RESEARCH ARTICLE

Constitutively active Notch1 converts cranial neural crest-derived frontonasal mesenchyme to perivascular cells in vivo

Sophie R. Miller*, Surangi N. Perera and Clare V. H. Baker‡

ABSTRACT

Perivascular/mural cells originate from either the mesoderm or the cranial neural crest. Regardless of their origin, Notch signalling is necessary for their formation. Furthermore, in both chicken and mouse, constitutive Notch1 activation (via expression of the Notch1 intracellular domain) is sufficient in vivo to convert trunk mesoderm-derived somite cells to perivascular cells, at the expense of skeletal muscle. In experiments originally designed to investigate the effect of premature Notch1 activation on the development of neural crest-derived olfactory ensheathing glial cells (OECs), we used in ovo electroporation to insert a tetracycline-inducible Notch1E construct (encoding a constitutively active mutant of mouse Notch1) into the genome of chicken cranial neural crest cell precursors, and activated Notch1E expression by doxycycline injection at embryonic day 4. Notch1E-targeted cells formed perivascular cells within the frontonasal mesenchyme, and expressed a perivascular marker on the olfactory nerve. Hence, constitutively activating Notch1 is sufficient in vivo to drive not only somite cells, but also neural crest-derived frontonasal mesenchyme and perhaps developing OECs, to a perivascular cell fate. These results also highlight the plasticity of neural crest-derived mesenchyme and glia.

KEY WORDS: Notch, Pericyte, Neural crest, Frontonasal mesenchyme, Olfactory ensheathing cells, Chick embryo

INTRODUCTION

Perivascular (mural) cells — pericytes and vascular smooth muscle cells — form the periendothelial (outer) wall of blood vessels: mature pericytes are embedded within the basement membrane of the endothelial cells in microvessels (capillaries, terminal arterioles, postcapillary venules), while vascular smooth muscle cells are found in multiple layers around larger vessels (reviewed by Armulik et al., 2011; Majesky et al., 2011). Perivascular cells in the trunk, and many in the head, originate from mesoderm, but quail-chick chimera experiments revealed that the cranial neural crest (including the cardiac neural crest, a subset of the cranial mesoderm, but quail-chick chimera experiments revealed that the cranial neural crest (e.g. Doi et al., 2006; Noseda et al., 2006; High et al., 2007, 2008; Liu et al., 2009, 2010; Chang et al., 2012; Manderfield et al., 2012, 2015; Wang et al., 2014; for reviews, see Gridley, 2007, 2010; Phng and Gerhardt, 2009; Boucher et al., 2012). Constitutive activation of the Notch pathway, via expression of the Notch1 intracellular domain (NICD), was sufficient to up-regulate smooth muscle myosin heavy chain (Myh11) and other smooth muscle marker genes in the C3H10T1/2 (mouse embryonic fibroblast) cell line (Doi et al., 2006). Physiological Notch activation, via co-culture with L cells stably expressing the Notch ligand Jagged1 (though not Delta-like 4), was also sufficient to up-regulate Myh11 in this fibroblast cell line (Doi et al., 2006). In contrast, NICD transfection did not up-regulate Myh11 in non-mesenchymal cell lines (mouse mammary gland epithelial cells, human umbilical vein endothelial cells, or human keratinocytes) (Doi et al., 2006). In vivo, NICD is sufficient to convert trunk mesoderm-derived somite cells to perivascular cells, at the expense of a muscle cell fate (Ben-Yair and Kalcheim, 2008; Mayeuf-Louchart et al., 2014). This was first demonstrated in chicken, by electroporating the lateral dermomyotome with NICD (Ben-Yair and Kalcheim, 2008), and more recently in mouse, by replacing one allele of the somite-expressed gene Pax3 with NICD (Mayeuf-Louchart et al., 2014).

