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The expression of aristaless-related homeobox in neural progenitors of gyrencephalic carnivore ferrets

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ABSTRACT

Aristaless-related homeobox (ARX) has important functions in the development of various organs including the brain. Mutations of the human *ARX* gene have been associated with malformations of the cerebral cortex such as microcephaly and lissencephaly. Although the expression patterns of ARX in the lissencephalic cerebral cortex of mice have been intensively investigated, those in expanded gyrencephalic brains remained unclear. Here, we show the expression patterns of ARX in the developing cerebral cortex of gyrencephalic carnivore ferrets. We found that ARX was expressed not only in intermediate progenitor (IP) cells but also in outer radial glial (oRG) cells, which are neural progenitors preferentially observed in the gyrencephalic cerebral cortex. We found that the majority of ARX-positive oRG cells expressed the proliferating cell marker Ki-67. These results may indicate that ARX in oRG cells mediates the expansion of the gyrencephalic cerebral cortex during development and evolution.

All procedures of animal experiments were approved by the Animal Care Committee of Kanazawa University.

1. Introduction

The aristaless-related homeobox (*ARX*) gene, located in the X chromosome, is highly conserved from rodents to primates [1,2]. ARX is expressed at various developmental stages and has important functions in many organs during development. For example, *ARX*-deficient mice die within 2 days after birth because of early-onset hypoglycemia [3]. This phenotype is induced by the loss of mature endocrine α cells in the pancreas because ARX is essential for the differentiation of endocrine progenitors into α cells. *ARX*-deficient mice also show impaired differentiation of Leydig cells in the testis and reduction of testosterone, resulting in smaller testes and hypoplastic seminal vesicles [4,5]. ARX also plays crucial roles in brain development [4]. ARX is expressed in interneurons derived from the ganglionic eminence and is important for the maturation and tangential migration of interneurons into the cerebral cortex [2,6,7]. ARX is also expressed in neural progenitors of glutamatergic neurons in the developing cerebral cortex and promotes the maintenance of neural progenitors [6,8]. Consistently, *ARX*-deficient male mice exhibit smaller brains and thinner upper layers of the cerebral cortex [4,8].

Many studies have revealed crucial roles of ARX in the development of the brain using mice. Mammalian brains, however, have changed dramatically during evolution, and the brains of mice are relatively smaller and simpler than those of primates and carnivores [9–15]. Therefore, the expression patterns and functions of ARX in the brains of primates and carnivores would be intriguing to examine. Indeed, mutations were found in the *ARX* gene of human patients with X-linked lissencephaly with abnormal genitalia (XLAG), who show microcephaly, lissencephaly, corpus callosum agenesis, a three-layered cortex and poorly myelinated white matter [16]. Another mutation in the *ARX* gene was identified in human patients with West syndrome [17]. These results raised the possibility that ARX is important for proper development of the complex brains of primates and carnivores.

Glutamatergic neurons in the cerebral cortex of mice are generated during development from neural progenitor cells such as radial glial (RG) cells in the ventricular zone (VZ) and intermediate progenitor (IP) cells in the subventricular zone (SVZ). In the developing cerebral cortex of primates and carnivores, the SVZ is further subdivided into the inner SVZ (ISVZ) and the outer SVZ (OSVZ), which contains additional neural progenitor cells called outer radial glial (oRG) cells [10–13,18–20]. Because it has been proposed that an increase in oRG cells during

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evolution resulted in the expansion and folding of the cerebral cortex in primates and carnivores [10–13,18–20], genes expressed in oRG cells would be of great interest. Here we examined the expression patterns of ARX in the cerebral cortex of gyrencephalic carnivore ferrets during development because the developing cerebral cortex of ferrets contains abundant oRG cells [21–24]. We found that ARX was expressed not only in IP cells but also in oRG cells in the OSVZ. Many ARX-positive oRG cells expressed the proliferating cell marker Ki-67, suggesting that ARX-positive oRG cells are indeed neural progenitors. It seems plausible that ARX plays important roles in oRG cells, which are crucial for the expansion of the gyrencephalic cerebral cortex during development and evolution.

