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—Original Article—

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

Hiroki UEHARU[1)]*, Masashi HIGUCHI[2)]*, Naoto NISHIMURA[1)], Saishu YOSHIDA[1)], Shiori SHIBUYA[1)], Kenta SENSUI[3)], Takako KATO[2)] and Yukio KATO[1–3)]

[1)]*Laboratory of Molecular Biology and Gene Regulation, Division of Life Science, Graduate School of Agriculture, Meiji University, Kanagawa 214-8571, Japan*
[2)]*Institute of Reproduction and Endocrinology, Meiji University, Kanagawa 214-8571, Japan*
[3)]*School of Agriculture, Meiji University, Kanagawa 214-8571, Japan*

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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The adenohypophysis (anterior and intermediate lobes), which develops from the oral ectoderm by an invagination at the early stage of the embryo, synthesizes and secretes various hormones to perform essential roles such as in growth, metabolism, reproduction, lactation, homeostasis and stress response. A number of transcription factors spatiotemporally appear and promote cell differentiation during pituitary organogenesis to allow production of each hormone in the respective specific cell that produces the hormones [1–6]. Stem/progenitor cells are crucially responsive for tissue maintenance as the cell resource of hormone-producing cells and nonhormone-producing cells, such as folliculo-stellate cells ([7] and references therein). We previously reported that the stem/progenitor marker SOX2 is expressed in all cells of the presumptive pituitary tissue and that the pituitary-specific transcription factor PROP1 starts to appear in SOX2-positive cells at rat embryonic day (E) 11.5, followed by occupation by all cells of the primordium Rathke's pouch at E13.5 [8, 9]. Hence, it follows that all of the pituitary cells once received the effects of PROP1. On the other hand, we most recently demonstrated that paired-related homeobox transcription factor PRRX1 (same as Mhox1, PRX1, PHOX1, PMX1 and K2) is expressed in PROP1/SOX2 double-positive cells and replaced with PROP1 during pituitary development [10–12]. PRRX2 (same as S8, PRX2, PMX2), a cognate factor of PRRX1, was identified in postnatal SOX2-positive cells, emerging behind *Prrx1* expression [13], indicating that the two cognate factors play their roles distinctively in the diverse stem/progenitor cells. Investigation of the function of the novel transcription factors PRRX1 and PRRX2 invariably served to unmask the pituitary stem/progenitor cells at the molecular level.

In previous studies, *Prrx1* and *Prrx2* were cloned as a binding protein for AT-rich elements in the muscle creatine kinase enhancer [14] and as a homeobox gene from the mouse adult spleen cDNA library [15], respectively. Double mutants of *Prrx1/Prrx2* revealed important roles for both gene products in craniofacial and limb morphogenesis [16–18]. *Prrx1* and *Prrx2* transcripts were largely observed to have overlapping expression profiles in the derivatives from the lateral plate mesoderm and in the neural crest-derived mesenchyme, which is where epithelio-mesenchymal interactions occur [16]. Interestingly, the *Prrx2* transcripts were first detected in the extraembryonic mesoderm at the primitive streak stage of the embryo, followed by strong expression in the initially formed intraembryonic mesoderm at the presomite stage [19]. Thus, PRRX1

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Correspondence: Y Kato (e-mail: yukato@isc.meiji.ac.jp)
* H Ueharu and M Higuchi contributed equally to this work.

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).

Transfection 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* (–1140/+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) or pET32a (Novagen, Madison, WI, 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 Ca^{2+}-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(dIdC) in 10 μl of binding buffer (10 mM Hepes buffer, pH 7.9, containing 0.4 mM $MgCl_2$, 0.4 mM DTT, 50 mM NaCl, 0.1 mM $ZnCl_2$, 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'-ACCAGACACTTCCGAAAGCA-3' and 5'-TCTTAGCCTGGAAGCCTCTTT-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 C_T method (DDC_T 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 ANOVAs 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

Table 1. List of primers used for construction of the 5' upstream region of *Prrx1* and *Prrx2*