Here, we show that constitutively active Notch1 is also sufficient in vivo to drive a perivascular cell fate in cranial neural crest-derived frontonasal mesenchyme, and perhaps also in developing olfactory ensheathing glial cells (OECs). We originally aimed to test the effect of prematurely activating Notch1 on the development of OECs, which are derived from the cranial neural crest cells that colonise the frontonasal mass before the olfactory placode forms (Barrau et al., 2010). OECs are first detected on the chicken olfactory nerve at embryonic day E3.5, via immunoreactivity for the early glial marker myelin protein zero (Mpz, P0) (Drapkin and Silverman, 1999). Two days later, at E5.5, Notch1 is up-regulated in developing OECs, and by E6.5, almost all developing OECs express Sox2 (Miller et al., 2016), which is a direct Notch/Rbpj target (Wakamatsu et al., 2004; Ehm et al., 2010; Li et al., 2012). In the development of Schwann cells, the glia of all other peripheral nerves, Notch signalling promotes the transition from Schwann cell precursors (which express Mpz) to immature Schwann cells (Woodhoo et al., 2009). To test the hypothesis that a similar Notch-mediated transition is important for OEC development, we aimed to activate Notch1 prematurely in developing chicken OECs, for which temporal control of the onset of Notch1 signalling would be required. Sato et al. (2008) previously used in ovo electroporation to insert into the genome of presomitic mesoderm cells both a construct that constitutively expresses the reverse (‘Tet-on’) tetracycline transactivator protein variant rtTA2S2M (Urlinger et al., 2000), and a tetracycline-inducible NotchIE construct, in which a single tetracycline-response element controls the bidirectional transcription of NotchIE (encoding a constitutively active extracellular deletion mutant of mouse Notch1; Kopan et al., 1996) and EGFP, whose expression was activated at somite stages by doxycycline injection. This resulted in the conversion of somite cells either to perivascular cells (also shown by electroporating a construct encoding NICD directly into the lateral dermomyotome; Ben-Yair and Kalcheim, 2008) or to OECs (Sato et al., 2008).
2008) or endothelial cells (Sato et al., 2008). Here, we used the conditional expression approach of Sato et al. (2008) to insert their tetracycline-inducible NotchΔE/EGFP construct into the genome of premigratory cranial neural crest cell precursors, and activate NotchΔE/EGFP expression from E4 (by doxycycline injection). 1.5 days before Notch1 is normally up-regulated in developing OECs (Miller et al., 2016). To our surprise, we saw a striking phenotype in the neural crest-derived frontonasal mesenchyme (most of which would normally form skeletal or connective tissue, as well as perivascular cells), namely the formation by NotchΔE/EGFP-targeted cells of ectopic perivascular cells. NotchΔE/EGFP-targeted cells on the olfactory nerve also upregulated a perivascular marker. Hence, constitutive activation of Notch1 is sufficient in vivo to convert not only trunk mesoderm-derived somite cells (Ben-Yair and Kalcheim, 2008; Sato et al., 2008; Mayeuf-Louchart et al., 2014), but also cranial neural crest-derived frontonasal mesenchyme (and perhaps developing olfactory glia) to perivascular cells. These results suggest that during normal development, vascular endothelial cells expressing developing olfactory glia to perivascular cells. These results suggest that during normal development, vascular endothelial cells expressing Notch ligands may recruit adjacent neural crest-derived frontonasal mesenchyme cells (and perhaps also developing olfactory glia) to form perivascular cells, via the sustained activation of Notch signalling. Furthermore, given that Notch signalling was not activated in targeted cranial neural crest-derived cells until after doxycycline was injected at E4, several days after the end of cranial neural crest migration, our data also speak to the plasticity of cranial neural crest-derived frontonasal mesenchyme and developing olfactory ensheathing glia.

RESULTS
We used the Tol2 transposase 'Tet-on' in ovo electroporation system (Sato et al., 2007; Watanabe et al., 2007), which inserts tetracycline-dependent constructs into the genome of targeted cells, to drive constitutively active Notch1 expression in cranial neural crest-derived cells from embryonic day (E)4 [Hamburger–Hamilton (HH) stage 24; Hamburger and Hamilton, 1951]. Our original intention was to investigate the effect of premature Notch1 activation on the development of olfactory ensheathing cells (OECs, the glial cells of the olfactory nerve), which up-regulate Notch1 from E5.5 (HH stage 24) (Miller et al., 2016). We therefore aimed to target the cranial neural crest precursors of OECs, which colonise the frontonasal mass before the olfactory placode forms (Barraud et al., 2010), with the Tol2-integratable, tetracycline-dependent construct pT2K-NotchΔE-BI-EGFP (Sato et al., 2008). In this construct, a single tetracycline-response element controls the bidirectional transcription of NotchΔE (encoding a constitutively active extracellular deletion mutant of mouse Notch1; Kopan et al., 1996) and EGFP (thus, EGFP labels cells successfully targeted with NotchΔE; Sato et al., 2008).

