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REGULAR ARTICLE

# PRRX1- and PRRX2-positive mesenchymal stem/progenitor cells are involved in vasculogenesis during rat embryonic pituitary development

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**Abstract** We have recently shown that cells positive for the paired-related homeobox transcription factors PRRX1 and PRRX2 occur in the rat pituitary, and that they are derived from two different origins: pituitary-derived cells positive for stem cell marker SOX2 and extra-pituitary-derived cells negative for SOX2. In this study, we have further characterized the PRRX1- and PRRX2-positive cells that originate from extra-pituitary cells. Immunohistochemical analyses were performed with specific antibodies against PRRX1 and PRRX2 in order to clarify their roles in pituitary vasculogenesis. PRRX1- and PRRX2-positive cells were found in Atwell's recess and at the periphery of the pituitary on embryonic day 15.5 (E15.5). Several PRRX1-positive cells then invaded the anterior lobe, together with a few PRRX2-positive cells, on E16.5. Some PRRX1-positive cells were also positive for mesenchymal stem cell marker NESTIN. Moreover, some PRRX1/NESTIN double-positive cells showed characteristics of vascular endothelial cells with an Isolectin-B4-binding capacity. PRRX1 co-localized with vascular smooth muscle cell/pericyte marker α-smooth muscle actin in the deep area of Atwell's recess. We confirmed the presence of PRRX2/NESTIN double-positive cells at an entry area in Atwell's recess and at the periphery of the pituitary, but PRRX2 did not co-localize with Isolectin B4 or α-smooth muscle actin. These data suggest that PRRX1- and PRRX2-positive mesenchymal stem/progenitor cells are present at the periphery of the embryonic pituitary and at the entry from Atwell's recess and participate in pituitary vasculogenesis by differentiation into vascular endothelial cells and pericytes, whereas the presence of PRRX2 indicates much higher stemness than PRRX1.

**Keywords** PRRX1 · PRRX2 · NESTIN-positive dividing mesenchymal stem/progenitor cell · Vasculogenesis · Embryonic pituitary development · Rat

**Abbreviations**

| | |
|---|---|
| PRRX1 | Paired-related homeobox 1 |
| PRRX2 | Paired-related homeobox 2 |
| SOX2 | Sex-determining region Y-box 2 |
| DAPI | 4'6-diamidino-2-phenylindole |
| NESTIN | Neuroepithelial stem cell |
| α-SMA | α-Smooth muscle actin |
| FITC | Fluorescein isothiocyanate |

## Introduction

The pituitary gland is an important endocrine organ that plays essential roles in physiological processes such as growth, metabolism, reproduction, lactation, homeostasis, and stress

Masashi Higuchi and Takako Kato contributed equally to this work.

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response, by secreting many hormones. The temporospatial appearance of multiple transcription factors leads to the specification of cell lineages that differentiate into each hormone-producing cell type during the development of the pituitary gland (Brinkmeier et al. 2009; Watkins-Chow and Camper 1998; Zhu et al. 2007; Zhu and Rosenfeld 2004). In vitro studies have demonstrated that cells expressing the gene of the HMG-box transcription factor SOX2 (sex-determining region Y-box 2), a stem/progenitor cell marker, are present in the pituitary gland and have the ability to generate all of the pituitary cell types (Chen et al. 2009; Fauquier et al. 2008).

Recently, the self-formation of the primitive anterior lobe of the pituitary gland, which is capable of producing pituitary hormones, has been succeeded by the three-dimensional culture of mouse embryonic stem cells (Suga et al. 2011). Although the development of the pituitary portal vein and vascular network in the anterior pituitary is crucial for the neuroendocrine control of the pituitary and for hormone secretion to target organs, the self-formation of the primitive pituitary in embryonic stem cell culture is not associated with the development of a vascular network. Pituitary vasculogenesis has been suggested to start by the invasion of extra-pituitary origin cells into the pituitary (Daikoku et al. 1981; Szabo and Csanyi 1982). Daikoku et al. (1981) have demonstrated that Atwell's recess, an intraglandular fossa that received several blood vessels, is located between the anterior lobe and rostral tip. The determination and description of the cells of extra-pituitary origin that might participate in vasculogenesis is an important issue for a better understanding of pituitary organogenesis.

We have recently reported that paired-related homeobox transcription factors, *Prrx1* (also known as *MHox*, *k2*, *Pmx*, and *Prx1*) and *Prrx2* (also known as *S8*, *Prx2*, and *Pmx2*), are expressed in the rat pituitary (Higuchi et al. 2014; Susa et al. 2012). They are known to be important factors for organogenesis of mesenchymal tissues and vasculogenesis (for a review, see Douville and Wigle 2007). Notably, *Prrx1*/*Prrx2* double-deficient mice demonstrate their crucial roles in craniofacial and limb morphogenesis (Balic et al. 2009; Meijlink et al. 1999; Mitchell et al. 2006). Immunohistochemical studies of the pituitary have shown that PRRX1- and PRRX2-positive cells have two different origins: SOX2-positive pituitary stem/progenitor cells and extra-pituitary cells negative to SOX2 (Higuchi et al. 2014).

We have demonstrated that PRRX1 is localized in SOX2-positive stem/progenitor cells, in committed cells, and in all kinds of anterior pituitary-hormone-producing cells, indicating that PRRX1 plays a role in pituitary organogenesis by being expressed in multipotent progenitor/committed cells and in cells in the early stage of terminal differentiation throughout life (Higuchi et al. 2014). However, PRRX2/SOX2 double-positive cells first appear in the postnatal stage of pituitary development.

