Hif1α-dependent hypoxia signaling contributes to the survival of deep-layer neurons and cortex formation in a mouse model

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Abstract
Hypoxia-inducible factor 1 α (Hif1α) plays a crucial role in brain development. To study the function of Hif1α in early brain development, we generated neuroepithelial cell-specific Hif1α-knockout mice. Hif1α-knockout mice died soon after birth; these mice exhibited an abnormal head shape, indicating the presence of brain defects. Morphological analysis revealed that Hif1α ablation reduced the overall size of the brain, especially affecting the telencephalon. Neuronal apoptosis predominantly occurred in deep-layer neurons, consequently the alignment of cortical layers was severely disorganized in Hif1α knockout mice. Furthermore, we demonstrated that Vegf signaling contributes to the survival of deep-layer neurons as a downstream effector of Hif1α-dependent hypoxia signaling. Taken together, our findings demonstrate that Hif1α plays a critical role in the early stages of telencephalon development.

Keywords: Hif1α, Hypoxia, Cortex, Telencephalon, Mouse

Introduction
In placental mammals, the embryo encounters drastic changes in the environmental oxygen concentration during development [1–4]. Even after the supply of oxygen from maternal blood, embryonic tissues, such as the neural tube, heart primordia, and intersomitic mesenchyme, continue to experience hypoxia [5, 6]. Proper development of mammalian embryos requires their adaptation to drastic changes in oxygen concentration. Therefore, mammalian embryos employ unique molecular pathways to enable gastrulation and organogenesis within a transient hypoxic environment. Indeed, it has been reported that culturing rodent embryos ex o utero under hyperoxic conditions leads to abnormal embryo morphology [7].

Hypoxia-inducible factor 1 α (Hif1α), a key transcription factor that forms a complex with Hif1β, is involved in the cellular response to anaerobic conditions [8–11]. The stability of the Hif1α–Hif1β complex is regulated by the enzyme prolyl hydroxylase 1–3 (PHD1-3) in an oxygen-dependent manner. Under anaerobic conditions, the Hif1α–Hif1β complex is not hydroxylated, and therefore, is not targeted for degradation. The stabilized Hif1α–Hif1β complex binds to the hypoxia response element (HRE) to activate the expression of genes involved in energy metabolism, erythropoiesis, and angiogenesis, thereby protecting cells and tissues from hypoxic stress [12, 13]. In mammalian embryos, a Hif1α-dependent hypoxic response is required not only for the prevention of damage caused by hypoxic stress but also for proper progression of embryonic development. Hif1α-deficient mouse embryos exhibit defects in cardiovascular formation, somitogenesis, and neural tube closure, resulting in developmental arrest and lethality by embryonic day (E) 11 [14–17]. Furthermore, studies have shown that Hif1α...
is required for the formation of the heart, cartilage, and limbs, using conditional \textit{Hif1a}–knockout mice [18–20]. Regarding the central nervous system (CNS), Christian et al. demonstrated that the ablation of \textit{Hif1a} using the Emx1-Cre driver causes apoptosis of neurons and cortical hypoplasia in the cerebral cortex [21]. However, the function of \textit{Hif1a} in cortical development has not been examined in detail. \textit{Hif1a}-knockout mice have been generated using Nestin-Cre driver to observe a more broad-spectrum effect on neural development. Studies using Nestin-Cre driver have revealed that the ablation of \textit{Hif1a} disrupts angiogenesis and consequently, expands a hypoxic region in the telencephalon at E16 [22]. Neural cell-specific ablation did not affect brain development until E15, but caused massive neuronal apoptosis in the telencephalon at E19, resulting in hydrocephalus at postnatal day (P) 70 [23]. Although Nestin-Cre mice express Cre mRNA throughout the CNS at E11.5, it is uncertain whether Cre is expressed before E11.5 [24]. In addition, Cre recombination activity is not detected in the ventricular zone (VZ) and subventricular zone (SVZ) at E12.5 and E14.5 in the commercially available Nestin-Cre line. Therefore, the Nestin-Cre line may be inefficient as a Cre-driver for recombination in embryonic neural progenitor cells [25]. Therefore, novel functions of \textit{Hif1a} in early brain development could be discovered using other Cre-driver mice that express Cre within embryonic neural progenitor cells before E11.5. Herein, we investigated the function of \textit{Hif1a} by neuroepithelial cell-specific ablation of \textit{Hif1a} using Sox1-Cre [26]. In the Sox1-Cre driver, Cre activity was observed in neuroepithelial cells at least from E8.5; thus, the crossing of \textit{Hif1a}-floxed mice with Sox1-Cre mice allow us to analyze the function of \textit{Hif1a} in early neural development. In the present study, we demonstrated that \textit{Hif1a} is required for neuronal survival, thereby facilitating the formation of cortical layers during telencephalon development.