We electroporated prospective cranial ectoderm in ovo at HH stages 6–8 (25–28 h of incubation) with pT2K-NotchΔE-BI-EGFP (hereafter NotchΔE/EGFP) or the Tol2-integratable control construct pT2K-CAGGS-EGFP, encoding EGFP only (Sato et al., 2007). Each of these constructs was co-electroporated with the Tol2-integratable construct pT2K-CAGGS-rTA2ΔM2 (Sato et al., 2007), encoding the reverse ('Tet-on') tetracycline transactivator protein rTA2 under the control of the synthetic CAGGS promoter (Niwa et al., 1991) (thus providing a continuous supply of rTA2 in targeted cells), plus the pCAGGS-T2TP construct, encoding Tol2 transposase (Sato et al., 2007) (to insert the rTA2 and NotchΔE/EGFP or control EGFP constructs into the genome of targeted cells). Doxycycline was injected into the yolk under the embryo at E4 (HH stage 24) to initiate NotchΔE/EGFP expression (the control EGFP is constitutively expressed). Embryos were collected 1–4 days later (E5–18; HH stages 27–34) for sectioning, followed by in situ hybridisation plus immunohistochemistry on sections.

Constitutive Notch activation from E4 may convert developing olfactory ensheathing cells into perivascular cells
In control EGFP-targeted embryos at E6 (two days after doxycycline injection), EGFP-positive developing OECs (which are neural crest-derived; Barraud et al., 2010) are distributed throughout the olfactory nerve at E6-7 express the perivascular cell marker Pdgfrβ (n = 3; Fig. 3F-F2), suggesting that, like NotchΔE/EGFP-targeted cells in the frontonasal mesenchyme, they may have been converted to perivascular cells. Several of the NotchΔE/EGFP-targeted cells on the olfactory nerve express the OEC marker Sox10 (Barraud et al., 2010) (Fig. 3F-E2), confirming that at least some developing OECs were targeted. Indeed, a few of the NotchΔE/EGFP-targeted cells co-express Sox10 and Pdgfrβ (yellow arrowheads, Fig. 3E-E2), suggesting they may have been caught in the process of changing fate. Some of the NotchΔE/EGFP-targeted cells on the olfactory nerve are Pdgfrβ-positive but Sox10-negative (black/white arrowheads, Fig. 3E-E2): these may have originated from NotchΔE/EGFP-targeted developing OECs that have already down-regulated Sox10 expression, or NotchΔE/EGFP-targeted frontonasal mesenchymal cells that have colonised the nerve. The endogenous olfactory nerve microvasculature is starting to form at this time; in situ hybridisation for Pdgfrβ and the vascular endothelial cell marker Lmo2 on sections of both NotchΔE/EGFP-targeted and wild-type embryos at E6.5–7 (n = 3) reveals some untargeted Pdgfrβ-positive cells (red arrowheads, Fig. 3E-E2) and a few Lmo2-positive cells (Fig. 3F-F2) within the olfactory nerve.

Constitutive Notch activation from E4 converts frontonasal mesenchyme cells to perivascular cells
At E6 (HH stage 29; two days after doxycycline injection) in control EGFP-targeted embryos (n = 2), EGFP-positive cells are distributed throughout the frontonasal mesenchyme and along peripheral nerves (Fig. 1A-B), with only a few EGFP-positive cells associated with Lmo2-positive vascular endothelium (Nagai and Sheng, 2008) (Fig. 1C-D). In contrast, in NotchΔE/EGFP-targeted embryos at E6-7 (HH stages 29–31; n = 8), most EGFP-positive cells are aggregated in rings in the mesenchyme (Fig. 1E-F1), encircling Lmo2-positive vascular endothelium (Fig. 1G-H). The same ‘ring-like’ distribution of EGFP-positive cells was also seen in NotchΔE/EGFP-targeted embryos at E5 (HH stage 27; n = 3) (not shown). Notch pathway activation in NotchΔE/EGFP-targeted cells at E6 was confirmed by co-immunostaining for EGFP and the cleaved Notch1 intracellular domain (n = 2; Fig. 1J).