Some reports suggest that the pituitary cell sub-population of Sca1$^{high}$ has properties of the stem/progenitor cells that have an endothelial or mesenchymal origin (Vankelecom 2010; Vankelecom and Gremeaux 2010). Immunohistochemistry with an antibody that recognizes both PRRX1 and PRRX2 has shown that the immune-reacted cells of the surrounding mesenchyme invade the pituitary anterior lobe and are positive to endothelial cell markers, platelet endothelial cell adhesion molecule-1 (PECAM-1, also called CD31), and neuroepithelial stem cell marker NESTIN before birth (Yako et al. 2013). In addition, we have recently demonstrated the presence of NESTIN-expressing dividing progenitor cells in the pituitary organ (Yoshida et al. 2013).

In the present study, we characterize the cells that invade the pituitary anterior lobe by using antibodies specific to PRRX1 and PRRX2, together with several markers for vascular cells, such as NESTIN (dividing stem/progenitor cell marker), Isolectin B4 (vascular endothelial cell marker), and α-smooth muscle actin (α-SMA, smooth muscle cell/pericyte marker). Immunohistochemistry has shown that the extra-pituitary cells are positive to PRRX1 and/or PRRX2. PRRX1 is present in NESTIN-positive dividing stem/progenitor cells, especially in Atwell's recess, and transiently in cells in the early stage of terminal differentiation into two types of vascular cells: vascular endothelial cells and pericytes. On the other hand, PRRX2 is also localized in NESTIN-positive mesenchymal stem cells that can be distinguished from terminally differentiated cells. Thus, we have demonstrated that PRRX1 and PRRX2 probably participate in pituitary vasculogenesis.

## Materials and methods

### Animals

Wistar-Imamichi 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 NIH Guidelines for the Care and Use of Laboratory Animals. Adult rats were paired, and the day that a vaginal plug was observed was designated as embryonic day 0.5 (E0.5). Pregnant rats were killed at the appropriate time point by cervical dislocation, and whole embryos at E15.5, E16.5, and E18.5 were surgically removed.

### Immunohistochemical analyses

Whole embryos were fixed with 4 % paraformaldehyde in 20 mM HEPES buffer (HEPES, pH 7.5) for 20 h at 4 °C and were immersed in 30 % trehalose in HEPES for 24 h at 4 °C, followed by embedding in O.C.T. compound (Sakura Finetek Japan, Tokyo, Japan) at −80 °C before being sectioned.

Frozen embryos were sectioned at a thickness of 8 μm in the sagittal or coronal planes. Frozen sections mounted on glass slides (Matsunami, Osaka, Japan) were activated by an Immunosaver (Nisshin EM, Tokyo, Japan) for 60 min at 80 °C and washed three times with HEPES containing 100 mM NaCl for 10 min, followed by blocking with 0.4 % Triton-X100 and 0.5 % bovine serum albumin in HEPES (blocking buffer) for 60 min at room temperature. The sections were then incubated with primary antibodies at the appropriate dilution for 16 h at 4 °C. The primary antibodies used were rabbit IgG against rat PRRX1 (dilution 1:1000), which was purified in this study, or rabbit antiserum against rat PRRX2 (dilution 1:1000; Higuchi et al. 2013), together with goat antibody against human SOX2 (dilution 1:400; Neuromics, Minn., USA) or mouse antibodies against rat NESTIN clone 25 (dilution 1:250; BD Bioscience, San Jose, Calif., USA) and human α-SMA (dilution 1:100; Dako, Glostrup, Denmark). Sections were stained with fluorescein isothiocyanate (FITC)-conjugated Isolectin B4 (dilution 1:100; Vector, Burlingame, Calif., USA) to identify non-primate mammalian endothelial cells. After the sections had been washed three times with HEPES for 5 min, they were incubated for 2 h at room temperature with Cy3-, Cy5-, or FITC-conjugated donkey anti-rabbit, anti-goat or anti-mouse IgG antibody (1:500 dilution; Jackson ImmunoResearch, West Grove, Pa., USA) as secondary antibodies in the blocking buffer. Finally, the sections were enclosed in a Vectashield mounting medium containing DAPI (4'6-diamidino-2-phenylindole; Vector), and immunofluorescence was observed by a fluorescence microscope (BZ-9000, Keyence, Osaka, Japan).

## Results

### Localization of PRRX1- and PRRX2-positive mesenchymal cells

We confirmed the localization of PRRX1- and PRRX2-positive cells in the rat embryonic pituitaries on E15.5 and E16.5 (Fig. 1). A number of PRRX1- and PRRX2-positive cells were present in Atwell's recess and at the periphery of the pituitary on E15.5 (Fig. 1a, d). Only 24 h later on E16.5, PRRX1-positive cells were scattered in the parenchyma of the anterior lobe, contains the dorsal and lateral regions, and at the boundary between the intermediate and posterior lobes and the periphery of the pituitary (Fig. 1b, c). On the other hand, PRRX2-positive cells were present at the periphery of the pituitary more abundantly than PRRX1, but not in the parenchyma, on E15.5 (Fig. 1d), whereas extremely low signals for PRRX2 were observed in fewer cells on E16.5 (Fig. 1e, f).

