'-aatacgctAGGTCCTGGACCTACCTGGTT-3’                     |
| –372 5'-aatacgctCAATTCGAGGCTAATCTGC-3’                        |
| Reverse primer                                               |
| +21 5'-accacatgegaGTGCCCGAGTCTCAAGTGCAGT-3’                   |

The uppercase and lowercase letters indicate the sequence of the gene to be amplified and adaptor containing the recognition sequence for restriction enzymes (MulI for the forward primer and NruI for the reverse primer, respectively).

| Table 2. List of primer sets to amplify full-length cDNA       |
|---------------------------------------------------------------|
| **Factor** | **Species** | **Accession Number** | **Forward primer** | **Reverse primer** |
| Prop1    | Rat         | NM_153627.1          | CGGAATTCGCCATGCGGTCACTACTCCAGCCA | CCGGCTCGAGTATCTTGGTCTCGTGGCTTAGGCC |
| Prrx1    | Rat         | NM_00105739.1        | CGGAATTCGCCATGCGGTCACTACTCCAGCCA | CCGGCTCGAGTATCTTGGTCTCGTGGCTTAGGCC |
| Prrx2    | Rat         | NM_00109181.1        | CGGAATTCGCCATGCGGTCACTACTCCAGCCA | CCGGCTCGAGTATCTTGGTCTCGTGGCTTAGGCC |
| SOX2     | Rat         | NM_00107103          | AGAGAATTCGCCATGCGGTCACTACTCCAGCCA | CCGGCTCGAGTATCTTGGTCTCGTGGCTTAGGCC |
| Sox2     | Rat         | NM_001085991.1       | AGAGAATTCGCCATGCGGTCACTACTCCAGCCA | CCGGCTCGAGTATCTTGGTCTCGTGGCTTAGGCC |
| Isl1     | Mouse       | NM_00124194.1        | AGAGAATTCGCCATGCGGTCACTACTCCAGCCA | CCGGCTCGAGTATCTTGGTCTCGTGGCTTAGGCC |
| Oct2     | Mouse       | NM_001286401.1       | AGAGAATTCGCCATGCGGTCACTACTCCAGCCA | CCGGCTCGAGTATCTTGGTCTCGTGGCTTAGGCC |
| Pax6     | Mouse       | NM_00124198.1        | AGAGAATTCGCCATGCGGTCACTACTCCAGCCA | CCGGCTCGAGTATCTTGGTCTCGTGGCTTAGGCC |
| Fosj1    | Mouse       | NM_00110702          | AGAGAATTCGCCATGCGGTCACTACTCCAGCCA | CCGGCTCGAGTATCTTGGTCTCGTGGCTTAGGCC |
| Hey1     | Mouse       | NM_010423.2          | AGAGAATTCGCCATGCGGTCACTACTCCAGCCA | CCGGCTCGAGTATCTTGGTCTCGTGGCTTAGGCC |
| Hey2     | Mouse       | NM_013904.1          | AGAGAATTCGCCATGCGGTCACTACTCCAGCCA | CCGGCTCGAGTATCTTGGTCTCGTGGCTTAGGCC |
| Id3      | Mouse       | NM_008321.2          | AGAGAATTCGCCATGCGGTCACTACTCCAGCCA | CCGGCTCGAGTATCTTGGTCTCGTGGCTTAGGCC |
| Kif6     | Mouse       | NM_0011083.2         | AGAGAATTCGCCATGCGGTCACTACTCCAGCCA | CCGGCTCGAGTATCTTGGTCTCGTGGCTTAGGCC |
| Nkb      | Mouse       | NM_008668.6          | AGAGAATTCGCCATGCGGTCACTACTCCAGCCA | CCGGCTCGAGTATCTTGGTCTCGTGGCTTAGGCC |
| Nnk      | Mouse       | NM_001291.2          | AGAGAATTCGCCATGCGGTCACTACTCCAGCCA | CCGGCTCGAGTATCTTGGTCTCGTGGCTTAGGCC |
| Runx1    | Mouse       | NM_00111021.2        | AGAGAATTCGCCATGCGGTCACTACTCCAGCCA | CCGGCTCGAGTATCTTGGTCTCGTGGCTTAGGCC |
| Sp1*     | Human       | BC062359.1           | AGAGAATTCGCCATGCGGTCACTACTCCAGCCA | CCGGCTCGAGTATCTTGGTCTCGTGGCTTAGGCC |
| Tal1     | Mouse       | NM_0011527.3         | AGAGAATTCGCCATGCGGTCACTACTCCAGCCA | CCGGCTCGAGTATCTTGGTCTCGTGGCTTAGGCC |
| Teif2    | Mouse       | NM_00114298.1        | AGAGAATTCGCCATGCGGTCACTACTCCAGCCA | CCGGCTCGAGTATCTTGGTCTCGTGGCTTAGGCC |
| Taf1     | Mouse       | NM_001285498.1       | AGAGAATTCGCCATGCGGTCACTACTCCAGCCA | CCGGCTCGAGTATCTTGGTCTCGTGGCTTAGGCC |
| Zipbl    | Mouse       | NM_0011756.4         | AGAGAATTCGCCATGCGGTCACTACTCCAGCCA | CCGGCTCGAGTATCTTGGTCTCGTGGCTTAGGCC |
| Zipbl1   | Mouse       | NM_007564.5          | AGAGAATTCGCCATGCGGTCACTACTCCAGCCA | CCGGCTCGAGTATCTTGGTCTCGTGGCTTAGGCC |