2. Materials and methods

2.1. Animals

Normally pigmented, sable ferrets (*Mustela putorius furo*) were purchased from Marshall Farms (North Rose, NY). Ferrets were maintained as described previously [25,26]. The day of conception and that of birth were counted as embryonic day 0 (E0) and postnatal day 0 (P0), respectively. All procedures were approved by the Animal Care Committee of Kanazawa University.

2.2. Preparation of sections

Sections were prepared as described previously with slight modifications [27,28]. Briefly, ferrets were deeply anesthetized and transcardially perfused with 4% paraformaldehyde (PFA), and the brains were dissected. The brains were cryoprotected by three-day immersion in 30% sucrose and embedded in OCT compound. Sections of 50 μm thickness were prepared using a cryostat.

2.3. Immunohistochemistry

Immunohistochemistry was performed as described previously with slight modifications [29,30]. Coronal sections were permeabilized with 0.3% Triton X-100/PBS and incubated overnight with primary antibodies, which included anti-Tbr2 (1:100, R&D Systems, RRID: AB_10569705), anti-Pax6 (1:200, Invitrogen, RRID: AB_10669586), anti-Ki-67 (1:1000, Invitrogen, RRID: AB_10853185), anti-reelin (1:500, Millipore, RRID: AB_2285132), anti-Sox2 (1:300, R&D, RRID: AB_355110), anti-phospho-histone H3 (1:1000, Millipore, RRID: AB_310016) and anti-ARX antibodies (1:1000, a gift from Dr. Kitamura) [4]. After incubation with secondary antibodies and Hoechst 33342, the sections were washed and mounted.

2.4. Microscopy

Images were obtained using a BIOREVO BZ-9000 (Keyence), an inverted Nikon Eclipse Ti2 confocal microscope (Nikon Instruments/Nikon Corp), an IX83 inverted microscope (Olympus) and an FV1000 confocal microscope (Olympus).

2.5. Cell counting

After background signals were removed using ImageJ software, the numbers of immunopositive cells were manually counted using the "cell counter" tool. The cell-dense layer next to the VZ was identified as the ISVZ, and the cell-sparse layer between the ISVZ and the IZ was identified as the OSVZ. In addition, the border between the ISVZ and the OSVZ was determined by the densities of Hoechst 33342-positive nuclei, Pax6-positive cells and Tbr2-positive cells. Statistical significance was determined using Student's *t*-test. "n" refers to the number of animals.

3. Results

3.1. The expression pattern of ARX in the germinal zones of the developing ferret cerebral cortex

To examine the expression of ARX in neural progenitors in the developing ferret cerebral cortex, we prepared coronal sections of the ferret cerebrum at embryonic day 40 (E40), when neural progenitors are abundantly distributed [21,22,24,31]. We then performed immunohistochemistry using anti-ARX antibody that has been shown to specifically recognize ARX in mice [4]. Consistent with previous studies using the rodent brain [4,32], ARX expression was observed in the SVZ of the ganglionic eminence, the cortical plate, the VZ and the ISVZ of the cerebral cortex in the ferret cerebrum (Fig. 1A and B), suggesting that this anti-ARX antibody also specifically recognizes ARX in the ferret cerebrum. ARX expression was also found in the marginal zone (MZ) of the cortex. Double immunostaining for ARX and reelin, which is a marker for Cajal-Retzius cells [33], showed that the majority of the reelin-positive Cajal-Retzius cells were negative for ARX (Fig. 1C). Previous studies showed that migrating interneurons are present in the MZ of the mouse cortex [34], leading us to conclude that the ARX-positive cells in the MZ are most likely migrating interneurons generated from the ganglionic eminence.

Interestingly, we found that ARX was also expressed in the OSVZ of the ferret cerebral cortex (Fig. 1B). Because the OSVZ is missing in the rodent cerebral cortex, we further investigated the detailed expression patterns of ARX in the OSVZ of the ferret cerebral cortex. Because previous studies demonstrated that neural progenitors in the OSVZ are reduced after birth [31], we examined ARX expression at a later stage. We performed ARX immunohistochemistry using coronal sections of the ferret cerebral cortex at P16 and found that ARX expression in the OSVZ was dramatically reduced between E40 and P16 (Fig. 1D). These results are consistent with the idea that ARX is expressed in neural progenitors in the OSVZ of the ferret cerebral cortex.