**Results**

**Generation of neuroepithelial cell-specific \textit{Hif1a}–knockout mice**

To address the biological significance of hypoxia in early brain development, we focused on deleting \textit{Hif1a}, a master regulator of cellular response to hypoxia [8–11]. We crossed \textit{Hif1a} \textit{floxed} mice [16] with Sox1-Cre\textsuperscript{+/-} mice [26] to selectively ablate \textit{Hif1a} in early neural progenitor cells, thereby affecting all CNS cells. The resulting \textit{Hif1a} gene carrying an exon 2 deletion encoded a malfunctioning \textit{Hif1a} protein that lost the ability to induce the expression of genes containing HRE [16]. We confirmed the tissue specificity of Cre expression driven by Sox1 promoter by generating Sox1-Cre\textsuperscript{+/-}; ROSA26/CAG-floxed STOP-tdTomato\textsuperscript{+/-} reporter mice. Cre expression was readily detected by the red fluorescent signal in developing cortical neurons, but not in vasculature at E16.5, indicating neuroepithelial cell-specific expression of Cre driven by Sox1 promoter (Additional file 1: Figure S1). Thus, we investigated the function of \textit{Hif1a}-dependent hypoxic response in neural progenitor cells and their progeny during brain development. Het erozygotes (\textit{Hif1a}\textsuperscript{floxed/wt}; Sox1-Cre\textsuperscript{+/-}) were viable and fertile, indicating that ablation of a single \textit{Hif1a} allele or the expression of Cre did not affect CNS development. In contrast, homozygotes (\textit{Hif1a}\textsuperscript{floxed/floxed}; Sox1-Cre\textsuperscript{+/-}) conditional mutants died within several hours after birth. Mice homozygous for the floxed \textit{Hif1a} allele with a Sox1-Cre allele (\textit{Hif1a}\textsuperscript{floxed/floxed}; Sox1-Cre\textsuperscript{+/-}) were used to generate conditional knockout mice, which are hereafter referred to as “KO” mice. Mice homozygous for the floxed \textit{Hif1a} allele without a Sox1-Cre allele (\textit{Hif1a}\textsuperscript{floxed/floxed}; Sox1-Cre\textsuperscript{+/-}) were used as controls and are hereafter referred to as “WT” mice.

We confirmed the deletion of \textit{Hif1a} using in situ hybridization with an antisense probe against exon 2. At E8.5, \textit{Hif1a} mRNA was predominantly expressed in neuroepithelial cells and mesenchymal cells of WT embryos. The expression patterns and levels of \textit{Hif1a} mRNA in KO embryos were comparable with those in WT embryos (Fig. 1A and B). At E9.5, \textit{Hif1a} continued to be expressed in neuroepithelial cells and mesenchymal cells in WT embryos (Fig. 1C). In contrast, \textit{Hif1a} expression diminished in the neural tube of KO embryos, although it remained unchanged in mesenchymal cells at E9.5 (Fig. 1D). To further validate the ablation of \textit{Hif1a}, the expression of \textit{Hif1a} and its target genes, \textit{Ldha}, \textit{Aldoa} (encoding a glycolytic enzyme) and \textit{Epo} (encoding erythropoietin), was analyzed using RT-qPCR. \textit{Hif1a} expression was slightly decreased by loss of \textit{Hif1a}, while \textit{Hif1a} target genes were expressed in KO embryos at levels similar to those in WT embryos at E8.5 (Fig. 1E). However, \textit{Hif1a} expression decreased by approximately 60% in KO embryos relative to that in WT embryos at E9.5 (Fig. 1F), which was consistent with the results of in situ hybridization showing the near-complete loss of \textit{Hif1a} transcripts in the neuroepithelium (Fig. 1D). Accordingly, the expression of \textit{Hif1a} target genes was downregulated in E9.5 KO embryos (Fig. 1F; \textit{Ldha}, reduced by 62%; \textit{Aldoa}, by 77%; and \textit{Epo}, by 59%). We isolated total RNA from whole mouse embryos and then prepared cDNA for RT-qPCR. Although it is preferred that total RNA is isolated exclusively from the neuroepithelium, this is technically challenging in early-stage embryos. Therefore, the reduced expression of \textit{Hif1a} and \textit{Hif1a} target genes would have been more severe if cDNA prepared from isolated neuroepithelium was used. As controls, we evaluated the expression of \textit{EpoR} (encoding erythropoietin
receptor) and Nqo1 (encoding electron respiratory chain enzyme), which are not dependent on Hif1α. The results showed that their expression was not altered in KO embryos at E9.5 (Fig. 1F); consequently, it was inferred that the decrease in Ldha, Aldoa, and Epo expression levels was not due to a deficit in respiratory metabolism and erythropoiesis, but instead reflected the loss of Hif1α-dependent gene regulation in the early neuroepithelium. Taken together, these results indicate that Hif1α was specifically ablated in neuroepithelial cells as early as E9.5 in KO embryos.