Since cranial neural crest cells normally give rise to perivascular cells in the blood vessels of the face and forebrain (Eschevers et al., 2001), we wished to use molecular markers to test whether the NotchΔE/EGFP-targeted cells encircling Lmo2-positive vascular endothelium in the frontonasal mesenchyme were indeed adopting a perivascular cell fate. There are no exclusive molecular markers for perivascular cells; furthermore, the expression levels of the various markers used can vary, depending on, for example, the developmental state of the cells (reviewed by Armulik et al., 2011). Nevertheless, one commonly used perivascular cell marker is platelet-derived growth factor receptor beta (Pdgfrβ) (reviewed by Armulik et al., 2011). After doxycycline injection at E4, control EGFP-targeted embryos at E6 show almost no co-localisation between EGFP and Pdgfrβ (n = 3), barring a few cells associated with the vasculature, as expected (Fig. 2A-B'). In contrast, most NotchΔE/EGFP-targeted cells in the frontonasal mesenchyme express Pdgfrβ at E6-7 (n = 5) (Fig. 2C-D'). Perivascular cells also express vascular endothelial growth factor A (Vegfa) (Darland et al., 2003; Parenti et al., 2004; Kale et al., 2005). After initiating Notch activity by injecting doxycycline at E4, we detected Vegfa expression in NotchΔE/EGFP-targeted cells at E6-E7 (n = 2; Fig. 2G-H'). Furthermore, immunoreactivity for the smooth muscle/myofibroblast marker alpha-smooth muscle actin (Acta2; reviewed by Armulik et al., 2011) was detected in some NotchΔE/EGFP-targeted cells associated with larger blood vessels at E5-E8 (n = 2; Fig. 2I-J').

Overall, these data suggest that constitutive Notch activation from E4 in cranial neural crest-derived frontonasal mesenchyme cells is sufficient to convert them to perivascular cells, identified by their location (i.e. encircling vascular endothelial cells in developing blood vessels) in combination with the expression of characteristic perivascular cell markers.
Vasculature containing NotchΔE/EGFP-targeted perivascular cells seems to attract peripheral axons and glia

In half of the NotchΔE/EGFP-targeted embryos at E5-8 (∼6 out of 12), olfactory and other peripheral axons and their accompanying OECs/Schwann cells seemed to project towards vasculature containing NotchΔE/EGFP-targeted cells, with some of the glial cells (identified by Sox10 expression; Barraud et al., 2010; Jacob, 2015) even found isolated from axons, in association with such cells. Fig. 4A-A3 shows an example at E5, in which the olfactory nerve is in contact with such a blood vessel, at which point olfactory axons seem to project in the wrong direction, away from the forebrain. Fig. 4B-B3 shows an example at E6, in which the olfactory nerve is close
contact with vasculature containing NotchΔE/EGFP-targeted cells, towards which untargeted Sox10-positive OECs seem to have migrated, leaving the olfactory nerve altogether. Fig. 4C-G² shows another example at E7, in which NotchΔE/EGFP-targeted, Pdgfrb-positive perivascular cells are closely associated with Sox10-positive glial cells (Fig. 4D¹, D²) and axons (and possibly neurons) caudal to the olfactory system (Fig. 4G¹, G²). In one NotchΔE/EGFP-targeted embryo at E8 (HH stage 34), the entire olfactory nerve on one side is misplaced laterally, outside the cartilage that normally encloses it, apparently projecting towards large blood vessels containing NotchΔE/EGFP-targeted cells (Fig. 4H). On a nearby section from the same embryo, many Sox10-positive OECs (both NotchΔE/EGFP-targeted and untargeted) seem to have migrated away from the olfactory nerve altogether, instead associating with blood vessels containing NotchΔE/EGFP-targeted cells (Fig. 4I-I³). Taken together, these results suggest that vasculature containing NotchΔE/EGFP-targeted cells attracts both peripheral axons and glia.

**DISCUSSION**

In experiments originally aimed at testing the effect on olfactory ensheathing cell (OEC) development of prematurely activating Notch1, which is normally expressed in developing chicken OECs from E5 (Miller et al., 2016), we used the Tol2 transposase/Tet-on system to drive NotchΔE, encoding a constitutively active form of mouse Notch1 (Kopan et al., 1996; Sato et al., 2008), in cranial neural crest-derived cells from E4. This proved to be sufficient to convert both frontonasal mesenchyme cells, and perhaps also developing OECs, to Pdgfrb-
Positive perivascular cells. \textit{Pdgfrb} encodes a receptor tyrosine kinase required in pericytes during angiogenesis, for their recruitment to sprouting capillaries and proliferation (Lindahl et al., 1997; Hellström et al., 1999; Winkler et al., 2010). In the frontonasal mesenchyme at E5-7, ectopic \textit{Notch}\textsubscript{ΔE}/\textit{EGFP}-targeted perivascular cells were found encircling \textit{Lmo2}-positive vascular endothelium. \textit{Fgfr2} (\textit{Flk1}, \textit{Kdr})-expressing angioblasts are found throughout the developing cranial mesenchyme in both chicken and mouse (Couly et al., 1995; Yoshida et al., 2008); in chicken, these initially dispersed \textit{Fgfr2}-positive cells have all incorporated into blood vessels by E3-4 (Couly et al., 1995). Hence, expression of constitutively active \textit{Notch1} from E4 in cranial neural crest-derived frontonasal mesenchyme cells causes them to adopt a perivascular cell fate and associate with the vascular endothelium of nearby blood vessels.