3.2. The expression of ARX in neural progenitors of the OSVZ in the ferret cerebral cortex

In the ferret OSVZ, there are two types of neural progenitors: oRG cells, defined as Pax6-positive/Tbr2-negative cells, and IP cells, which are Tbr2-positive [23,24,35]. To examine if ARX is expressed in neural progenitors in the OSVZ, we performed triple immunohistochemistry with anti-Pax6, anti-Tbr2 and anti-ARX antibodies (Fig. 2A). We found that ARX was expressed in both Pax6-positive/Tbr2-negative cells and Tbr2-positive cells in the OSVZ of the ferret cerebral cortex at E40 (Fig. 2B). We also found that many ARX-positive cells in the OSVZ were positive for Sox2, which is another oRG marker (Fig. 2C). These results suggest that ARX is expressed in both oRG cells and IP cells.

We then quantified the percentages of oRG cells and IP cells co-expressing ARX (Fig. 2D). Interestingly, we found that the percentage of ARX-positive cells was significantly higher in Pax6-positive/Tbr2-negative oRG cells than in Tbr2-positive IP cells (oRG, 43.0% ± 2.6; IP cells, 23.0% ± 3.4; $p = 0.003$; Student's *t*-test). This result suggests that ARX is more important in oRG cells than in IP cells in the ferret OSVZ.

3.3. The expression of cell proliferation markers in ARX-positive neural progenitors

It has been reported that ARX is important for the promotion of cell proliferation by regulating cell cycle exit [6,8]. To confirm that ARX-positive cells have cell proliferation activities, we performed quadruple immunohistochemistry with anti-Ki-67, anti-Pax6, anti-Tbr2 and anti-ARX antibodies (Fig. 3A). We found that many ARX-positive oRG cells and ARX-positive IP cells also expressed Ki-67 (Fig. 3B), suggesting that they are indeed proliferating cells. We then quantified the