**Hif1α ablation causes severe brain defects**

Next, we examined the gross morphology of embryos and pups. Although KO embryos were indistinguishable from WT embryos in appearance until E12.5, they exhibited an abnormal-shaped head at E14.5. The length of the head along the sagittal axis was substantially reduced in KO embryos compared to that in WT...
embryos at E14.5 (Fig. 2A and B, curved two direction arrow). At E16.5, the parietal region of the head was flattened in KO embryos (Fig. 2D, arrowhead), which became more evident by P0 (Fig. 2F, arrowhead). Head abnormalities typically indicate brain hypoplasia; thus, we next examined the morphology of the brain. The brain of KO embryos was smaller than that of their WT counterparts, particularly the telencephalon at E14.5 and E16.5 (Fig. 2G–J). The brain of P0 KO pups exhibited considerable hypoplasia compared to that of WTs. Notably, the cerebrum of KO pups was extremely small compared to that of WT littermates (Fig. 2K and L). Collectively, these results demonstrate the requirement of Hif1α for normal development of the brain, especially that of the cerebrum.

**Neurons undergo massive apoptosis in Hif1α-ablated embryos**

Because the telencephalon was most acutely affected by the loss of Hif1α, we investigated the role of Hif1α-dependent hypoxia signaling in telencephalic development. The ablation of Hif1α has also been reported to induce apoptosis of neuronal cells in the telencephalon [21, 23]. Thus, we examined apoptosis using immunofluorescent labeling of cleaved caspase3, a marker of apoptotic cells. Some apoptotic cells were detected in the

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*Fig. 2* Conditional ablation of Hif1α in neuroepithelial cells results in a smaller telencephalon. Morphology of the head of WT (A, C, and E) and KO (B, D, and F) mice at E14.5, E16.5, and P0 are shown. Abnormally flattened parietal region is indicated by arrowheads. Scale bars, 1 mm. Morphology of whole-brain of WT (G, I, and K) and KO (H, J, and L) mice at E14.5, E16.5, and P0 are shown. Scale bars, 1 mm. WT, wild type; KO, knockout.
dorsal telencephalon of KO embryos at E12.5 (Fig. 3B). By E14.5, KO embryos displayed enhanced levels of apoptotic cells in the pallium (dorsal telencephalon) and the subpallium (ventral telencephalon) (Fig. 3D). The number of apoptotic cells in KO embryos was further increased in both the pallium and subpallium by E16.5 and E18.5 (Fig. 3F and H).

Cerebral cortex layers are disorganized by the loss of Hif1α

We further analyzed apoptosis in the KO cortex at P0. A large proportion of apoptotic cells resided in the Tuj1+ neuronal layer of the cortex, and few apoptotic cells were detected in the intermediate, subventricular, and VZ (Fig. 4B). This observation suggests that Hif1α loss led to the preferential elimination of postmitotic neurons, and consequently led to the reduction of the size of the cerebrum. We counted the number of Satb2+ and Ctip2+ cortical neurons in four arbitrarily defined regions (100 μm-wide) in the P0 cortex. Satb2 is intensely expressed in upper-layer neurons (layers II–IV), whereas Ctip2 is mainly expressed in deep-layer neurons (layers V and VI) in the cortex. The number of Satb2+ and Ctip2+ cells in the P0 cortex was reduced by approximately 88% and 49%, respectively, compared to WT controls (Table 1). This indicated that deep-layer neurons are more sensitive to Hif1α ablation than upper-layer neurons. Remarkably, apoptotic cells formed aggregates in the cortex (Fig. 4B), implying a disruption of the cortical layer formation in the KO cortex. The cortex consists of molecularly distinct Satb2+ upper layer and Ctip2+ deep layer projection neurons in the cerebrum of WT embryos (Fig. 4C). In contrast, each cortical layer was obscured, and Satb2+ and Ctip2+ cells were broadly scattered throughout the KO cortex (Fig. 4D).

The disorganized cortical layers was reminiscent of the phenotype of Reelin-deficient mice, reeler [27]. Cajal-Retzius (C-R) cells, which occur in the most superficial marginal zone layer of the cerebral cortex, regulate
cortical layer formation through the secretion of Reelin [28, 29]. Therefore, the Sox1-Cre-mediated deletion of Hif1α during early telencephalic development may have affected the survival of C-R cells. However, C-R cells were detected in the most superficial layer (layer I) of the cortex, underneath the pia, in both WT and KO telencephalons (Fig. 4E and F), suggesting that the Reelin-mediated guidance of postmitotic neurons is intact in the KO cortex.