Prrx1		
Forward primer	–2297	5'-aatacgcgtTCTAGAACAATGGGGGAG-3'
	–1400	5'-aatacgcgtAGTGGCTGGAGCGAGGCG-3'
	–1140	5'-aatacgcgtTCTCATAGCTACAGGAGAAG-3'
	–450	5'-aatacgcgtTCTCCGCCAAAACAAAGCTG-3'
Reverse primer	+103	5'-acacactcgcgaTCCACTTAATAGGAGCCTGTA-3'
Prrx2		
Forward primer	–5060	5'-aatacgcgtAGGAGGATTTGTGTGGCTTG-3'
	–3567	5'-aatacgcgtGGGCACCAGGAACATTTGTA-3'
	–1752	5'-aatacgcgtACACACCAGAAGAGGGCATC-3'
	–1091	5'-aatacgcgtAGGCTCTGGACCTCACTTTG-3'
	–372	5'-aatacgcgtCAAATTCGAGGCTAATCTGC-3'
Reverse primer	+21	5'-acacactcgcgaGTGCCGGATCTCAAGTCAGT-3'

The uppercase and lowercase letters indicate the sequence of the gene to be amplified and adaptor containing the recognition sequence for restriction enzymes (*Mul* I for the forward primer and *Nru* I 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	CCGGCTCGAGTTACTCTTGCTTCCGTTGCTTAGCC
Prrx1	Rat	NM_153821.1	GAGAATTCGCGATGTTTTATTTCCACTG	GGAGCTCGAGTTCATCCTTGTGTCATATC
Prrx2	Rat	NM_001105739.1	CCGGGAATTCGGCATGGACAGCGCGGCCGCCGCCTT	CCGGCTCGAGTGTCAGTTCACTGTGGGCACC
SOX2	Rat	NM_001109181.1	GGAGAATTCGCCATGTATAACATGATGGAGACG	GGACTCGAGTCACATGTGCGACAGGGG
Lhx2	Mouse	NM_010710.3	AGAGAGAATTCGCGATGCTGTTCCACAGTC	ACACACTCGAGTTGGGGGGTGGCGAGTC
Lhx3	Rat	XM_001059910	GGAATTCGCCATGGAAGCTCGCGGGGAGCT	TCTCTCGAGTCAGAACTGAGCATGGTCTAC
Isl1	Mouse	NM_021459.4	AGAGACAATTGGATATGGGAGACATGGGCGA	TGTGTCTCGAGCCTCATGCCTCAATAGGACTGG
Otx2	Mouse	NM_001286481.1	AGAGAGAATTCAGCATGATGTCTTATCTAAAGCAACCGC	TGTGTCTCGAGACAGGTCTTCACAAAACCTGGAA
Pax6	Mouse	NM_001244198.1	GACAATTGAGCATGCAGAACAGTCACAGC	ACGCTCGAGCTCTCTCCTTCTCTCTTTA
Foxj1	Mouse	NM_008240.3	GACAATTGGACATGGCGGAGAGCTGGCTG	ACGCTCGAGACCTTTTACAAGAAGGCAC
Hey1	Mouse	NM_010423.2	GAGAATTCACCATGAAGAGAGCTCACCCAG	ACGCTCGAGCATCAGTTCTTTAGAAAGC
Hey2	Mouse	NM_013904.1	GAGAATTCGCGATGAAGCGCCCTTGTGAGG	ACGCTCGAGATTCAAGAATAAGTTAAAAG
Id3	Mouse	NM_008321.2	AGAGAGAATTCAACATGAAGGCGCTGAGCCC	TGTGTCTCGAGTTCGGGAGGTGCCAGGACGAC
Klf6	Mouse	NM_011803.2	GAGAATTCAGCATGAAACTTTCACCTGCG	ACGCTCGAGCCTACAGGATTCGTCCCTC
Nfib	Mouse	NM_008687.6	GAGAATTCGTCATGATGTATTCTCCCATCTGTCTCACTCAGGATGAG	GCGTCGACCAAGCTAGCCCAGGTACC
Nkx3-1	Mouse	NM_010921.3	AGAGAGAATTCGGGATGCTTAGGGTAGCGG	TGTGTCTCGAGTTGCTACCAGAAAGATGGATGC
Runx1	Mouse	NM_001111021.2	GAGAATTCAAGAGTGCGAGTCTGCCTGTG	ACGCTCGAGTCCCTCGATGGCGATGGCGC
*Sp1**	Human	BC062539.1	GAGAATTCACCATGAGCGACCAAGATCACT	TCCTCGAGCTACCTTGCATCCCGGG
Tal1	Mouse	NM_011527.3	AGAGAGAATTCACGATGACGGAGCGGCCGCCGAGC	TGTGTCTCGAGTCACATAAGTCCATTGACCTGCTT
Tcf7l2	Mouse	NM_001142918.1	AGAGAGAATTCAAAATGCCGCAGCTGAACGGC	TGTGTCTCGAGCCTAGCAGATGCGGTG
Tead2	Mouse	NM_001285498.1	GAGAATTCAAGATGGGGGATCCCCGGACTG	ACGCTCGAGCTCCATCAGTCCCTGACCAGG
Zfp36	Mouse	NM_011756.4	GAGAATTCACCATGGATCTCTCTGCCATC	ACGCTCGAGGCACTTGTCACTCAGAGAC
Zfp36l1	Mouse	NM_007564.5	GAGAATTCAGCATGATCCGCGGCGCCCCGGCAC	ACGCTCGAAGTCAGTGTGAGGGCTACTGGCGGAG