Many PRRX1-positive cells were observed in Atwell's recess, penetrating deep into the anterior lobe. Although PRRX2-positive cells were initially found in the deep region of Atwell's recess at E15.5, they thereafter remained in the entry region of Atwell's recess and the periphery of the

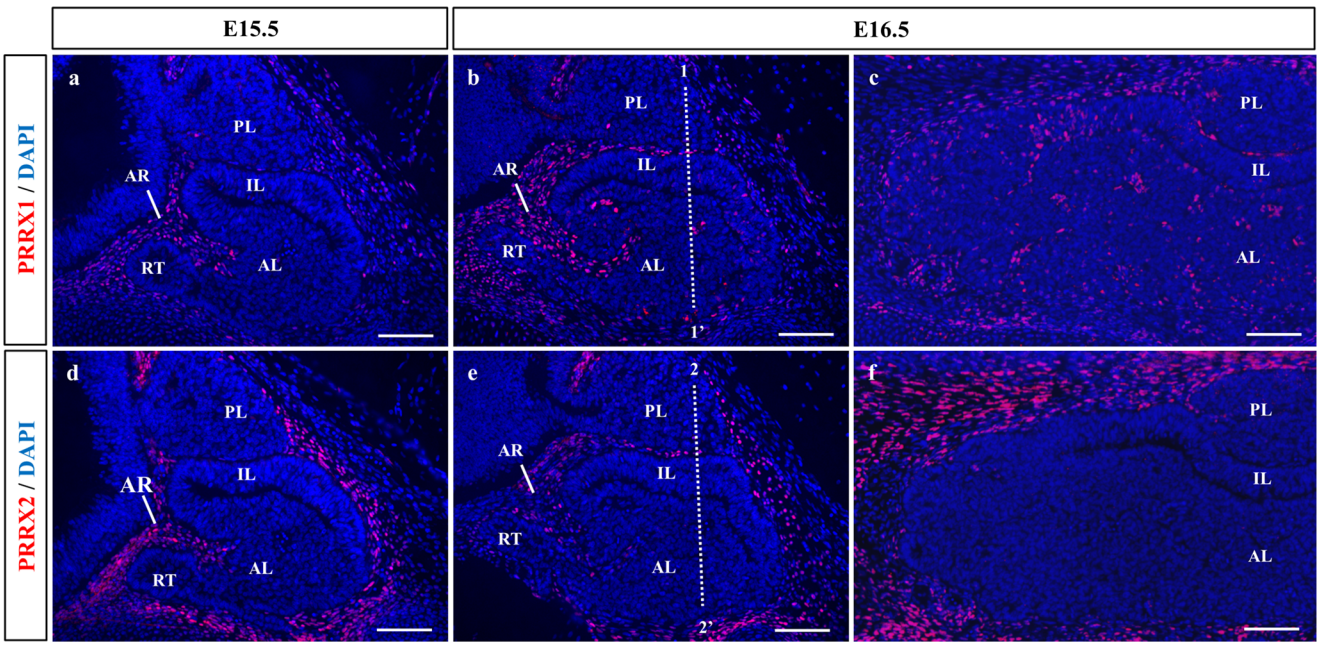


**Fig. 1** Localization of PRRX1- and PRRX2-positive cells inside and outside the embryonic pituitary. Frozen sagittal (**a**, **b**, **d**, **e**) and coronal (**c**, **f**; view of right half portion of the pituitary) sections of rat pituitaries at E15.5 and E16.5 were reacted with anti-PRRX1 IgG (**a–c**) and anti-PRRX2 antiserum (**d–f**), followed by visualization with Cy3 (*red*). Merged images with DAPI (*blue*) are shown. *Dotted lines* labeled *1-1'* in **b** and *2-2'* in **e** indicate the direction of the coronal plane shown in **c**, **f**, respectively (*AL* anterior lobe, *IL* intermediate lobe, *PL* posterior lobe, *RT* rostral tip, *AR* Atwell's recess). *Bars* 100 μm

pituitary, being only rarely present in the deep region of Atwell's recess on E16.5.

## Absence of SOX2 in invading mesenchymal PRRX1- and PRRX2-positive cells

We previously observed that SOX2 occupied all cells of the pituitary primordium called Rathke's pouch at E13.5 (Yoshida et al. 2009), and that PRRX1/PRRX2 appeared in the SOX2-positive cells of the anterior lobe around E15.5 (Higuchi et al. 2013; Susa et al. 2012). To investigate mesenchymal cells invading Atwell's recess, triple-immunohistochemistry of PRRX1 or PRRX2 with SOX2 and NESTIN was performed for the embryonic pituitary at E16.5 (Fig. 2). Whereas PRRX1/SOX2/NESITN triple-positive cells were present in the anterior lobe (Fig. 2a–d, arrowheads), many PRRX1/NESTIN double-positive cells negative for SOX2 were observed in Atwell's recess (Fig. 2e–j, arrows), the sinusoid of the anterior lobe (Fig. 2k–m), and the periphery of the pituitary (Fig. 2n–p), together with a few PRRX1 or NESTIN single-positive cells.

However, PRRX2 was not detected in SOX2-positive cells of the anterior lobe (Fig. 2q–t). PRRX2-positive cells negative for SOX2 were positive for NESTIN and located in Atwell's recess (Fig. 2u–z, arrows), the sinusoid (Fig. 2aa–ac), and the periphery of the pituitary (Fig. 2ad–af), together with many NESTIN single-positive cells. PRRX2 single-positive cells were almost never observed in Atwell's recess and the sinusoid. The number of PRRX2-positive cells invading the anterior lobe was fewer than that of PRRX1-positive cells.