**Neuronal migration is impaired in Hif1α-ablated cortex**

To explore the mechanism inducing neuronal apoptosis by loss of Hif1α, we examined whether upper- or deep-layer neurons undergo apoptosis via immunofluorescent staining using anti-cleaved caspase3 and anti-Satb2 or anti-Ctip2 antibodies (Fig. 5A–D). We observed that many Ctip2+ cells were co-labeled with cleaved caspase3 (Fig. 5D, inset). The number of Satb2+/cleaved caspase3+ double-labelled cells was lower than that of

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**Table 1** The number of Satb2- and Ctip2-positive cells in P0 cortex

|               | Satb2+ cells (%) | Ctip2+ cells (%) |
|---------------|------------------|------------------|
| WT            | 130.13 ± 8.74 (100) | 32.13 ± 7.08 (100) |
| KO            | 114.25 ± 7.31 (87.80) | 15.63 ± 3.08 (48.64) |

The number of Satb2- and Ctip2-positive cells were counted in P0 cortex (100 μm width) on 8 histological sections obtained from 4 different pups.
Ctip2+/cleaved caspase3+ cells in the E18.5 KO cortex (Fig. 5B, inset). Specifically, approximately 8.4% and 35.1% of total cleaved caspase3+ cells were co-labeled with Satb2 and Ctip2, respectively (Fig. 5E). This demonstrated that deep-layer neurons preferentially undergo apoptosis in the absence of Hif1α, which was consistent with data showing a predominant reduction of deep-layer neurons in the KO cortex (Table 1). Approximately 56% of all cells were cleaved caspase3+ cells, thereby explaining the severe loss of layer marker expression.

Many Satb2+ upper-layer neurons were detected around apoptotic cell aggregates, especially on the apical side of the KO cortex (Fig. 5A, yellow arrowheads). Consistent with this, Satb2+ cells were sparsely distributed on the pial surface side of apoptotic cell aggregates. Conversely, Ctip2+ neurons were abnormally detected near the pial surface side where the upper layers were formed in the normal cortex (Fig. 5C, white arrowheads). The cerebral cortex consists of six neuronal layers that develop in an inside-out manner, i.e., early-born neurons settle in the deep layers, whereas late-born neurons migrate through the deep-layer neurons and form more superficial layers (upper-layers) [30]. Thus, we hypothesized that the migration of the upper-layer neurons from the VZ to the pial surface was impeded by apoptosis of deep-layer neurons, resulting in a partial reversal of the cortical layer in the KO cortex. To test this hypothesis, we performed a BrdU birthdating assay. To label the upper-layer neurons, BrdU was injected into pregnant mice at E14.5, when upper-layer neurons are being born, and then the position of BrdU-labeled neurons were compared between KO and WT cortices at E18.5. A large proportion of BrdU+ neurons were located in the upper-layers of the WT cortex (corresponding to bins 1 and 2; Fig. 6A and B). In contrast, many BrdU+ neurons were detected in the deeper-layers of the KO cortex. Importantly, most BrdU+ neurons were negative for apoptosis markers (Fig. 6C and D), which is consistent with the results shown in Fig. 5B and E. The distribution of BrdU+ neurons born at E14.5 was quantified in five
Fig. 6 Neural migration of upper-layer neurons is impaired by loss of Hif1α. Apoptotic cells and BrdU-incorporated neurons in coronal sections of WT and KO cortices at E18.5 are detected with anti-cleaved caspase3 and anti-BrdU antibodies (A–D). Scale bars, 100 μm (A–D). Three independent experiments are performed and one representative image is shown, respectively. The percentage of BrdU+ cells in the cortex (bins 1–5, 500 μm-wide) is presented as a histogram (G). The total number of BrdU+ cells in the cortex (500 μm-wide region) is presented as a bar graph (H). Gray bar, WT; black bar, KO. Data are the mean ± S.E.M. of four sections. Statistical differences were assessed using Student’s t-test, *p < 0.05. WT, wild type; KO, knockout.
arbitrarily defined regions (500 μm-wide) in the cortex. In the WT cortex, approximately 72% of BrdU+ neurons were present in the upper-layers (bins 1 and 2). In contrast, the percentage of BrdU+ neurons in the presumptive area of the upper-layers (bins 1 and 2) was reduced to approximately 36% in the KO cortex (Fig. 6G). Approximately 42% of BrdU+ neurons populated the middle area (bins 3 and 4) in the KO cortex, where a large number of apoptotic cells were observed. In contrast, approximately 21% of BrdU+ neurons were detected in the same region of the WT cortex (Fig. 6G). Notably, the total number of BrdU+ neurons in the KO cortex was comparable with that of BrdU+ neurons in the WT cortex, suggesting that the proliferation of neural progenitors of upper-layer neurons was not altered by the loss of Hif1α (Fig. 6H). Taken together, these results strongly support our hypothesis that the migration of upper-layer neurons was impeded by massive apoptosis of deep-layer neurons in the KO cortex.