* The human SP1 cDNA clone was kindly provided by Dr. R. Tjian of the University of California at Berkeley.
of basic promoter elements in the proximal region. Notably, the promoter activity of Prrx1 in the NIH3T3 cells did not show much alteration over –450 b, while that in the TtT/GF cells showed a different response depending on the upstream length (Fig. 2A). On the other hand, the promoter activity of Prrx2 showed an increase in the NIH3T3 cells and a decrease in the TtT/GF cells with different responses depending on the upstream length (Fig. 2B). These data indicate that Prrx2 expression is cell-type dependent with unique regulatory factors. We subsequently examined expression of Prrx2 and observed that the expression level of Prrx1 and Prrx2 by RT-PCR and confirmed that NIH3T3 and TtT/GF cells express both genes but that CHO does not (Fig. 2C).

Next, we performed co-transfection with several transcription factors sorted by their characteristic expression and/or function for organogenesis from a microarray analyses of RNAs prepared from the rat pituitary on E15.5 and pituitary S100β-positive pituitary cells. Immunohistochemistry for PrrxXs (A, PRRX1; B, PRRX2) and SOX2 was performed using frozen sections of rat pituitary at embryonic day 16.5 (E16.5) and postnatal day 30 (P30). Areas of PrrxXs and SOX2 in open boxes, which were visualized with Cy3 (red) and fluorescein isothiocyanate (green), were enlarged as shown below together with the merged image. The arrow and dotted line indicate cells double positive for PRRXs and SOX2 as shown below together with the merged image. The arrow and dotted line indicate cells double positive for PRRXs and SOX2 in open boxes, which were visualized with Cy3 (red) and fluorescein isothiocyanate (green), were enlarged as shown below together with the merged image. The arrow and dotted line indicate cells double positive for PRRXs and SOX2 in open boxes, which were visualized with Cy3 (red) and fluorescein isothiocyanate (green), were enlarged as shown below together with the merged image.

**Fig. 1.** Localization of PRRX1 and PRRX2 in pituitary stem/progenitor cells. Immunohistochemistry for PRRXs (A, PRRX1; B, PRRX2) and SOX2 was performed using frozen sections of rat pituitary at embryonic day 16.5 (E16.5) and postnatal day 30 (P30). Areas of PRRXs and SOX2 in open boxes, which were visualized with Cy3 (red) and fluorescein isothiocyanate (green), were enlarged as shown below together with the merged image. The arrow and dotted line indicate cells double positive for PRRXs and SOX2 and the marginal cell layer, respectively. AL, anterior lobe; IL, intermediate lobe; PL, posterior lobe; RC, Rathke’s cleft. Scale bars: 50 µm and 10 µm (enlarged images).