* The human SP1 cDNA clone was kindly provided by Dr. R. Tijan of the University of California at Berkeley.

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

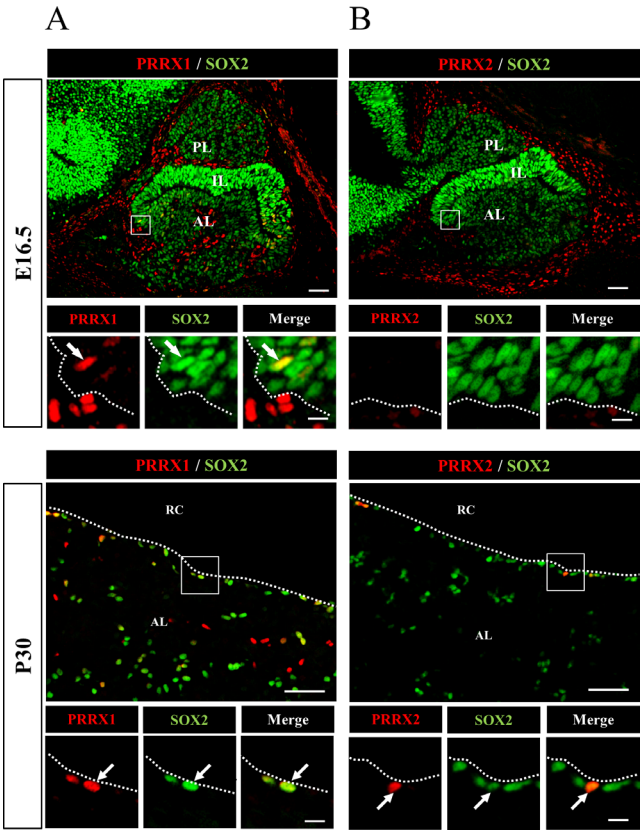

Fig. 1. Localization of PRRX1 and PRRX2 in pituitary stem/progenitor cells. Immunohistochemistry for PRRXs (A, PRRX1; B, PRRX2) and SOX2 was performed using frozen sections of rat pituitary at embryonic day 16.5 (E16.5) and postnatal day 30 (P30). Areas of PRRXs and SOX2 in open boxes, which were visualized 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).

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 *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 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 *Prrx*2 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 *Prrx*2 expression.

Immunohistochemistry of KLF6

Finally, to verify whether KLF6 is present in the pituitary stem/progenitor cells, triple-immunostaining of KLF6, SOX2 and PRRX2