Double-immunohistochemistry of PRRX1 or PRRX2 with NESTIN was performed for coronal sections of the rat pituitary on E16.5 (Fig. 3a, e). Although PRRX1/NESTIN double-positive cells were observed in the periphery (Fig. 3b), they were also present inside the anterior lobe (Fig. 3c), in the boundary between the intermediate and posterior lobes, and inside the posterior lobe (Fig. 3d). Markedly, PRRX1/NESTIN double-positive cells in the parenchyma formed clumps (Fig. 3a, arrowheads) in which PRRX1 or NESTIN single-positive cells were also found (Fig. 3c). On the other hand, many PRRX2/NESTIN double-positive cells were detected at the periphery of the anterior lobe (Fig. 3e, f), but none

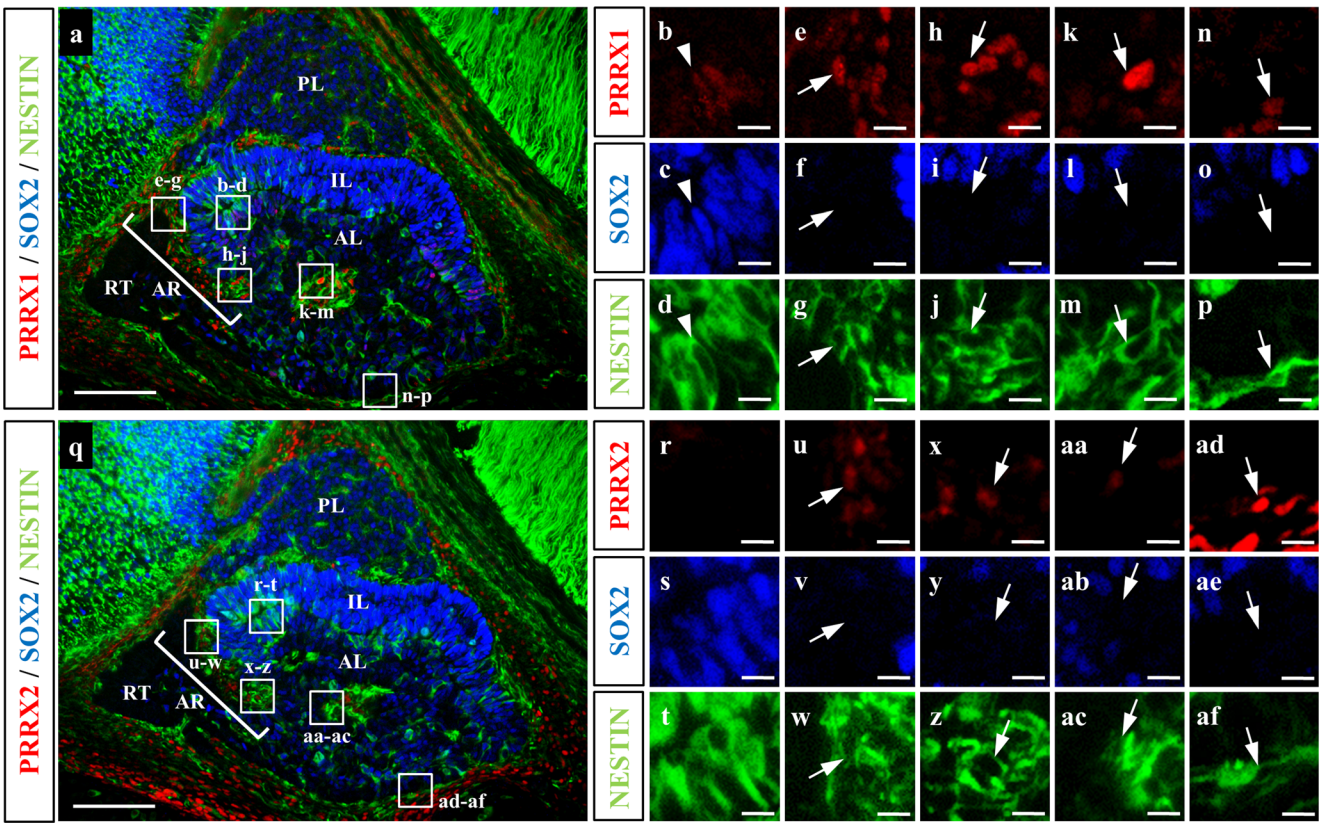


**Fig. 2** Triple-immunohistochemistry of PRRX1 or PRRX2 with SOX2 and NESTIN in rat embryonic pituitary. Immunohistochemical merged images of PRRX1 or PRRX2 (Cy3, *red*), with SOX2 (fluorescein isothiocyanate [FITC], *blue*) and NESTIN (Cy5, *green*) in sagittal sections of rat pituitary at E16.5 (**a** PRRX1, **q** PRRX2). *Boxed areas* are enlarged in **b–p** for PRRX1 and in **r–af** for PRRX2 (*arrows* PRRX1- or PRRX2-positive cells positive for NESTIN but negative for SOX2, *arrowheads* PRRX1/SOX2/NESTIN triple-positive cells, *AL* anterior lobe, *IL* intermediate lobe, *PL* posterior lobe, *RT* rostral tip, *AR* Atwell's recess). *Bars* 100 μm (**a**, **q**), 10 μm (**b–p**, **r–af**)