Postmitotic neurons migrate towards the pia along the radial glial cell fiber spanning the length of the developing cortical plate, and is important for proper ‘inside-out’ neural migration [27, 28, 30]. Almost radial glial fibers spanned the entire cortex in both of WT and KO telencephalon (Additional file 2: Figure S2). This result suggests that the radial glial fibers-dependent neural migration is intact in the KO cortex.

Possible mechanism underlying the role of Hif1α in cortical development

Previous studies demonstrated that vascular endothelial growth factor (encoded by Vegf), a downstream target of Hif1α, functions not only as an angiogenic growth factor but also as a neurotrophic factor [31, 32]. It has also been demonstrated that neuronal cell-specific Vegf homozygous knockout mice die within 24 h after birth due to the dysmorphogenesis of the cortex caused by massive neuronal apoptosis [33, 34]. Moreover, as apoptotic cells seem to accumulate in the middle area of the cortex [33], the Vegf homozygous knockout phenotype resembles that of our Hif1α homozygous knockout mice. We therefore investigated if the reduced Vegf expression was associated with reduced survival of deep-layer neurons upon Hif1α loss. Vegf is expressed in the ventral layer of the brain at E14.5 [35]. First, we analyzed Vegf expression levels in WT and KO telencephalons at E13.5, when deep-layer neurons are born. To analyze this, the dorsal part of the telencephalon, including the cortex, was surgically isolated, and RNA was prepared for RT-qPCR. We confirmed a significant reduction in Vegf expression in the cortex of KO mice (Fig. 7A). Next, we examined whether Vegf signaling activity is required for the survival of deep-layer neurons. Although the suppression of Vegf receptor function is the ideal method to inhibit the input of Vegf signaling, known receptors for Vegf, such as Flk1, Flt1, Nrp1, and Nrp2, do not appear to be involved in cortical development and thus unlikely targets to manipulate Vegf signaling in the developing brain [34, 36–38]. Therefore, we took advantage of the ligand-binding domain of the Vegf receptor, sFlt1, to interfere with Vegf signaling [39]. It has been reported that the overexpression of sFlt1 inhibits Vegf signaling in vivo [40]. We thus hypothesized that secreted sFlt1 functions as a decoy Vegf receptor and inhibits paracrine and/or autocrine Vegf signaling inputs to neural progenitors and/or neurons of deep layers. The expression construct of mouse sFlt1 was introduced into neural progenitor cells of E13.5 WT embryos using in utero electroporation along with the EGFP expression construct. At E18.5, numerous EGFP+sFlt1-overexpressing cells were co-labeled with apoptotic markers, while no apoptosis was observed in the control cortex (Fig. 7B–F). However, we could not detect apoptotic cell aggregates, which may be due to the variation in transfection efficiency of sFlt1 (Fig. 7D and E). Next, we investigated cortical layer formation in the sFlt1-transfected cortex. Satb2+ neurons were dispersed in the apical side of the cortex where deep-layer neurons normally settle in the control cortex (Fig. 7G, H, K, L). The Ctip2+ neuron layer was considerably shifted to the pial surface in the transfected region of sFlt1, consistent
with the accumulation of upper-layer neurons in the deep layers of the KO cortex (Fig. 7I, J, M, N). In contrast, apoptosis and cortical layer defects were not induced when the sFlt1-expressing construct was electroporated into neural progenitor cells at E15.5 (Fig. 7O–T), suggesting that Vegf signaling is not required for the survival of...
upper-layer neurons. Taken together, inhibition of Vegf signaling by the overexpression of sFlt1 mimics cortical development defects seen in KO embryos.

As we postulated that neuronal apoptosis is due to the decrease in Vegf levels in the KO cortex, it is important to identify the source of Vegf in the telencephalon. Unfortunately, we could not detect the expression of Vegf mRNA and Vegf protein in the telencephalon using in situ hybridization and immunofluorescence (data not shown). Instead of that, we tried to determine whether Vegf functions in cell-autonomous or non-cell-autonomous manner. The expression construct of Cre recombinase was introduced into E13.5 Hif1α flox/flox telencephalon using in utero electroporation. At E18.5, EGFP ‑Hif1α ‑ablated cells did not undergo apoptosis (Fig. 8A), suggesting the non-cell-autonomous function of Vegf for the survival of deep-layer neurons. Notable, many EGFP + Hif1α ‑ablated cells were localized more apical side compared to EGFP+ cells in control cortex (Fig. 8B and E). This result indicates that, besides regulating the survival of deep-layer neurons in non-cell-autonomous manner, Hif1α functions cell-autonomously in neuronal migration.