Finally, to verify whether KLF6 is present in the pituitary stem/progenitor cells, triple-immunostaining of KLF6, SOX2 and PRRX2 of an S100β-GFP rat (unpublished data). The SEAP activities for Prrx1 (~2297/+103), Prrx1 (~450/+103), Prrx2 (~5060/+21) and Prrx2 (~372/+21) were then assayed (Table 3). The results showed that transcription of Prrx1 is regulated repressively by FOXJ1, HEY1, NF1B and ZFP36 by less than 0.6-fold and stimulated by SOX2, LHX2, ISL1 and PAX6 by more than 1.9-fold. Transcription of Prrx2 was repressed by HEY1, HEY2, PROP1, OTX2, ID3, ZFP36 and ZFP36L1 by less than 0.6-fold and stimulated by PRRX2, SOX2, PAX6 and KLF6 by more than 1.9-fold. Among factors affecting commonly or distinctively both gene expressions, it was interesting that PRRX2 stimulated its own gene expression and that SOX2 had opposite effects for two genes. Thus, several transcription factors that participate in pituitary organogenesis have regulatory potency with respect to Prrx1 and Prrx2 expression. We were ultimately interested in KLF6, since it potently stimulated only Prrx2 and its role is little noticed in pituitary development. To verify evidence of correlation between PRRX2 and KLF6, we performed the following experiments.

**Binding of KLF6 on Prrx2 promoter region**

Since reporter assays showed a specific regulation of KLF6 for the Prrx2 promoter, we surveyed the consensus recognition sequence of KLF6, CCNCNCCC including CACCC and GC-rich elements. As shown in Fig. 3A, the putative binding site for KLF6 was found in 8 positions in the 372 base length of the proximal upstream region. To confirm the KLF6 binding to the upstream region of Prrx2, EMSA was performed using a FAM labelled probe (~372/+21). The mixture of probe and protein gave broad shift bands with high molecular sizes in contrast to the high mobility band obtained with the probe alone (Fig. 3B), showing a potential ability of KLF6 with respect to Prrx2 regulation. When unlabelled competitors were added in increasing molar amounts, the molecular sizes and intensities of the shift bands decreased and those of the bands of the probe increased. Addition of an 80-molar excess amount caused most of the shift band to fade, indicating decomposition of the specific binding between KLF6 and the probe.

**Knockdown with Klf6 siRNA**

Knockdown of Klf6 mRNA using siRNAs in TtT/GF cells, which expresses Klf6 (Fig. 2C), was performed to confirm whether KLF6 modulates expression of Prrx2 and Prrx1. Cell proliferation was decreased by about 50% at 72 h after siRNA transfection (Fig. 4A). Microscopy after staining nuclei with Diff-Quik showed a decrease in cell density for the Klf6 siRNA-transfected cells in comparison with the control. In addition, we observed that siRNA treatment resulted in enlargement of nuclei and cytoplasm and a decreased number of cells (insets in Fig. 4B).

Quantitative real-time PCR of RNAs from cells confirmed knockdown of the Klf6 mRNA level by transfection of Klf6 siRNAs at about 90% efficiency (Fig. 4C). Then we measured the expression levels of Prrx1 and Prrx2 and observed that the expression level of Prrx1 did not change, whereas that of Prrx2 decreased significantly by about 55% (Fig. 4C), showing a Klf6 siRNA-dependent repression of Prrx2 expression.

**Immunohistochemistry of KLF6**

Finally, to verify whether KLF6 is present in the pituitary stem/progenitor cells, triple-immunostaining of KLF6, SOX2 and PRRX2
KLF6 was found in the cells lining the marginal cell layer (MCL) of the anterior and intermediate lobes (Fig. 5A and B). Although KLF6-negative cells were found among PRRX2/SOX2 double-positive cells (Fig. 5A arrowhead), immunohistochemistry confirmed that all positive signals of KLF6 were observed in the PRRX2/SOX2 double-positive cells of pituitary stem/progenitor cells.