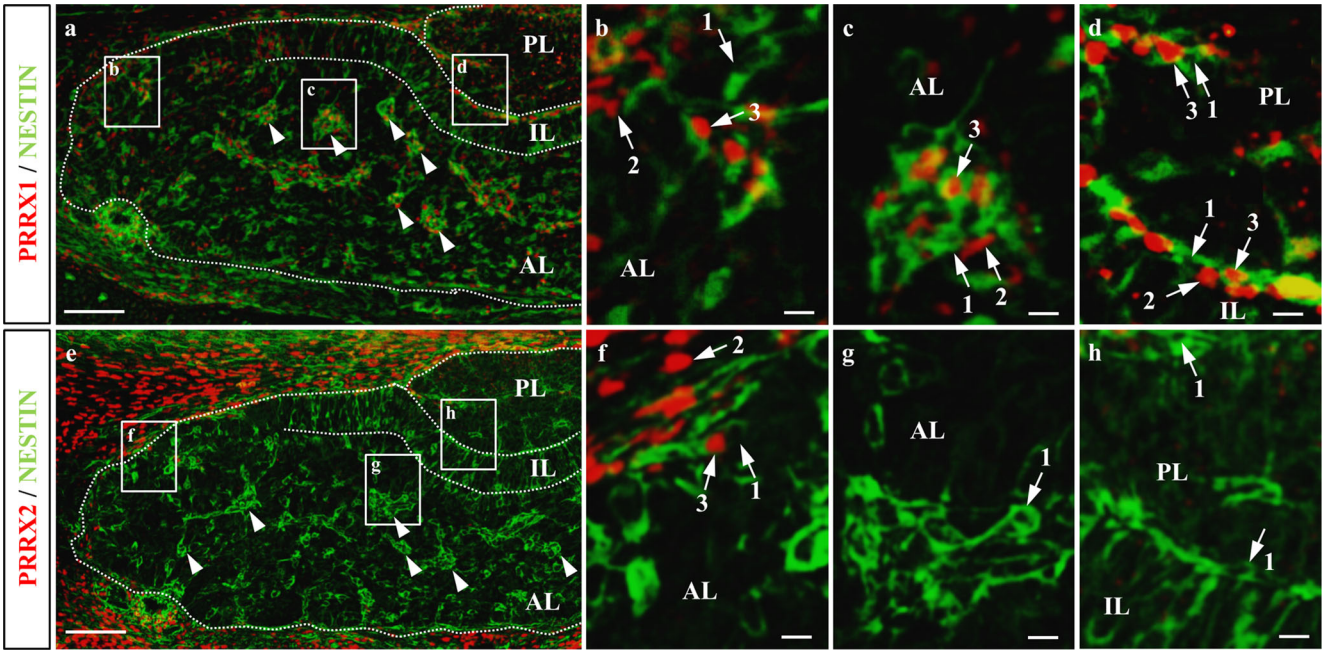


**Fig. 3** Localization of PRRX1- and PRRX2-positive dividing mesenchymal stem/progenitor cells. Double-immunostaining of PRRX1 or PRRX2 (Cy3, *red*) and NESTIN (Cy5, *green*) was performed for coronal sections of rat pituitary at E16.5. Merged images (**a**; PRRX1, **e**; PRRX2) are shown, and *boxed areas* are enlarged in **b–d** (PRRX1) and **f–h** (PRRX2). *Arrows* with *numbers* indicate cell types singly positive for NESTIN (*1*), singly positive for PRRX1 or PRRX2 (*2*), or doubly positive for PRRX1 (PRRX2)/NESTIN (*3*). *Arrowheads* indicate clumps of NESTIN-positive cells (*AL* anterior lobe, *IL* intermediate lobe, *PL* posterior lobe). *Bars* 100 μm (**a**, **e**), 10 μm (**b–d**, **f–h**)

were seen in either the anterior lobe or the boundary between the intermediate and posterior lobes (Fig. 3g, h).

## Differentiation into vascular endothelial cells from PRRX1- and PRRX2-positive cells

Triple-immunostaining of PRRX1 or PRRX2 with NESTIN and Isolectin B4, together with DAPI staining, was performed for the rat pituitary on E15.5 (Fig. 4a–d, i–l). By superimposing the four stained images of Atwell's recess (Fig. 4e–h, m–p), six populations were observed: Type 1, NESTIN single-positive; Type 2, PRRXs (PRRX1 or PRRX2) single-positive; Type 3, PRRXs/NESTIN double-positive; Type 4, PRRXs/NESTIN/Isolectin B4 triple-positive; Type 5, NESTIN/Isolectin B4 double-positive; and Type 6, Isolectin B4 single-positive.

All cell types are shown in Fig. 4e–h. Notably, Type 4 (PRRX1/NESTIN/Isolectin B4 triple-positive) was found in the deep region of Atwell's recess. These data indicate that some of the PRRX1-positive cells with or without the expression of NESTIN invade the anterior lobe of the embryonic pituitary, and then PRRX1/NESTIN double-positive cells differentiate into vascular endothelial cells that have an Isolectin-B4-binding capacity. As shown in Fig. 4i–p, however, we could not find the Type 4 (PRRX2/NESTIN/Isolectin B4 triple-positive) population.

We further characterized PRRX1-positive cells invading the deep parenchyma on E16.5 and E18.5 (Fig. 5a–e). A number of PRRX1 single-positive cells were present in Atwell's recess, together with PRRX1/Isolectin B4 double-positive cells (Fig. 5c) and Isolectin B4 single-positive cells (Fig. 5b, arrowhead). In the deep part region of Atwell's recess, PRRX1/Isolectin B4 double-positive cells were detected (Fig. 5c, arrow) together with Isolectin B4 single-positive cells (Fig. 5c, arrowhead). At E18.5, the number of Isolectin-B4-positive cells increased, as if they were involved in the formation of the vessel wall together with PRRX1 single-positive cells, which penetrated to the dorsal region of the anterior lobe (Fig. 5d, e). On E16.5, some PRRX2-positive cells in the periphery were Isolectin-B4-positive, and only Isolectin B4 single-positive cells were observed inside the anterior lobe (Fig. 5f–h).