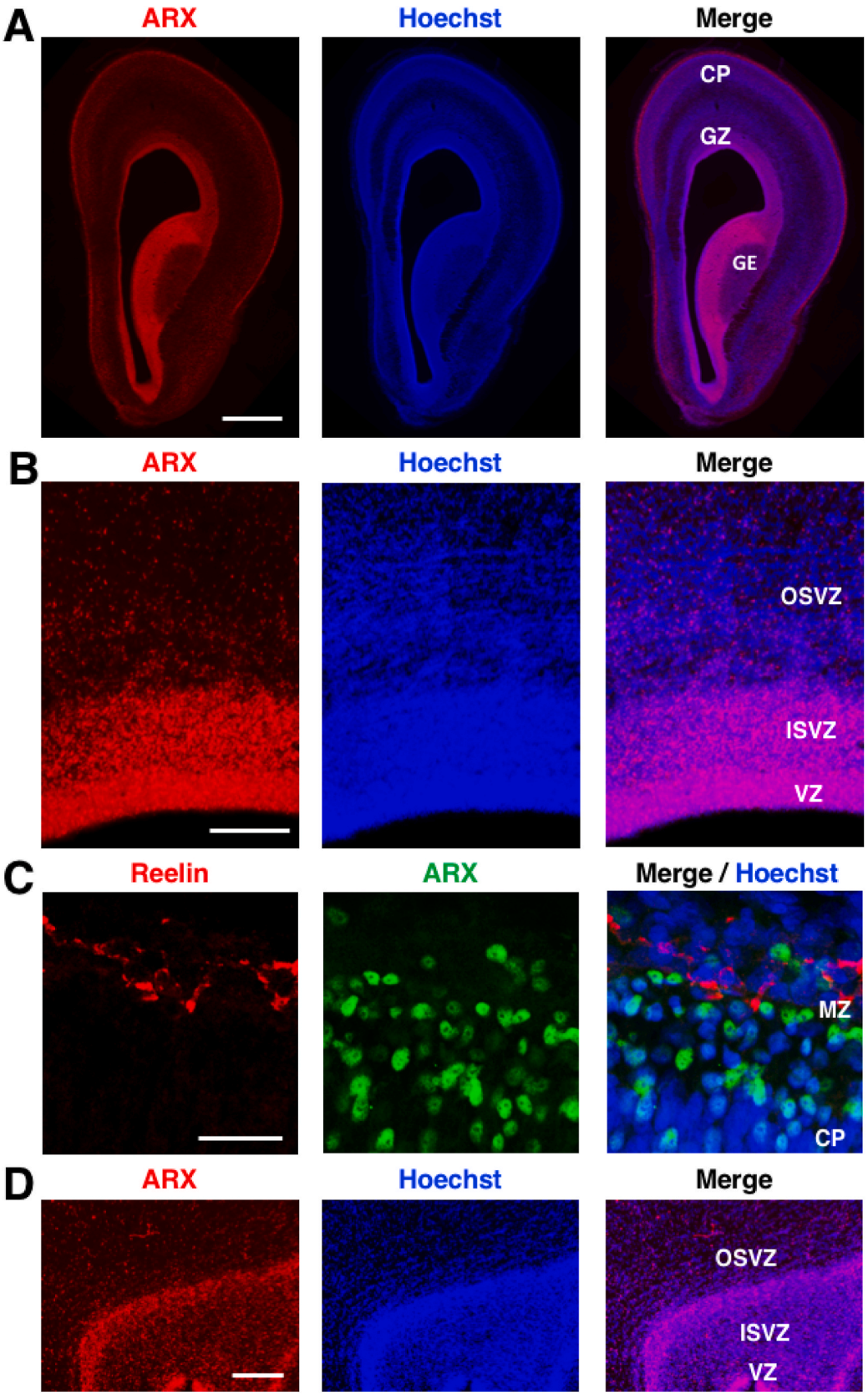

Fig. 1. The distribution of ARX expression in the germinal zones of the ferret cerebral cortex. **(A, B)** Coronal sections of the ferret cerebrum at E40 were stained using anti-ARX antibody (red) and Hoechst 33342 (blue). Lower magnification images (**A**) and higher magnification images of the germinal zones of the cerebral cortex (**B**) are shown. (**C**) Coronal sections of the ferret cerebrum at E40 were stained using anti-ARX antibody (green), anti-reelin antibody (red) and Hoechst 33342 (blue). High magnification images of the marginal zone are shown. **(D)** Coronal sections of the ferret cerebral cortex at P16 were stained using anti-ARX antibody (red) and Hoechst 33342 (blue). Higher magnification images of the germinal zones of the cerebral cortex are shown. MZ, marginal zone; CP, cortical plate; GZ, germinal zone; GE, ganglionic eminence; VZ, ventricular zone; ISVZ, inner subventricular zone; OSVZ, outer subventricular zone. Scale bars = 1 mm (**A**), 200 μm (**B, D**) and 30 μm (**C**). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