\textit{Notch}\textsubscript{ΔE}/\textit{EGFP}-targeted \textit{Pdgfrb}-positive cells were also seen within the olfactory nerve, suggesting that constitutive \textit{Notch1} activation from E4 within developing OECs (which can first be identified at E3.5, by myelin protein zero immunoreactivity; Drapkin and Silverman, 1999) could be sufficient to convert them to a perivascular cell fate. Indeed, some of the \textit{Notch}\textsubscript{ΔE}/\textit{EGFP}-targeted, \textit{Pdgfrb}-positive cells on the olfactory nerve co-expressed the OEC marker \textit{Sox10} (Barraud et al., 2010), suggesting they were in the process of changing fate. Most \textit{Notch}\textsubscript{ΔE}/\textit{EGFP}-targeted cells seemed to be excluded from the interior of the olfactory nerve and instead aggregated together at the edges,

**Fig. 3. Constitutive Notch activation from E4 converts developing olfactory ensheathing cells into perivascular cells.** Parasagittal sections of the olfactory region from chicken embryos in which the cranial ectoderm had been targeted in ovo at E1 with \textit{EGFP} alone (control) or \textit{Notch}\textsubscript{ΔE}/\textit{EGFP}, using the Tol2 transposase/‘Tet-on’ electroporation system. Eggs were injected with doxycycline at E4. Dotted lines demarcate the olfactory nerve. (A-B\textsuperscript{1}) A control \textit{EGFP}-targeted embryo at E6, in which the olfactory placode was not targeted. \textit{EGFP}-targeted neural crest-derived cells are found throughout the frontonasal mesenchyme and associated with \textit{Tubb3}-positive olfactory axons, presumably developing OECs. (C-D\textsuperscript{1}) A \textit{Notch}\textsubscript{ΔE}/\textit{EGFP}-targeted embryo at E6. \textit{Notch}\textsubscript{ΔE}/\textit{EGFP}-targeted cells associated with the olfactory nerve are aggregated at the edges of the nerve, rather than being found throughout the nerve, and form processes extending away from it (arrowhead in D, D\textsuperscript{1}). (E-E\textsuperscript{2}) In a \textit{Notch}\textsubscript{ΔE}/\textit{EGFP}-targeted embryo at E7, \textit{in situ} hybridisation for the perivascular marker \textit{Pdgfrb} (shown as a false-colour overlay in E\textsuperscript{1},E\textsuperscript{2}), combined with immunostaining for the OEC marker \textit{Sox10}, shows that some \textit{Notch}\textsubscript{ΔE}/\textit{EGFP}-targeted cells are developing OECs; a few of these co-express \textit{Pdgfrb} (yellow arrowheads), suggesting they may be undergoing fate conversion. Some \textit{Notch}\textsubscript{ΔE}/\textit{EGFP}-targeted cells express \textit{Pdgfrb} but not \textit{Sox10} (black/white arrowheads). Some untargeted cells express \textit{Pdgfrb} (red arrowheads). (F-F\textsuperscript{2}) In a nearby section from the same \textit{Notch}\textsubscript{ΔE}/\textit{EGFP}-targeted embryo at E7, \textit{in situ} hybridisation for \textit{Lmo2} reveals a few weakly \textit{Lmo2}-positive vascular endothelial cells on the olfactory nerve (arrowheads). \textit{bv}, blood vessel; \textit{EGFP}, enhanced GFP; \textit{fb}, forebrain; \textit{oe}, olfactory epithelium; \textit{on}, olfactory nerve; \textit{pn}, peripheral nerve. Scale bars: 100 μm.