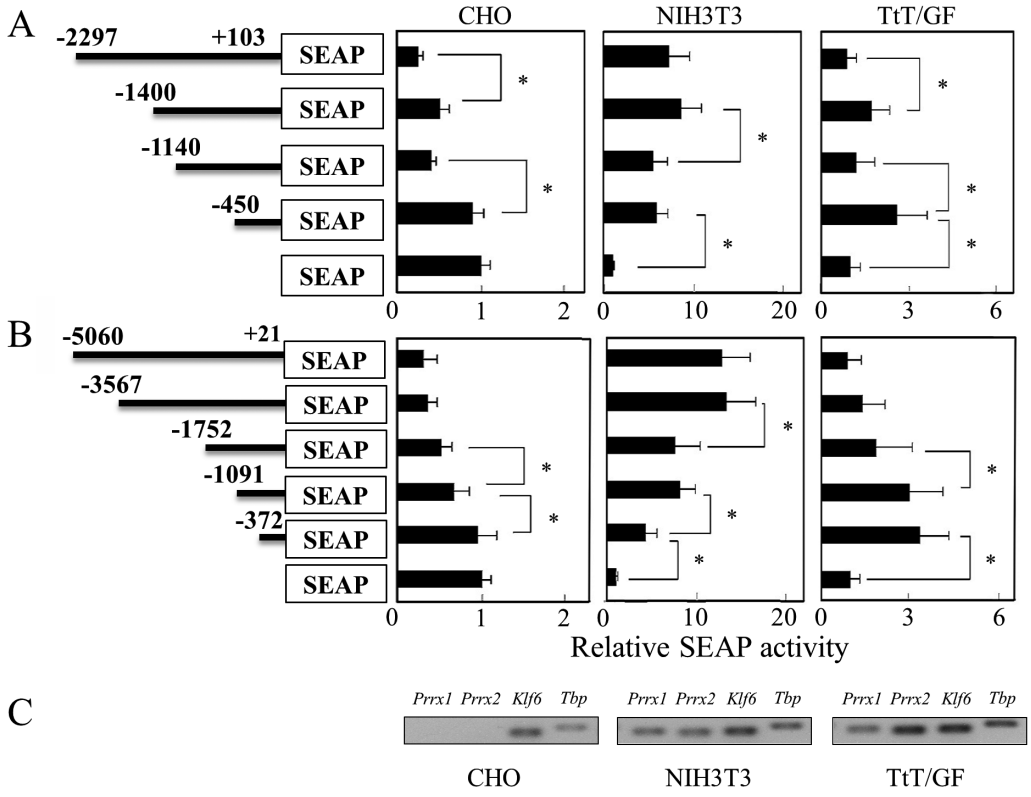

Fig. 2. Transient transfection assay of mouse *Prrx1* and *Prrx2* promoters. Reporter vectors of *Prrx1* (A) and *Prrx2* (B) were transfected into CHO, NIH3T3 and TtT/GF cells. An aliquot of cultured medium was used for the SEAP assay. Reporter gene activities are indicated relative to that of the pcDNA3.1 vector. Representative data (mean ± SD) are shown from means of quadruplicate transfections from two independent experiments. Asterisks indicate statistical significance by one-way ANOVAs with Dunnett's test (*P<0.05). (C) RT-PCR was performed for *Prrx1*, *Prrx2*, *Klf6* and *Tbp* (TATA-box binding protein).

was performed for the adult rat pituitary (P30). 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 SP1 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

Table 3. Transcriptional activity of the 5' upstream region of *Prrx1* and *Prrx2* in the presence of transcription factors