Discussion
In the present study, we elucidated the role of Hif1α in early-stage telencephalic development. We observed that neuroepithelial cell ‑specific ablation of Hif1α causes massive apoptosis of neurons in the telencephalon and cerebral cortex, which manifests as reduced brain size. We demonstrated that deep-layer neurons predominantly undergo apoptosis by Hif1α ‑ablation, and their loss affects the radial migration of upper-layer neurons and subsequent cortical layer formation. Furthermore, we showed that Vegf acts as a neurotrophic factor likely acting downstream of Hif1α to promote the survival of deep-layer neurons (Fig. 9).

Neural cell-specific knockout of Hif1α using Nestin ‑Cre driver causes massive apoptosis in the cerebrum at E19, resulting in hydrocephalus at P70, while no gross morphological differences can be observed in E15 embryos [23]. In our KO embryos, apoptosis occurred as early as

![Caspase3](image1)

**Fig. 8** Vegf functions as the survival factor for deep-layer neurons in non-cell-autonomous manner. pEGFP-CAGS (control) or pCAG-Cre:GFP (Cre:GFP) plasmid was electroporated into neural progenitor cells in one side of telencephalic semisphere of Hif1α flox/flox embryo at E13.5. Cleaved caspase3+ apoptotic cells (magenta) and EGFP+ transfected cells (green) were detected in coronal sections of the cortex at E18.5. Scale bars, 100 μm. Three independent experiments are performed and one representative image is shown, respectively.
E12.5, and gross morphological abnormalities of the head were apparent at E14.5 (Fig. 2). These phenotypic differences are presumably the result of the timing of Cre-mediated recombination in the Nestin-Cre vs. Sox1-Cre driver alleles. In the Nestin-Cre mouse line, Cre mRNA expression is detected by E11.5; however, it is unclear if Cre is expressed at earlier stages [24]. In contrast, Cre-mediated recombination was evident as early as E9.5 in our Sox1-Cre-mediated Hif1α KO embryos (Fig. 1D and F). Liang et al. indicated that Cre-mediated recombination occurs at sufficient levels in postmitotic neuronal cells, and not only in neural progenitors in Nestin-Cre embryos [25]. Thus, by restricting the recombination to early neuroepithelial cells using the Sox1-Cre driver, we produced a more complete neural-specific ablation of Hif1α and revealed a specific function for the gene in early telencephalic development. Sox1 mRNA expression begins at the anterior neuroepithelium of wild-type mouse embryos at E8.0 [41]. Consistent with this, Cre recombination activity has been detected in the neuroepithelium of Sox1-Cre; ROSA26R-EYFP, at least E8.5 onward [26]. However, Cre-mediated recombination was not detected at E8.5 in Sox1-Cre-mediated Hif1α KO embryos using in situ hybridization. The delay in Cre activity may be related to the difference in recombination efficiency of the ROSA26R allele compared to that of the Hif1α flox/flox allele. Dorsal forebrain radial glial cell-specific knockout of Hif1α using Emx1-Cre driver also causes apoptosis in the telencephalon. Emx1-Cre-mediated homozygous Hif1α mutant mice are viable and fertile, similar with Nestin-Cre-mediated homozygous Hif1α mutant mice. Notably, heterozygous ablation of Hif1α using Emx1-Cre driver causes precocious neurogenic differentiation of neural progenitor cells, resulting in mild
microcephaly [21]. In contrast, heterozygous ablation of Hif1α using Sox1-Cre did not alter the number of Sox2+ apical neural progenitor cells and Tbr2+ basal neural progenitor cells (Additional file 3: Figure S3). We thus speculate that these phenotypic differences are due to the timing, or the level of Cre expression in the Emx1-Cre vs. Sox1-Cre driver alleles.