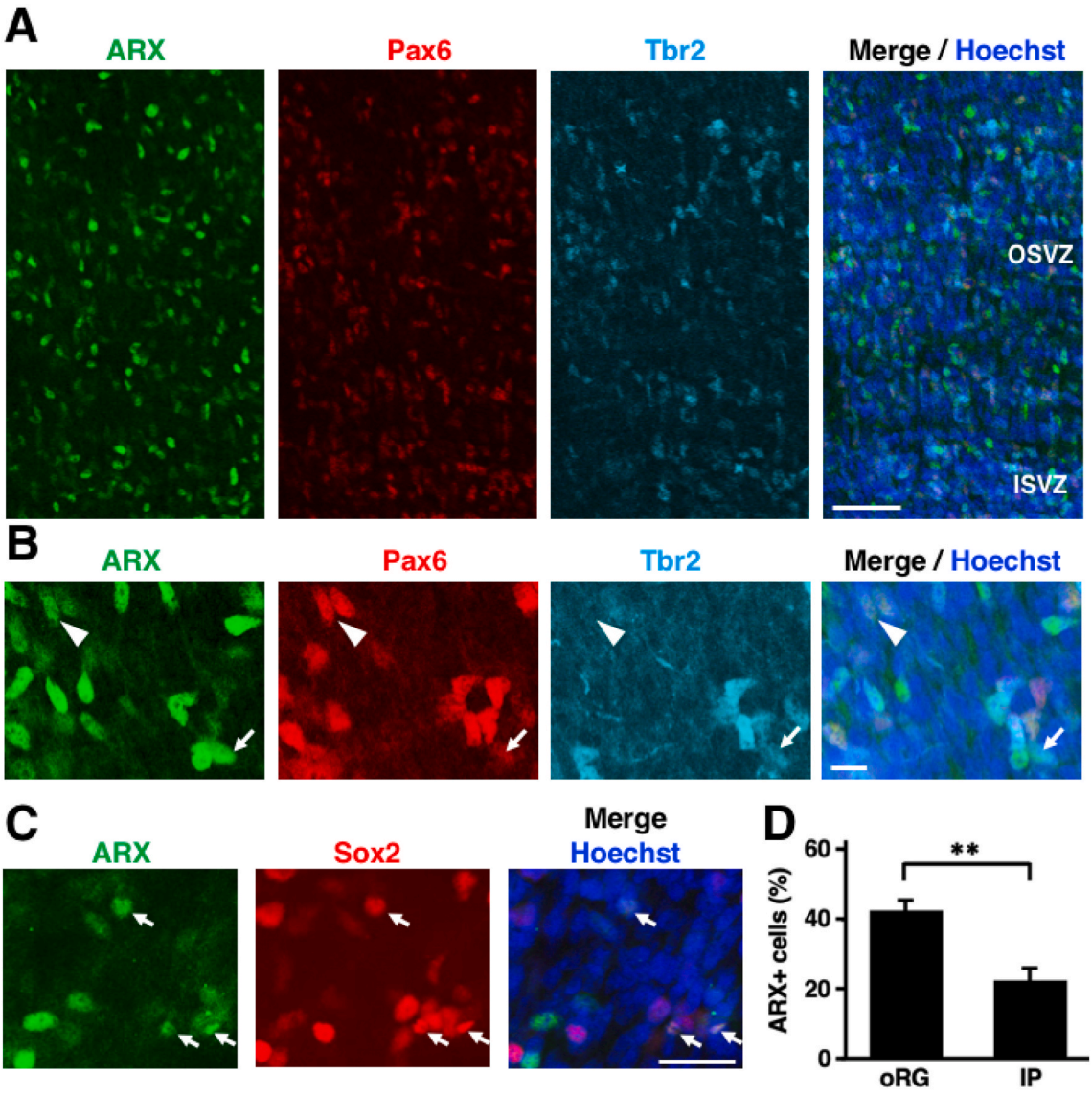

Fig. 2. ARX expression in neural progenitors in the ferret OSVZ. **(A)** Coronal sections of the ferret cerebral cortex at E40 were stained using Hoechst 33342, anti-ARX (green), anti-Pax6 (red) and anti-Tbr2 (cyan) antibodies. Images corresponding to the germinal zones are shown. **(B)** High magnification images of the OSVZ. Arrowheads and arrows indicate ARX-positive oRG cells and ARX-positive IP cells, respectively. (**C**) Coronal sections of the ferret cerebral cortex at E40 were stained using Hoechst 33342, anti-ARX antibody (green) and anti-Sox2 antibody (red). High magnification images of the OSVZ are shown. Arrows indicate ARX- and Sox2-positive cells. **(D)** The percentages of oRG cells and IP cells co-expressing ARX. Bars represent mean ± SD. Student's *t*-test; $^{**}p < 0.01$; n = 4 for each condition. Scale bars = 50 μm (**A**), 10 μm (**B**) and 25 μm (**C**). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