	Prrx1		*Prrx2*	
	–2297	–450	–5060	–372
PROP1	1.29 ± 0.20	0.87 ± 0.15	0.23 ± 0.29**	0.42 ± 0.23**
PRRX1	0.80 ± 0.20	0.53 ± 0.17**	0.90 ± 0.17	0.51 ± 0.20**
PRRX2	1.75 ± 0.21**	0.94 ± 0.26	4.11 ± 0.22**	0.79 ± 0.10
SOX2	3.31 ± 0.13**	0.89 ± 0.18	0.39 ± 0.10	0.46 ± 0.20**
LHX2	1.74 ± 0.17*	1.31 ± 0.17	1.08 ± 0.22	0.93 ± 0.11
LHX3	1.41 ± 0.21	1.54 ± 0.26*	0.62 ± 0.18**	1.44 ± 0.24
ISL1	3.94 ± 0.16**	1.67 ± 0.02**	1.01 ± 0.26	1.12 ± 0.11
OTX2	1.73 ± 0.11**	1.41 ± 0.16*	0.49 ± 0.04**	0.60 ± 0.29*
PAX6	2.21 ± 0.17**	1.39 ± 0.14	2.72 ± 0.12**	1.40 ± 0.02**
FOXJ1	0.27 ± 0.11**	0.61 ± 0.06**	0.16 ± 0.10**	0.53 ± 0.05**
HEY1	0.52 ± 0.16**	0.59 ± 0.10**	0.06 ± 0.16**	0.39 ± 0.08**
HEY2	0.52 ± 0.08**	0.94 ± 0.11	0.16 ± 0.27**	0.43 ± 0.06**
ID3	1.09 ± 0.28	0.81 ± 0.25	0.43 ± 0.21**	0.38 ± 0.11*
KLF6	0.75 ± 0.07**	1.05 ± 0.13	18.23 ± 0.13**	6.36 ± 0.21*
NFIB	1.05 ± 0.16	0.55 ± 0.22**	0.75 ± 0.23**	1.07 ± 0.13
NKX3-1	1.40 ± 0.22	0.83 ± 0.11	1.52 ± 0.20*	0.80 ± 0.10
RUNX1	0.87 ± 0.08	0.88 ± 0.21	0.80 ± 0.12	1.13 ± 0.20
SP1	3.69 ± 0.20**	2.21 ± 0.20**	7.87 ± 0.16**	4.83 ± 0.07**
TAL1	0.79 ± 0.17*	0.80 ± 0.08*	0.97 ± 0.20	0.89 ± 0.04
TCF7L2	1.21 ± 0.11*	0.83 ± 0.28	1.86 ± 0.06**	0.88 ± 0.11
TEAD2	0.58 ± 0.22**	0.71 ± 0.14**	0.31 ± 0.07**	0.79 ± 0.24
ZFP36	0.44 ± 0.18**	0.39 ± 0.26**	0.25 ± 0.14**	0.69 ± 0.20**
ZFP36L1	0.63 ± 0.14**	0.89 ± 0.24	0.40 ± 0.15**	1.03 ± 0.04
KLF6+SP1	1.04 ± 0.15	0.77 ± 0.07*	26.27 ± 0.12**	6.40 ± 0.14**

Transcriptional activity was assayed in CHO cells by transfection of reporter vectors, *Prrx1* (–2297/+103), *Prrx1* (–405/+103), *Prrx2* (–5060/+21) and *Prrx2* (–372/+21), and an expression vector harboring cDNA encoding the transcription factors listed in the Table. Data are indicated as the relative activity against that without the expression vector, and representative data are shown as means ± SD of quadruplicate transfections in two independent experiments. Asterisks indicate statistical significance by Student's *t*-test (*P<0.05; **P<0.01).

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 development 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

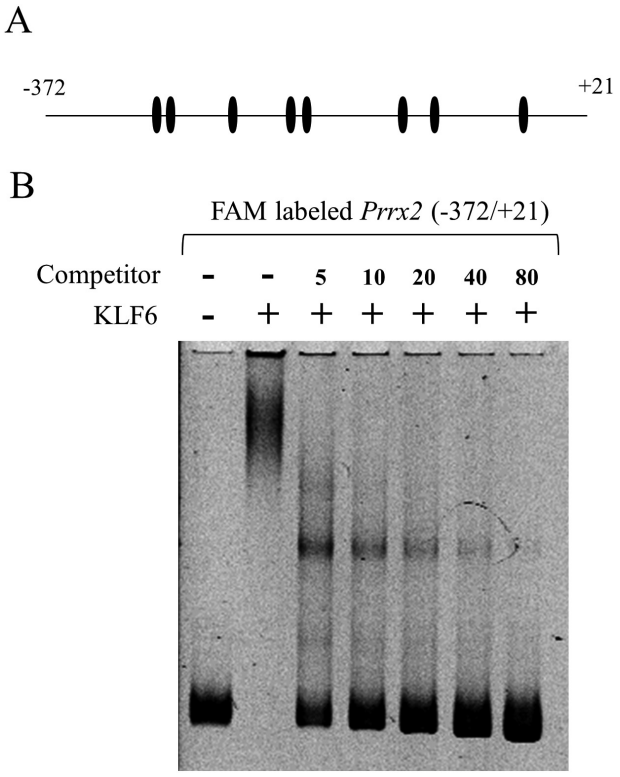

Fig. 3. Electrophoretic mobility shift assay (EMSA) for KLF6. (A) A diagram of the 5' upstream (–372/+21) region of *Prrx2*, which was used as a binding probe, is shown. A putative binding site (CCNCNCCN including GC element and CACCC) of KLF6 is shown with a closed ellipse. (B) EMSA was performed using 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.