## Differentiation into pericytes from PRRX1- and PRRX2-positive cells

α-SMA, which recognizes a microfilament in vascular smooth muscle cells and pericytes (Herman and D'Amore 1985; Skalli et al. 1989), is considered as an early vascular marker (Boyd et al. 2007). Double-immunohistochemistry for PRRX1 or PRRX2 with α-SMA was performed on rat pituitaries at E16.5 (Fig. 6a, d). PRRX1-positive mesenchymal cells in the entry region over Atwell's recess were negative for α-SMA (Fig. 6b). Notably, in the deep region of Atwell's recess, a few PRRX1-positive cells showed positive signals for α-SMA (Fig. 6c). In addition, we observed a weak α-SMA

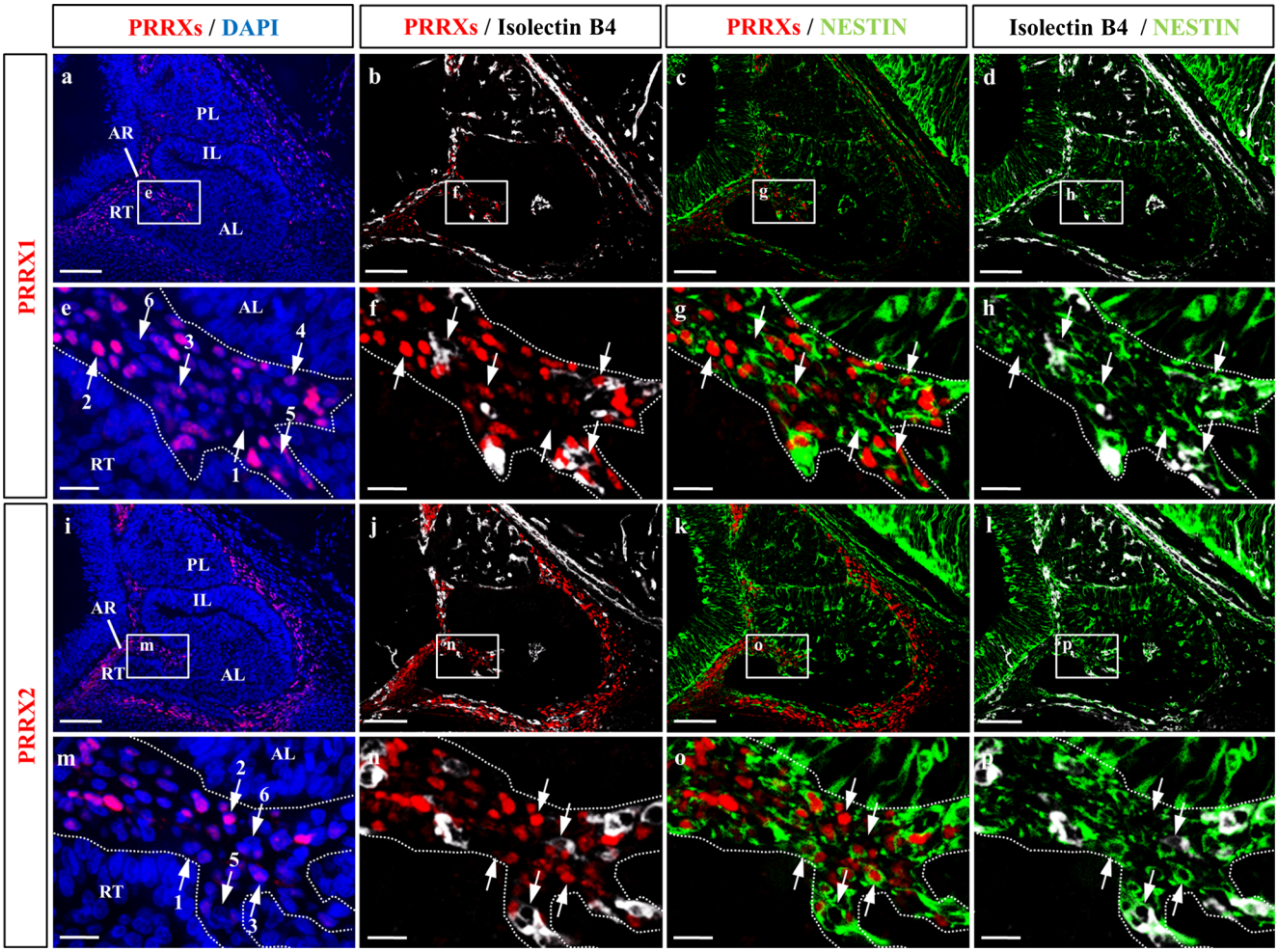


**Fig. 4** Co-localization of PRRX1 or PRRX2 with mesenchymal stem cell marker NESTIN and vascular endothelial marker Isolectin B4. **a**, **e**, **i**, **m** Merged images of PRRX1 or PRRX2 (Cy3, *red*) and DAPI (*blue*) in the sagittal plane of rat pituitary at E15.5. **b–d**, **j–l** Merged images of PRRX1 (**b–d**) or PRRX2 (**j–l**) and NESTIN (Cy5, *green*) with FITC-conjugated Isolectin B4 (*white*) are shown. *Boxed areas* are enlarged in **e–h** (PRRX1) and **m–p** (PRRX2). *Arrows* with *numbers* indicate cell types positive to PRRXs, NESTIN, and/or Isolectin B4 (*1* NESTIN single-positive, *2* PRRXs single-positive, *3* PRRXs/NESTIN double-positive, *4* PRRXs/NESTIN/Isolectin B4 triple-positive, *5* NESTIN/Isolectin B4 double-positive, *6* Isolectin B4 single-positive, *AL* anterior lobe, *IL* intermediate lobe, *PL* posterior lobe, *RT* rostral tip, *AR* Atwell's recess). *Bars* 100 μm (**a–d**, **i–l**), 20 μm (**e–h**, **m–p**)

signal inside the anterior lobe on E18.5 (data not shown). These results indicate that at least some PRRX1-positive mesenchymal cells differentiate into pericytes during the invasion. On the other hand, all the PRRX2-positive cells were negative for α-SMA (Fig. 6d–f).