RESEARCH ARTICLE

Biology Open
projecting away from the nerve. This may reflect the lack of blood vessels inside developing nerves until relatively late in development, given that we did not see many Lmo2-positive vascular endothelial cells inside the chicken olfactory nerve at E6.5-7 (in the rat sciatic nerve, blood vessels are first seen only at E18; Wanner et al., 2006). The presence of some untargeted Pdgfrb-positive cells within the olfactory nerve at E6.5-7 also suggests that perivascular cells are normally beginning to differentiate at this stage. Taken together, these data may

Fig. 4. Peripheral axons and glia seem to be attracted to blood vessels containing Notch.ΔE/EGFP-targeted cells. Parasagittal (A-G) and coronal (H-I) sections from embryos in which the cranial ectoderm had been targeted in ovo at E1 with Notch.ΔE/EGFP, using the Tol2 transposase/Tet-on electroporation system. Eggs were injected with doxycycline at E4. (A) In an E5 embryo, in situ hybridisation for Sox10 reveals developing OECs on the olfactory nerve. In the same section as A, immunostained for EGFP and Tubb3, with Sox10 shown as a false-colour overlay in A2,A3. A thin nerve branch (arrow) deviates from the olfactory nerve away from the forebrain (for orientation, see low-power inset in A2). The branch-point is near a developing blood vessel, whose wall contains Notch.ΔE/EGFP-targeted cells. (B-B3) In an E6 embryo, several untargeted Sox10-positive cells (arrowheads), presumably developing OECs, are found isolated in the mesenchyme at some distance from the olfactory nerve, near Notch.ΔE/EGFP-targeted cells. (C-D) In an E7 embryo, in situ hybridisation for Pdgfrb followed by immunostaining for EGFP and Sox10 reveals that many Notch.ΔE/EGFP-targeted cells have formed Pdgfrb-positive perivascular cells, with which many Sox10-positive cells (presumably peripheral glial cells) are associated. This is far from the olfactory nerve: note the location of the olfactory epithelium at the top right. (E-G) A nearby section of the same E7 embryo, shown at low-power in E-E3 for orientation (note the position of the olfactory epithelium and olfactory nerve towards the top right, and the forebrain and adenohypophysis towards the top left). In situ hybridisation for Lmo2 and immunostaining for EGFP and Tubb3 confirm the presence of peripheral axons (and possibly neurons) close to a large concentration of Notch.ΔE/EGFP-targeted cells that are associated with Lmo2-positive vascular endothelium. (H) In an E8 embryo (coronal section), the entire olfactory nerve on one side is misplaced laterally (yellow arrow) towards several large blood vessels whose walls contain Notch.ΔE/EGFP-targeted cells. The displaced olfactory nerve is in contact with another peripheral nerve, and no longer surrounded by cartilage (identified by immunostaining with an anti-Sox9 antibody that also cross-reacts with other SoxE family members), unlike the olfactory nerve on the other side. (I-I3) In a nearby section of the same E8 embryo, in situ hybridisation for Sox10 and immunostaining for EGFP and Tubb3 show that some Sox10-positive OECs – both untargeted (black/white arrowheads) and Notch.ΔE/EGFP-targeted (yellow arrowheads) – are found at a distance from axons, associated instead with blood vessels whose walls contain Notch.ΔE/EGFP-targeted cells. ah, adenohypophysis; bv, blood vessel; EGFP, enhanced GFP; fb, forebrain; oe, olfactory epithelium; on, olfactory nerve; pn, peripheral nerve. Scale bars: 100 μm.
also suggest that at least some of the perivascular cells of the olfactory nerve vasculature derive from developing OECs, in response to sustained Notch1 activation. This is in contrast to the trunk, where only endoneurial fibroblasts, and not endoneurial perivascular cells, derive from Schwann cell precursors (Joseph et al., 2004). Furthermore, since expression of the constitutively active Notch1 mutant protein was only activated in targeted cranial neural crest-derived cells following doxycycline injection at E4, our findings also reveal the plasticity of cranial neural crest-derived frontonasal mesenchyme and developing olfactory ensheathing glia.

Our results are consistent with previous work showing that constitutive Notch1 activation (via expression of the Notch1 intracellular domain) in trunk mesoderm-derived somite cells promotes adoption of a perivascular fate at the expense of a skeletal muscle fate (Ben-Yair and Kalcheim, 2009; Gridley, 2010; Boucher et al., 2012). Our data suggest that constitutive Notch1 signalling from E4 in cranial neural crest-derived frontonasal mesenchyme and developing OECs promotes a perivascular cell fate. Since Notch signalling is required for neural crest-derived perivascular cell formation (High et al., 2007, 2008; Chang et al., 2012; Manderfield et al., 2012; Wang et al., 2014; Manderfield et al., 2015), this likely reflects a normal developmental process, whereby vascular endothelial cells expressing Notch ligands recruit adjacent frontonasal mesenchyme cells to form perivascular cells through sustained activation of Notch signalling.