**Discussion**

The present study examined the transcriptional activity of several transcription factors for Prrx1 and Prrx2, which are present in pituitary stem/progenitor cells [10], and revealed that Prrx1 and Prrx2 expression are regulated stimulative or repressive with common factors, such as PAX6, FOXJ1, HEY1, TEAD2 and ZFP36; by contrast, Prrx1 is distinctly regulated by SOX2, LHX2 and ISL1, and Prrx2 is distinctly regulated by PRRX2, HEY2, ID3, KLF6 and TCF7L2, respectively, which are known to play crucial roles in pituitary stem/progenitor cells and organogenesis [1, 5, 6, 25]. We focused attention on KLF6, which participates in modulation of Prrx2 expression, using in vitro experiments and demonstrated by immunohistochemistry that KLF6 is specifically present in the PRRX2-positive stem/progenitor cells of the anterior lobe of the rat postnatal pituitary gland. The results provide important clues to uncovering the adult pituitary stem/progenitor cells at the molecular level.

In the present study, we confirmed the distinct temporospatial localizations of PRRX1 and PRRX2 in the embryonic and postnatal pituitaries. Recently, we observed the appearance of PRRX1 and PRRX2 in postnatal SOX2-positive cells but not postnatal PROP1-negative cells present in a stem cell niche of the pituitary, the marginal cell layer [13]. Interestingly, PRRX2 is not present in the embryonic pituitary. Our data showing that KLF6 co-localized with PRRX2 in cells of the marginal cell layer at the postnatal stage suggest that KLF6 might regulate Prrx2 expression in an adult pituitary stem cell.

KLF6 is a member of the Krüppel-like factors with three zinc fingers characteristically in its carboxyl region. The family consists of 17 proteins, 9 of which are included the SPI superfamily [26]. KLF6 is known to play a role in differentiation and development of tissues, in addition to its role as a tumor suppressor gene [26]. Klf6 (Klf6−/−) knockout mice died by E12.5 and were associated with markedly reduced hematopoiesis and poorly organized yolk sac vascularization [27]. They generated Klf6−/− embryonic stem (ES) cells and demonstrated that Klf6−/− ES cells have significant hematopoietic
defects associated with delayed expression of differentiation markers, 
*Brachyury*, Klf1 and Gata1, followed by differentiation into embryoid 
bodies (EBs), and that forced expression of KLF6 enhances the 
 hematopoietic potential of wild-type EBs, implicating the role of 
Klf6 in ES-cell differentiation and hematopoiesis. Interestingly, 
the role of KLF6 was demonstrated by knockdown analysis, and 
it was found that this protein is likely involved in phosphorylation 
of retinoblastoma protein along with upregulation of cyclin D1 and 
cdk4, which are required for the cell cycle G1/S transition [28] 
however, cyclin D1 and cdk4 were not examined in this study. These 
data indicate that KLF6 plays roles in differentiation and develop-
tment through cell cycle progression [26]. Taken together with the 
accumulated reports, our finding that KLF6 is present in pituitary 
stem/progenitor cells indicates its important role in maintaining 
stemness and/or progression of differentiation.

This study showed the remarkable regulatory activity of KLF6 
on the promoter of *Prrx2*. Presumably, the presence of putative 
KLF-binding sites at 8 positions in the proximal region within the 
372 b upstream might serve to massively bind and activate the 
expression of a reporter gene. Notably, it has been reported that 
KLF6 has the ability to interact with other transcription factors, 
such as Sp1, KLF4, p53 and aryl hydrocarbon receptor, in a cell 
type- and/or tissue-dependent manner [29–31]. We observed that 
expression of Sp1 is higher than that of Klf6 in the pituitary (data 
not shown), making it thereby sufficient for *Prrx2* to be regulated 
with a KLF6-SP1 complex. However, SP1-dependent activation 
between KLF6 and SP1 was additive but not synergistic with respect 
to *Prrx2* expression (Table 1). Assay of the promoter activity in the 
three cell lines showed that *Prrx2* as well as *Prrx1* is responsive with 
cell type- and the promoter length-dependent. Therefore, a further 
search for cooperative factors in pituitary stem/progenitor cells is 
important to clarify the function of KLF6.