## Discussion

Vasculogenesis is an essential event in pituitary organogenesis and in the development of pituitary cells into hormone-producing cells. Although it is often pointed out that this event starts with the invasion of extra-pituitary cells from Atwell's recess (Daikoku et al. 1981), the types of cells that invade from the recess have remained unclear. Recently, we have demonstrated that PRRX1 and PRRX2, which are known as important factors in mesenchymal cell differentiation at the craniofacial region (Balic et al. 2009; Meijlink et al. 1999), participate in the development of ectodermal hormone-producing cells (Higuchi et al. 2013, 2014). Consequently, the present study has especially focused on whether PRRX1- and PRRX2-positive cells of extra-pituitary origin are involved in the vasculogenesis of the rat embryonic pituitary.

In the present study, we have demonstrated that many PRRX1-positive cells that are present in Atwell's recess invade the anterior lobe and participate in the development of blood vessels. PRRX1/NESTIN double-positive cells can be found around the recess, in addition to PRRX1 single-positive and NESTIN single-positive cells, indicating that either or both single-positive cells can transform their characteristics during the invasion. Since NESTIN, a class IV intermediate

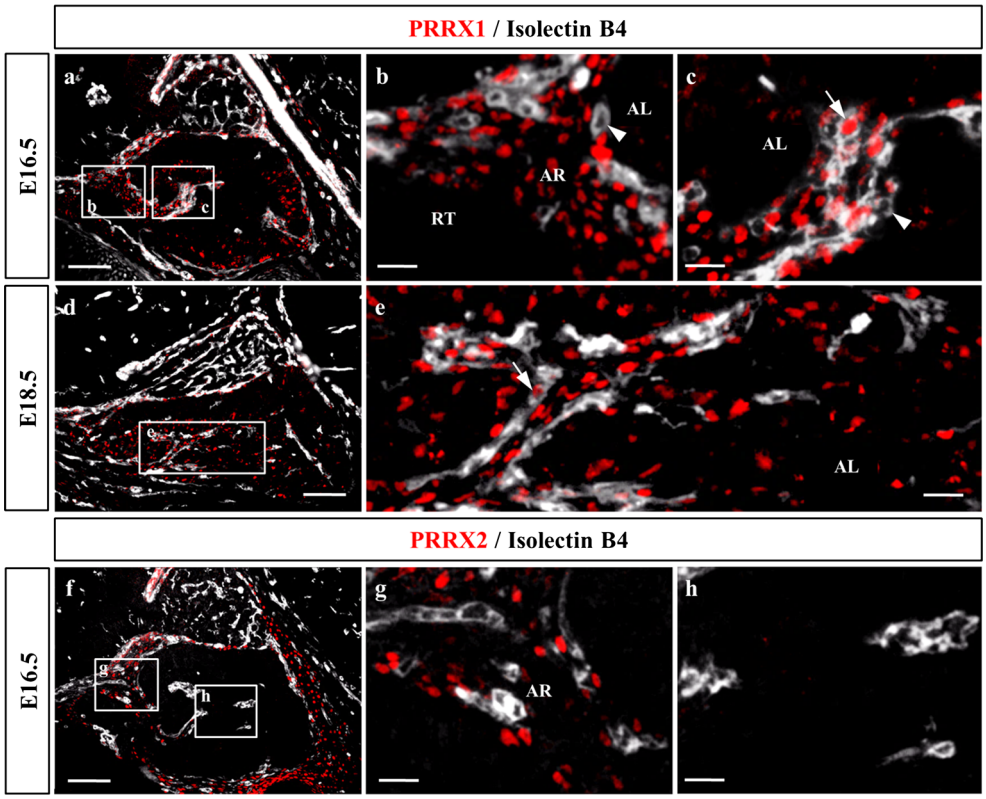


**Fig. 5** Appearance of PRRX1 and PRRX2 in vascular endothelial cells. After immunostaining with PRRX1 or PRRX2 (Cy3, *red*), fluorescent labeling by binding of FITC-conjugated Isolectin B4 (*white*) was performed. Merged images of rat pituitaries at E16.5 (**a**, **f**) and at E18.5 (**d**) are shown in the sagittal plane. *Boxed areas* are enlarged in **b**, **c**, **e** (PRRX1), and **g**, **h** (PRRX2). *Arrows* and *arrowheads* indicate PRRX1/Isolectin B4 double-positive and Isolectin B4 single-positive cells, respectively (*AL* anterior lobe, *RT* rostral tip, *AR* Atwell's recess). *Bars* 100 μm (**a**, **d**, **f**), 20 μm (**b**, **c**, **e**, **g**, **h**)

filament protein, is known to be expressed in dividing cells (Sunabori et al. 2008) and plays a role in mesenchymal and hematopoietic stem/progenitor cells (Mendez-Ferrer et al. 2010; Shih et al. 2001) and neural stem/progenitor cells (Hunziker and Stein 2000), our observation that heterogeneous cells invade the anterior lobe is important for understanding pituitary vasculogenesis. We have further demonstrated that some PRRX1/NESTIN double-positive cells have the characteristic of vascular endothelial cells in that they can bind to Isolectin B4. In addition, we have observed that some PRRX1-positive cells, which invaded the pituitary, are positive for α-SMA at E16.5 before angiogenesis. We assume that cells positive for both PRRX1 and α-SMA are in the early stage of angiogenesis. Interestingly, α-SMA has not been observed in the posterior lobe, which is composed of highly developed capillary. Thus, we suggest that the PRRX1-positive cells of the mesenchymal lineage are composed of dividing multipotent stem/progenitor cells expressing