percentages of ARX-positive oRG cells and ARX-positive IP cells co-expressing Ki-67 (Fig. 3C). More than half of ARX-positive oRG cells and ARX-positive IP cells co-expressed Ki-67 (ARX-positive oRG cells, 52.8% ± 9.4; ARX-positive IP cells, 63.4% ± 6.1). Furthermore, we performed double immunostaining for ARX and phospho-histone H3 (pHH3), which is found in the nuclei of cells during M phase. We found that almost all ARX-positive cells were negative for pHH3, suggesting that ARX is not expressed in M-phase progenitor cells (Fig. 3D and E). This result is consistent with previous studies showing that ARX is important for maintaining the length of S phase, but not M phase [6,8]. These results may indicate that ARX was important for cell proliferation of neural progenitors in the OSVZ of the ferret cerebral cortex. Because primates and carnivores have an expanded OSVZ compared to rodents, it seems plausible that acquisition of ARX expression in the OSVZ led to the development of the OSVZ in primates and carnivores during evolution.

4. Discussion

Here we have shown that ARX is predominantly expressed in oRG cells in the OSVZ of the developing ferret cerebral cortex. Furthermore, ARX-positive neural progenitors exhibit high cell proliferation activity. Previous studies have shown that ARX is important for the maintenance of the cell cycle of neural progenitors in the mouse cerebral cortex [6,8]. In cerebral cortex-specific *ARX* knock out mice, cell proliferation of RG cells and IP cells was reduced, and as a result, their numbers decreased [8]. Targeted inhibition of ARX causes neural progenitors to exit the cell cycle prematurely and to adopt the neuronal fate in the mouse cerebral cortex [6]. It seems therefore conceivable that ARX also regulates cell proliferation of oRG cells in the OSVZ of the ferret cerebral cortex. In addition, oRG cells have a longer S-phase compared with IP cells in the ferret OSVZ [36]. Because our results showed that ARX is more abundantly expressed in oRG cells than in IP cells, ARX may regulate the