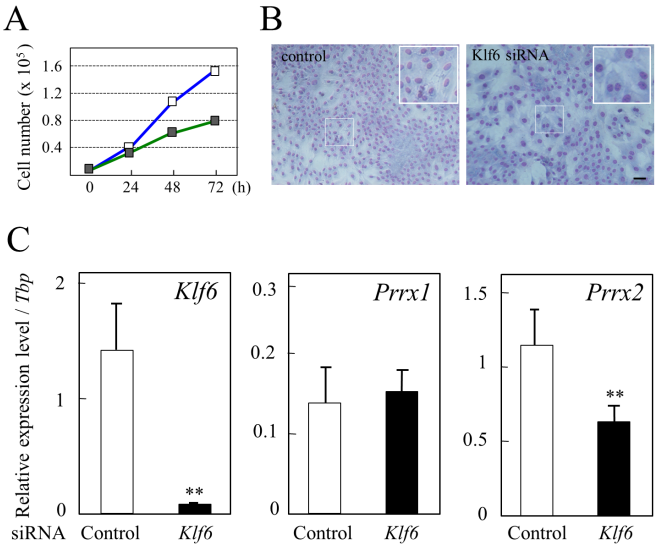

Fig. 4. Knockdown analysis of the *Klf6* mRNA level using siRNA. A: The *Klf6* mRNA level was knocked down for TtT/GF cells by transfection of *Klf6* siRNAs, and cell proliferation was measured. The respective cell numbers of the control (open boxes) and siRNA-transfected cells (closed boxes) were measured. 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).

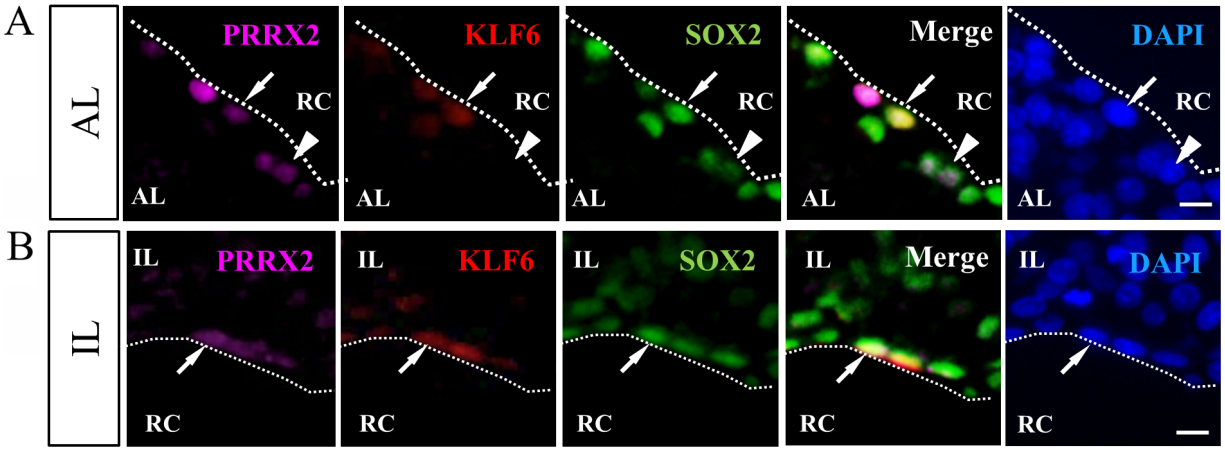

Fig. 5. Immunohistochemistry of KLF6 together with PRRX2 and SOX2. Immunohistochemistry for KLF6 was performed using frozen sections of rat pituitary at P20. Note that immunohistochemical images of KLF6, PRRX2 and SOX2 were visualized with Cy3 (red), Cy5 (purple) and fluorescein isothiocyanate (green) 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 elements might form a large complex by multiple binding of 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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