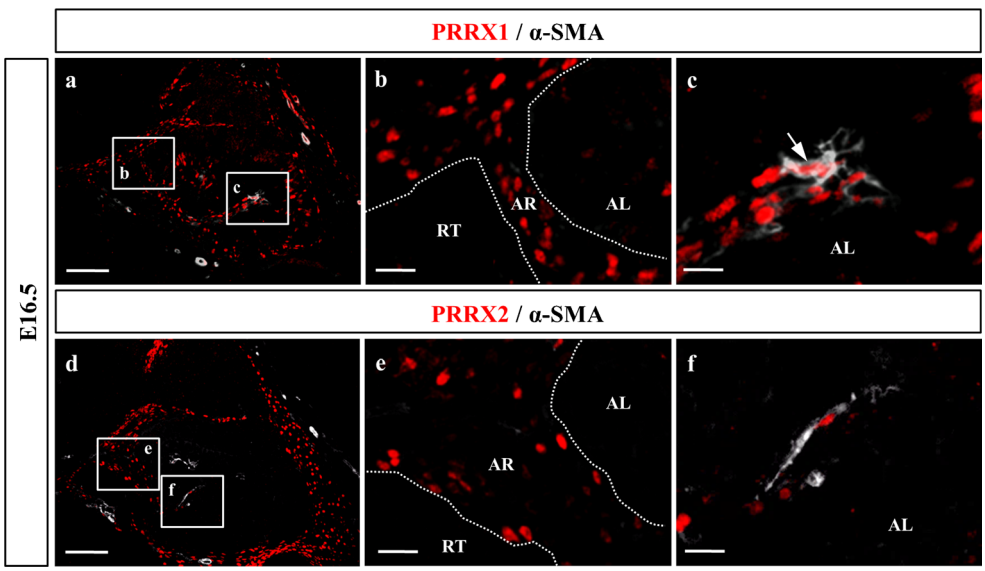


**Fig. 6** Co-localization of PRRX1 or PRRX2 with α-SMA. Double-immunostaining for PRRX1 or PRRX2 (Cy3, *red*) and α-SMA (FITC, *white*) in rat pituitary at E16.5 in the sagittal plane. Merged images are shown in **a**, **d**. *Boxed areas* are enlarged in **b**, **c** (PRRX1) and in **e**, **f** (PRRX2). *Arrow* indicates PRRX1/α-SMA double-positive cells (*AL* anterior lobe, *RT* rostral tip, *AR* Atwell's recess). *Bars* 100 μm (**a**, **d**), 20 μm (**b**, **c**, **e**, **f**)

NESTIN, for differentiation into the vascular endothelial cells and pericytes.

In contrast to PRRX1-positive cells, PRRX2-positive cells have been observed in the entry region of Atwell's recess and are mostly Isolectin-B4-negative. Notably, PRRX2-positive cells markedly decrease in number before proceeding to the deep part of the anterior lobe at E15.5 and are absent thereafter. Since some of the PRRX2-positive cells are obviously positive for NESTIN, PRRX2-positive cells might invade concurrently with their differentiation. Although we consider that PRRX2-positive cells, including those that are NESTIN-positive, also differentiate into vascular endothelial cells and/or pericytes with proliferation, the fate of these cells is as yet unclear and remains to be resolved. Meanwhile, we have observed that the ectodermal lineage PRRX2-positive cells emerge as cells positive for SOX2 in the anterior and intermediate lobe sides of the postnatal marginal cell layer, a pituitary stem/progenitor niche (Higuchi et al. 2014); we therefore assume that PRRX2-positive cells of the mesenchymal stem/progenitor cell lineage also possess much higher stemness than PRRX1-positive cells.

Our present and past studies (Higuchi et al. 2013, 2014) address the various roles of PRRX1 and PRRX2 and the clarification of their differences. More recently, we have demonstrated that *Prrx1* and *Prrx2* are expressed in various pituitary stem/progenitor cells and are modulated by several pituitary transcription factors (Ueharu et al. 2014). Among the factors, KLF6 (Krüppel-like transcription factor 6), which is known as a regulator of proliferation, differentiation, and development, is mostly present in PRRX2-positive cells and shows specific and remarkable stimulation of the expression of *Prrx2*, but not of *Prrx1*. On the other hand, we have found that TtT/GF cells, a pituitary tumor-derived cell line, express both *Prrx1* and *Prrx2* (Susa et al. 2012). Using this cell line, we have demonstrated that PRRX1 and PRRX2 modulate cell growth distinctly by means of *p21* expression. Notably, TtT/GF cells show a higher expression of *Nestin* (Yoshida et al. 2014) and stemness markers, ABC transporter subfamily G2 (*Abcg2*), and stem cell antigen 1 (*Sca1*; Mitsuishi et al. 2013). In addition, another pituitary-tumor-derived cell line, Tpit/F1, expresses *Prrx1* and *Prrx2* in addition to stemness markers (Yoshida et al. 2014). Thus, the different roles of PRRX1 and PRRX2 might be understood by using TtT/GF as a model cell line.

In conclusion, we have revealed the presence of PRRX1- and PRRX2-positive cells in the periphery of the embryonic pituitary and found that many of them invade the pituitary in Atwell's recess. Some invading cells expressing PRRX1 and NESTIN are mesenchymal progenitor cells, suggesting that they have stemness and proliferative capacity. These findings indicate that PRRX1 plays important roles in the differentiation of invading cells into vascular endothelial cells and pericytes. In contrast, some of the cells expressing PRRX2 and NESTIN might function as dividing mesenchymal stem cells, but their role is as yet not fully understood. Further study, such as tracing cell fate, is needed to reveal the differentiation and role of extra-pituitary-derived PRRX1- and PRRX2-positive cells and of pituitary stem/progenitor cells.

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