Massive apoptosis of deep-layer neurons appears to be the major cause of cortical layer formation deficiency in the KO cortex. However, the mechanism by which apoptotic elimination disturbs the migration of upper-layer neurons is still elusive. One possibility is the loss of the chemotactic gradient for upper-layer neurons toward the pia. For example, the endogenous gradient of Semaphorin-3A (Sema3A) functions as a guidance factor and regulates the migration of upper-layer neurons [42]. Sema3A mRNA is highly expressed in outer cortical layer neurons of the rat cortex from E14 (nearly equal to E12 mouse cortex) which correspond with deep-layer neurons. Thereafter, Sema3A protein is detected across the telencephalon, blood vessels regressed and formed punctate structures in the proximity of apoptotic cell aggregates, instead of elongated networks (Additional file 4: Figure S4). A similar phenotype is observed in the Nestin-Cre-mediated Hif1α KO embryos, suggesting that a deficiency of vascular network formation impacted on the survival of neurons [23]. Therefore, it is difficult at present to define the cellular mechanism of Vegf signaling in deep-layer neuron survival. However, our overexpression experiment showed that vascular networks normally develop whereas cortical layer formation is disturbed in the sFlt1-transfected cortex (Additional file 5: Figure S5). These data suggest the primary role of Vegf as a survival factor for neurons rather than an endothelial cell growth factor during cortical development. In support of our findings, Lange et al. demonstrated that Gpr124-LacZ knock-in null embryos do not exhibit any neuronal apoptosis, even though the vasculature is largely diminished in the cortex [21]. In addition, the formation of the cortical vascular network is non-cell autonomously regulated by Vegf, which is released from neurons [36]. Collectively we speculate that Vegf expression might reduce by neuronal apoptosis, and the deficiency in Vegf affect vascular outgrowth and extension in cortical tissue of KO brain. Our findings emphasize that the function of Vegf for the survival of neurons is essential for cortical layer formation. However, we can not exclude the possibility that Vegf regulates neural migration as well. In future studies it will be important to confirm this possibility.

In conclusion, our findings provide insights into the molecular mechanisms underlying the development of murine embryonic telencephalon, which is regulated by Hif1α-dependent hypoxia signaling.

Methods
Mice

All mice were maintained on a C57BL/6 background. Hif1α flox/flox mice (Stock No. 007561) and ROSA26/CAG-floxed STOP-tdTomato mice (Stock No. 007905) were obtained from The Jackson Laboratory, and Sox1-Cre+/- mice from the RIKEN Bioresource Research Center (Accession No. CDB0525K, http://www2.clst.riken.jp/arg/mutant%20mice%20list.html) [26]. Hif1α flox/flox mice and heterozygotes (Hif1α flox/wt, Sox1-Cre+/-) were crossed to obtain homozygotes (Hif1α flox/flox, Sox1-Cre+/-).
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For embryonic staging, the morning on which the vaginal plug was observed was designated as E0.5.

RT-qPCR
Total RNA was extracted from E8.5 and E9.5 WT and KO embryos, or the cortex of E13.5 WT and KO brains using the RNeasy Mini Kit (Qiagen) according to the manufacturer’s instructions. Isolated RNA was then reverse-transcribed to cDNA using PrimeScript 1st strand cDNA Synthesis Kit (Takara) according to the manufacturer’s instructions. qPCR using SYBR green was performed on a LightCycler Nano System (Roche). Gene expression was normalized to Gapdh expression levels. Each sample was analyzed at least in triplicate. The relative fold change was calculated using the $2^{-\Delta \Delta Ct}$ method. For detection of Hif1α, Ldha, Aldoa, Epo, EpoR, Nqo1, Vegfa, and Gapdh expression levels, the following primers were used: Hif1α FW: TGAGCTTGCTCATCAGTTCG, Hif1α RV: CAT AACAGAAGCTTTATCAAAGATGTGA, Ldha FW: GGC ACTGACGCAGACAAG, Ldha RV: TGATCACCCTCGT AGGCACGTG; Aldoa FW: TGGGAAAGAGGAGAAGCT GA, Aldoa RV: GACAAGCGAGGCTTGGG; Epo FW: TCTCGCACAGTCGAGTCTG, Epo RV: CTTCGCA CAACCCATCGT; EpoR FW: GTCCTCATCTCGTC TGTCT, EpoR RV: ATGCCAGCGCAGATCTCTCT; Nqo1 FW: AGCGTTCGTATTACGATCC, Nqo1 RV: AGT ACAATCAGGGCTTCTTCTG; Vegfa FW: CTGTGT CAGAGCGGAGAAAGC, Vegfa RV: ACACCTGCAAGT ACGTTCGTT; Gapdh FW: CATGTTCAGATGTA CTCCACTC, Gapdh RV: GGCCTCACCCCATTGTG.

In situ hybridization
A part of the mouse Hif1α sequence was PCR-amplified using the following primer set: FW-Hif1α: AAAGAG TCTGAGGTTTTATGAG and RV-Hif1α: CTTCTT AAGCTTATCAAAAAAGCCA. The PCR products were cloned into the pGEM-T Easy vector (Promega) to synthesize the cRNA probe.

Whole-mount and cryosection in situ hybridization was performed as described previously [44, 45].