Consistent with this hypothesis, sustained activation of Notch signalling [via exposure to Delta-like 4 (Dl4) from endothelial cells] is both sufficient and necessary for conversion of skeletal myoblasts to pericytes in vitro: silencing of Dl4 restores myogenesis (Cappellari et al., 2013). In vivo, expression of the Notch1 intracellular domain in MyoD-positive muscle cells also drives a pericyte fate, while occasional perivascular cells in wild-type embryos are derived from Myf5- or MyoD-expressing precursors (Cappellari et al., 2013). This suggests that Notch ligand production from vascular endothelium in skeletal muscle may sometimes induce a fate switch in adjacent myoblasts. Sustained Notch signalling is also required in vascular smooth muscle cells to suppress alternative fates and maintain the perivascular fate: in the absence of the Notch ligand Jagged1, mouse somite-derived vascular smooth muscle cells adopt a chondrocyte fate, which can lead to vessel ossification (Briot et al., 2014). Thus, sustained Notch signalling appears not only to promote, but also maintain, the perivascular cell fate.

We also found that vasculature containing NotchΔE/EGFP-targeted perivascular cells seemed to attract peripheral axons and their associated glia (OECs on the olfactory nerve; Schwann cells on all other nerves), with perivascular cells seemed to attract peripheral axons and their associated glia (OECs on the olfactory nerve; Schwann cells on all other nerves), with perivascular cells attracting OECs/Schwann cells, and at least in some cases olfactory axons, towards the vasculature. Overall, our data support and extend previous work showing that the Notch pathway is necessary for the formation of perivascular cells from the cranial neural crest (High et al., 2007, 2008; Chang et al., 2012; Manderfield et al., 2012; Wang et al., 2014; Manderfield et al., 2015), by showing that constitutively active Notch1 promotes a perivascular cell fate in frontonasal mesenchyme, and perhaps also in glial progenitors on the olfactory nerve, several days after the end of cranial neural crest migration. Intriguingly, constitutive activation of Notch signalling via expression of the Notch3 intracellular domain seems to promote the proliferation, but not the specification, of brain pericytes in zebrafish (Wang et al., 2014), suggesting that the activation of distinct Notch signalling pathways may have different outcomes during the development of perivascular cells.

**MATERIALS AND METHODS**

**Electroporation constructs**

All electroporation constructs were kind gifts of Yoshiko Takahashi (Kyoto University, Kyoto, Japan); the pT2K-NotchΔE-BI-EGFP construct (Sato et al., 2008) was used with the kind permission of Raphael Kopan (Washington University, St Louis, MO, USA). Constructs were prepared using the EndoFree Plasmid Maxi kit (Qiagen) to a stock concentration of 5 µg/µl. pCAGGS-T2TP (Kawakami and Noda, 2004; Sato et al., 2007) encodes Tol2 transposase under the control of the synthetic CAGGS promoter (Niwa et al., 1991); the Tol2-integratable pT2K-CAGGS-T2TP construct (Sato et al., 2007) encodes the reverse (‘Tet-on’) tetracycline transactivator transactivator protein variant rTA2M2 (Uhringer et al., 2000); the Tol2-integratable, tetracycline-dependent pT2K-NotchΔE-BI-EGFP construct (Sato et al., 2008) encodes a constitutively active extracellular deletion mutant of mouse Notch1 (NotchΔE; Kopan et al., 1996) and EGFP, bidirectionally transcribed under the control of a single tetracycline-response element; the Tol2-integratable pT2K-CAGGS-EGFP control construct (Sato et al., 2007) encodes EGFP alone.