EMSA showed an unexpected smear pattern with high molecular 
complexes between the labelled probe and KLF6, the specific 
binding of which was verified by addition of a non-labelled probe. 
However, the reason why the complex showed the smear pattern 
remains unclear. Previously, we observed that LIM-homeodomain 
factor resulted in a large molecular size for the complex [32]. In that 
case, the LIM domain formed protein-protein interactions, resulting 
in catenation of protein-DNA complexes. However, there is no report
Fig. 3. Electrophoretic mobility shift assay (EMSA) for KLF6. (A) A diagram of the 5’ upstream (−372/+21) region of Prrx2, which was used as a binding probe, is shown. A putative binding site (CCNCNCCN including GC element and CACCC) of KLF6 is shown with a closed ellipse. (B) EMSA was performed using a 100 fmol FAM-labelled fragment (−372/+21) without and with a 5–80 molar excess amount of non-labelled fragment (−372/+21) as a competitor to confirm the specific DNA/protein complex. Electrophoresis of the FAM-labelled fragment (probe) alone is shown at the left of the panel.

Fig. 4. Knockdown analysis of the Klf6 mRNA level using siRNA. A: The Klf6 mRNA level was knocked down for TtT/GF cells by transfection of Klf6 siRNAs, and cell proliferation was measured. The respective cell numbers of the control (open boxes) and siRNA-transfected cells (closed boxes) were measured. Data are shown as means ± SD for two independent experiments. B: Microscopy observation of cells after 72 h of culture. Upper and lower panels show photographs of the control and siRNA-transfected cells, respectively. An enlarged image of the boxed area is shown at the upper right. Scale bars: 100 μm. C: Real-time PCR was performed for Klf6 (left panel), Prrx1 (middle panel) and Prrx2 (right panel) using total RNAs prepared from cells cultured for 24 h after transfection. All quantified data are shown as the relative expression level against that of TATA-box binding protein (Tbp). Open and closed bars indicate data of cells transfected with control siRNA and Klf6 siRNA, respectively. Asterisks indicate statistical significance by Student’s t-test (**P<0.01).

Fig. 5. Immunohistochemistry of KLF6 together with PRRX2 and SOX2. Immunohistochemistry for KLF6 was performed using frozen sections of rat pituitary at P20. Note that immunohistochemical images of KLF6, PRRX2 and SOX2 were visualized with Cy3 (red), Cy5 (purple) and fluorescein isothiocyanate (green) and counterstained with DAPI (blue). Dotted lines indicate the marginal cell layer (MCL). AL, anterior lobe; IL, intermediate lobe; RC, Rathke’s cleft. Scale bars: 10 μm.
of a protein-protein interaction domain in KLF6. On the other hand, KLF6 has three C2H2-type Zn-fingers, which bound on CACCC and GC-elements. In addition, there are 8 binding sequences for KLF6 in the probe used. Thus, binding of 8 KLF6 molecules to the probe may result in a large molecular complex. An alternative explanation is that the binding between the three zinc fingers and 8 binding sequences for KLF6 to the probe DNA and/or by catenation of the probe DNA through the three zinc fingers. At least, this massive KLF6 binding may induce marked activation of Prrx2 expression.

In summary, the present study demonstrated that the pituitary transcription factors PRRX1 and PRRX2 present in stem/progenitor cells are regulated by many transcription factors participating in pituitary organogenesis. Particularly, KLF6 was identified as stimulating distinctively Prrx2 expression but not Prrx1 expression. In addition, in vivo evidence of the localization of KLF6 and Prrx2 in the stem/progenitor cells of the pituitary gland was demonstrated. This KLF6 finding provides a novel clue to resolve the role of the pituitary stem/progenitor cells and maintenance of this tissue.

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