Immunofluorescence
Mouse brains were fixed with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for 3 h at 4 °C and cryopreserved in 30% sucrose in PBS. Brains were embedded in optimal cutting temperature compound (OCT) and stored at −80 °C until further use. The cryostat sections were cut at 10 μm and adhered onto glass slides. The sections were washed with PBS and then incubated with 10 mM citric acid (pH 6.0) for 30 min at 80 °C for antigen retrieval. After washing with PBS briefly, sections were incubated with 0.5% Triton X-100 in PBS for 15 min at room temperature and then with blocking buffer (3% BSA in TBST) for 30 min at room temperature. Further, the sections were incubated at 4 °C overnight with primary antibodies against Nestin (RC2, DSHB; 1/100), Sox2 (AF2018, R&D systems; 1/200), Tbr2 (ab23345, Abcam; 1/1000), cleaved caspase3 (9661, Cell Signaling; 1/200), Reelin (MAB5364, Chemicon; 1/1000), β-tubulin class III (TuJ1, PRB-435P, Covance; 1/1000), Satb2 (ab51502, Abcam; 1/500), and Ctip2 (ab18465, Abcam; 1/500). To visualize the vasculature, IB4-FITC (Sigma, 1/100) was used. Sections were washed thrice with TBST for 10 min and then incubated with the appropriate secondary antibodies conjugated with Alexa Fluor 488 or 546 (A11001, A21208, Invitrogen; 1/300) for 1 h at room temperature. Nuclei were stained with DAPI. Fluorescence microscopy was performed on a BX51 microscope equipped with a DP30BW CCD camera (Olympus) using 10 × and 20 × objective lenses. Images were acquired using the DP controller software (Olympus).

BrdU birthdating assay
Pregnant mice (E14.5) were injected intraperitoneally with BrdU at 0.1 mg/kg of body weight and euthanized at E18.5. Brains were fixed with 4% PFA solution in PBS for 3 h at 4 °C and cryopreserved in 30% sucrose in PBS. Brains were embedded in OCT and stored at −80 °C until further use. The cryostat sections were cut at 12 μm and adhered onto glass slides. Then, the sections were washed with PBS and incubated with 10 mM citric acid (pH 6.0) for 30 min at 80 °C for antigen retrieval. The sections were incubated with 0.1 N HCl for 1 h at 37 °C. After incubation and brief washing with PBS, the sections were incubated with 0.5% Triton X-100 in PBS for 15 min at room temperature, and then incubated with blocking buffer (10% horse serum in TBST) for 30 min at room temperature. Further, the sections were incubated at 4 °C overnight with primary antibodies against BrdU (347,580, BD Bioscience; 1/100) and cleaved caspase3 (9661, Cell Signaling; 1/200), washed twice with TBST for 10 min, and incubated with the appropriate secondary antibodies conjugated with Alexa 488 or 546 (A11001, A21208, Invitrogen; 1/300) for 1 h at room temperature. Fluorescence microscopy was performed on the same microscope using a DP30BW CCD camera (Olympus) using 10 × and 20 × objective lenses. Images were processed using the DP controller software (Olympus).

In utero electroporation
In utero electroporation was performed as previously described, with slight modifications [46]. Briefly, pregnant mice at E13.5 or E15.5 were anesthetized by intraperitoneal (IP) injection of saline containing...
butorphanol (5 mg/kg body weight), midazolam (4 mg/kg body weight), and medetomidine (0.3 mg per kg body weight), and then the uterine horns were exposed. Approximately 1 μL of plasmid DNA mixture with 0.01% Fast Green solution was injected into the lateral ventricle of embryos using a fine-tip glass micropipette (G-1.0, Narishige). The final concentration of plasmid DNA was as follows: pEGFP-CAGS (0.5 or 1 μg/μL), m-sFlt1-pCI (1 μg/μL), pCI (1 μg/μL) and pCAG-Cre-GFP (1 μg/μL). Electric pulses (50 V, 1 pulse and 35 V, 4 pulses; 50 ms on, 950 ms off) were delivered to the dorsomedial part of the cortex using a forceps-type electrode (5 mm diameter, BEX) through CUY21 EDIT II (BEX). After wound closure, anesthesia was terminated by IP injection of atipamezole (3 mg/kg body weight). The embryos were then allowed to develop to E18.5.

Statistics
For statistical analysis, two-tailed Student’s t-test was used to determine p-values, and values with p less than 0.05 were considered statistically significant.

Supplementary Information
The online version contains supplementary material available at https://doi.org/10.1186/s13041-022-00911-0.

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Authors’ contributions
DS and HS designed the study; DS, TS, TK, and YM performed experiments; MT and HM analyzed the RT-qPCR data; HS‑H, TH, and HS interpreted the data and the final version of the manuscript. DS wrote the paper. All authors read and approved the manuscript.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations
Ethics approval and consent to participate
All animal experiments were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of Kanazawa Medical University and Doshisha University. A minimum sample size of five individuals was used in each assay, unless otherwise stated.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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