**In ovo electroporation**

Fertilised chicken (Gallus gallus domesticus) eggs were obtained from commercial sources. All work with chicken embryos was conducted in accordance with the UK Animals (Scientific Procedures) Act 1986. Eggs were incubated in a humidified atmosphere at 38°C for 25-28 h to reach Hamburger–Hamilton stages 6-8 (Hamburger and Hamilton, 1951) (between the head-fold stage and the 4-somite stage). Black ink (Font India, Pelikan) was diluted to 1% in filtered phosphate-buffered saline (PBS) and injected underneath the blastoderm to visualise the embryo. The cranial ectoderm and neural folds were co-electroporated with 1:1:1 pCAGGS-T2TP, pT2K-CAGGS-rTA2M2 and either pT2K-NotchΔE-BI-EGFP or control pT2K-CAGGS-EGFP, to a final concentration of 0.9 µg/µl each, mixed with Fast Green to a final dilution of 2% and sucrose to a final concentration of 8%. The positive electrode was placed in the yolk underneath the head process and perpendicular to the cranial–caudal axis of the embryo. The plasmid solution was micro-pipetted over the cranial ectoderm and the negative ‘spoon-type’ electrode brought down over the embryo, as described (Brown et al., 2012).

An ECM 830 Square Wave Pulse generator (BTX Instrument Division, Harvard Apparatus, Inc.) was used to apply five 50-ms 5 V pulses at 100 ms intervals. The egg was sealed with Parafilm and returned to the incubator. At embryonic day (E4), 500 µl of doxycycline solution (100 µg/ml doxycycline in water) was injected under the embryo. The egg was re-sealed and returned to the incubator until the desired stage. Surviving embryos were fixed in modified Carnoy’s (6 volumes ethanol, 3 volumes 37% formaldehyde, 1 volume glacial acetic acid), dehydrated into ethanol, cleared in Histosol (National Diagnostics) and embedded in paraffin wax for sectioning at 6 µm on a rotary microtome (Microm).

**Riboprobes**

Chicken Lmo2 (Nakazawa et al., 2006) was a kind gift of Guojun Sheng (RIKEN Center for Developmental Biology, Kobe, Japan). Chicken Sox10 (Cheng et al., 2000) was a kind gift of Marianne Bronner (Caltech, Pasadena, CA, USA). An 803-bp fragment of chicken Pdgfrb cDNA,
corresponding to base-pairs 1486-2288 (NCBI reference sequence XM_001233829.3) was PCR-amplified from cDNA (forward primer TA-ACGTGCTCTGCTGAAGGG; reverse primer CAGGAAAGTGTTGTGTTGTGCC), cloned into pDrive (Qiagen) using the Qiagen PCR cloning kit and sequenced (Biochemistry Department DNA Sequencing Facility, Cambridge, UK). A 608-bp fragment of chicken Vegfa cDNA, corresponding to base-pairs 631-1238 (NCBI reference sequence NM_205042.2) was similarly PCR-amplified (forward primer GCGGAAGCCCAAGAAGTTA; reverse primer GTTCCAGGCGAGAATACCGG) and cloned into pDrive. PCR primers were designed and specificity checked using Primer-BLAST software from NCBI (Ye et al., 2012). The OligoCalc program (Kibbe, 2007) (http://www.basic.northwestern.edu/biotools/oligocalc.html) was used to check primer melting temperature and self-complementarity.

In situ hybridisation on sections

Slides were de-waxed in Histolsol (National Diagnostics) and rehydrated through a graded ethanol series into diethylpyrocarbonate (DEPC)-treated PBS. Digoxigenin-labelled antisense riboprobes were generated as described (Henrique et al., 1995).

Immunohistochemistry

Whether after fixation following in situ hybridisation as described in the preceding section, or after de-waxing and rehydrating untreated slides as described in the preceding section, slides were rinsed in PBS, blocked for 1 h at room temperature in 10% sheep serum in PBS with 0.1% Triton X-100 and then incubated overnight at 4°C with primary antibodies in blocking solution. Whether after fixation following in situ hybridisation as described in the preceding section, or after de-waxing and rehydrating untreated slides as described in the preceding section, slides were rinsed in PBS, blocked for 1 h at room temperature in 10% sheep serum in PBS with 0.1% Triton X-100 and then incubated overnight at 4°C with primary antibodies in blocking solution. Whether after fixation following in situ hybridisation as described in the preceding section, or after de-waxing and rehydrating untreated slides as described in the preceding section, slides were rinsed in PBS, blocked for 1 h at room temperature in 10% sheep serum in PBS with 0.1% Triton X-100 and then incubated overnight at 4°C with primary antibodies in blocking solution. Whether after fixation following in situ hybridisation as described in the preceding section, or after de-waxing and rehydrating untreated slides as described in the preceding section, slides were rinsed in PBS, blocked for 1 h at room temperature in 10% sheep serum in PBS with 0.1% Triton X-100 and then incubated overnight at 4°C with primary antibodies in blocking solution.