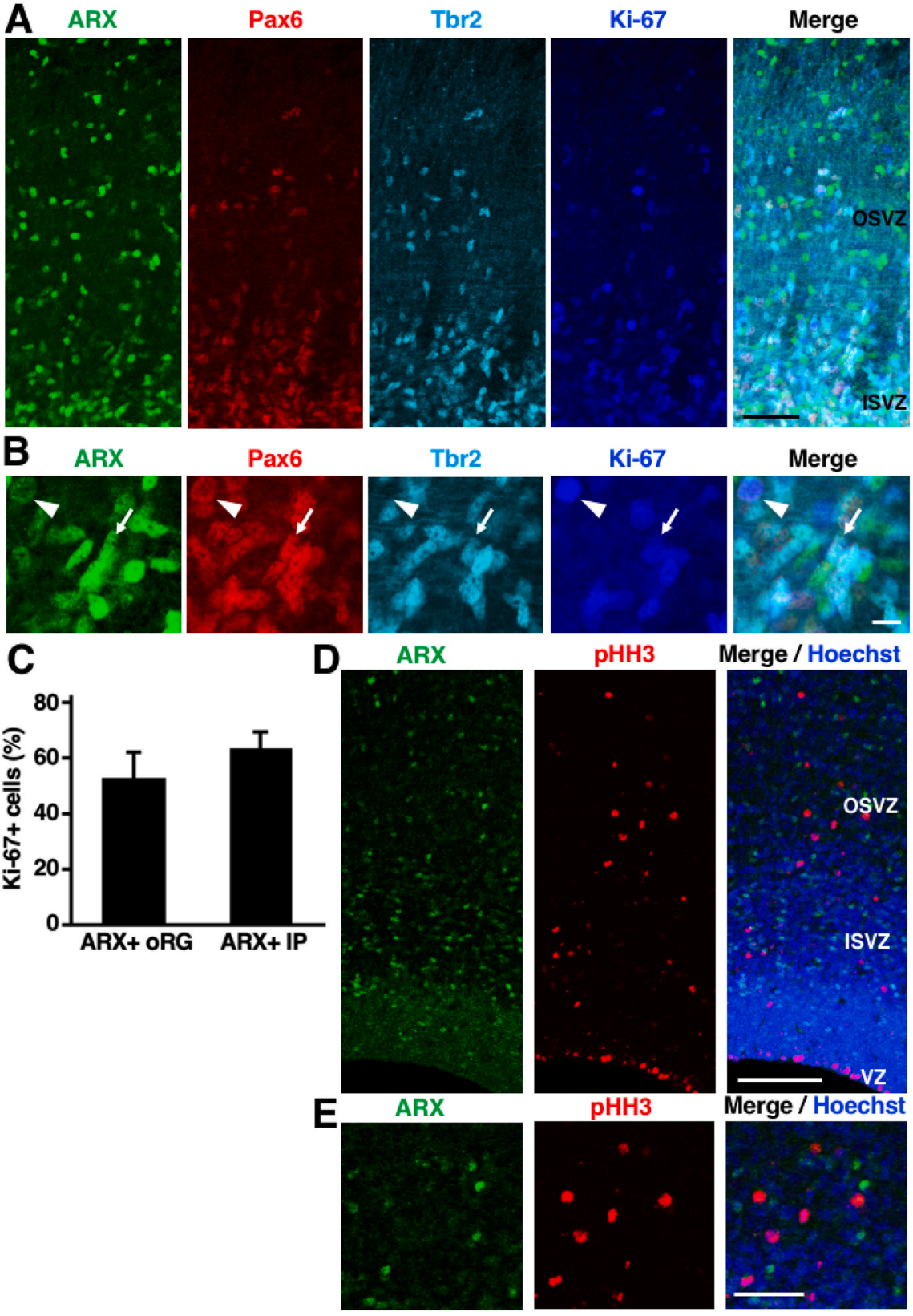

Fig. 3. The expression of Ki-67 in ARX-positive neural progenitors in the ferret OSVZ. **(A)** Coronal sections of the ferret cerebral cortex at E40 were stained using anti-ARX (green), anti-Pax6 (red), anti-Tbr2 (cyan) and anti-Ki-67 (blue) antibodies. Images corresponding to the germinal zones are shown. **(B)** High magnification images of the OSVZ. Arrowheads and arrows indicate ARX-positive oRG cells and ARX-positive IP cells co-expressing Ki-67. **(C)** The percentages of ARX-positive oRG cells and ARX-positive IP cells co-expressing Ki-67. **(D)** Coronal sections of the ferret cerebral cortex at E40 were stained using Hoechst 33342, anti-ARX antibody (green) and anti-pHH3 antibody (red). Images corresponding to the germinal zones are shown. **(E)** High magnification images of the OSVZ. Bars represent mean ± SD. Scale bars = 50 μm (**A**), 10 μm (**B**), 200 μm (**D**) and 25 μm (E). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

length of S-phase in neural progenitors.

Because it is difficult to investigate the roles of ARX in oRG cells using mice, ferrets should be useful to address this issue for the following reasons. First, we recently established genetic manipulation techniques for the ferret cerebral cortex using *in utero* electroporation and the CRISPR/Cas9 system [21,37,38]. These techniques enabled us to investigate the molecular mechanisms underlying cortical folding using ferrets [22,38–41] and should be applicable to examining the role of ARX in the developing ferret cerebral cortex. Second, one pregnant ferret mother usually gives birth to 6 or more babies. This large number of babies from one pregnant mother allows us to perform analyses under various experimental conditions and to obtain an adequate number of experimental samples.

However, in order to examine the roles of ARX in oRG cells, we need to overcome one limitation. It has been known that when plasmids are introduced into the cerebral cortex using *in utero* electroporation, gene expression is affected not only in oRG cells but also in RG cells and in IP cells. Because ARX is also expressed in RG cells and IP cells, and because oRG cells are produced from RG cells, genetic manipulation using *in utero* electroporation would affect ARX expression in both oRG cells and RG cells. Therefore, it is difficult to distinguish between cell-autonomous effects of *ARX* knockout in oRG cells and non-cell-autonomous effects of *ARX* knockout in RG cells on oRG cells. It would be intriguing to find an oRG cell-specific promoter to manipulate ARX expression in oRG cells specifically.

CRediT authorship contribution statement

Hiroki Maeyama: performed experiments. **Yohei Shinmyo:** and. **Hiroshi Kawasaki:** Writing – original draft.

Declaration of competing interest

We declare that we do not have competing financial interests.

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