Progenitor cell heterogeneity in the adult carotid body germinal niche

Verónica Sobrino*,[1], Valentina Annese*,[1], Ricardo Pardal#,[1]

[1] Instituto de Biomedicina de Sevilla (IBiS), Hospital Universitario Virgen del Rocío/CSIC/Universidad de Sevilla, Dpto. de Fisiología Médica y Biofísica. Avda. Manuel Siurot, s/n. E-41013 Sevilla (SPAIN).

* These authors contributed equally to this work.

Correspondence: rpardal@us.es. Instituto de Biomedicina de Sevilla (IBiS), Laboratory 103, Hospital Universitario Virgen del Rocío/CSIC/Universidad de Sevilla, Dpto. de Fisiología Médica y Biofísica. Avda. Manuel Siurot, s/n. E-41013 Sevilla (SPAIN).

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Abstract

Somatic stem cells confer plasticity to adult tissues, permitting their maintenance, repair and adaptation to a changing environment. Adult germinal niches supporting somatic stem cells have been thoroughly characterized throughout the organism, including in central and peripheral nervous systems. Stem cells do not reside alone within their niches, but they are rather accompanied by multiple progenitor cells that not only contribute to the progression of stem cell lineage but also regulate their behavior. Understanding the mechanisms underlying these interactions within the niche is crucial to comprehend associated pathologies and to use stem cells in cell therapy. We have described a stunning germinal niche in the adult peripheral nervous system: the carotid body. This is a chemoreceptor organ with a crucial function during physiological adaptation to hypoxia. We have shown the presence of multipotent stem cells within this niche, escorted by multiple restricted progenitor cell types that contribute to niche physiology and hence organismal adaptation to the lack of oxygen. Herein, we discuss new and existing data about the nature of all these stem and progenitor cell types present in the carotid body germinal niche, discussing their role in physiology and their clinical relevance for the treatment of diverse pathologies.

1. Introduction

Somatic stem cells enable functional and structural plasticity of adult tissues. These cells are responsible for tissue repair and maintenance, and in some cases, like in the central nervous system (CNS), they contribute to functional plasticity and adaptability to a changing environment. Neural stem cells within the brain give rise to new neurons and glia throughout life (Kempermann and Gage, 1999), to contribute to seminal functions such as learning and memory (Bond et al., 2015). Since their discovery (Reynolds and Weiss, 1992), research on neural stem cells has created a dynamic and expanding field that has profoundly inspired neuroscience and regenerative medicine against neurological disorders. Adult brain stem cells have been shown to self-renew and to exploit multipotency by differentiating into glial and neuronal cells (Reynolds and Weiss, 1992). Understanding proliferation and differentiation mechanisms employed by neural stem cells to achieve their functions is critical for our capacity to use these cells in our own benefit.

Adult stem cells reside in specific niches where they are subjected to fine regulation of their behavior (Scadden, 2014). These germinal niches have been shown to include both cellular and non-cellular elements, and their integrity and complexity ensure a correct functioning of stem cells and avoids the appearance of potential hyperproliferation and conversion into cancer stem cells (Li and Neaves, 2006). A deep comprehension of germinal niches is also fundamental to understand stem cell biology and behavior, and to be able to use these cells in our fight against disease.

Stem cells within germinal niches are not alone, but rather they are accompanied by multiple cell types that participate in the regulation of niche functioning. Most of these escorting cell types are actually derived from the stem cells themselves. They are proliferative progenitors, some of them multipotent, some others more restricted to

specific cell lineages, and they contribute to niche functioning both by being part of cell lineage progression and by regulating stem cell behavior. This heterogeneity of progenitor cell types is observed in every germinal niche that is thoroughly characterized in the adult nervous system. For instance, in the subventricular zone (SVZ) of the adult forebrain, multipotent astrocyte-like quiescent stem cells or type B cells give rise to proliferative intermediate progenitors or type C cells, which eventually progress through specification to generate neuroblasts or type A cells (Garcia-Verdugo et al., 1998). At the end, neuroblasts migrate through the dorsal migratory stream towards the olfactory bulb to generate new interneurons that participate in olfaction plasticity (Ming and Song, 2011). All these intermediate cells have been shown to modulate stem cell activity and neurogenesis through soluble molecules, constituting a critical component of the niche functioning (Liu et al., 2005). Therefore, adult germinal niche functioning relies on the participation of a heterogeneous group of stem and progenitor cells, which manage to survey the environment to be able to respond to maintenance, injury, or physiological adaptation requirements.

Adult germinal niches have also been described in the peripheral nervous system (PNS), where multipotent neural crest-derived stem cells (NCSCs) are able to persist into adulthood to contribute to tissue maintenance, repair, and adaptation. Not as exhaustively characterized as their counterparts in the CNS, adult multipotent NCSCs have been described to reside in the enteric ganglia (Laranjeira et al., 2011), and in some other target tissues of NCSC migration, such as bone marrow (Nagoshi et al., 2008), cornea (Yoshida et al., 2006), heart (Tomita et al., 2005), or skin (Wong et al., 2006). Little is known in general about the functioning and regulation of these stem cell niches in the adult PNS, including the characterization of diverse stem and progenitor cell types potentially present.

We have recently described the existence of an enticing germinal niche within a specific area of the PNS, the adult carotid bodies (CB). These are chemoreceptor organs that play a crucial role in the detection of changes in chemical variables in the blood, informing the CNS to enable the triggering of counter regulatory responses (Weir et al., 2005). The most important parameter change perceived by the carotid body is a decrease in blood PO2. In the case of acute hypoxemia, the carotid body immediately increases the firing rate towards the CNS, so that respiratory centers in the brain-stem can trigger hyperventilation through a sympathetic discharge (López-Barneo et al., 2001). If the stimulus is prolonged, like in high altitude dwellers, the carotid bodies are crucial facilitating organismal acclimation to the decrease in environmental oxygen (Joseph and Pequignot, 2009; Weir et al., 2005). This well-described physiological adaptation response relies on an increase of carotid body size, including a rise in the number of chemoreceptor neuronal cells (Arias-Stella and Valcarcel, 1976; McGregor et al., 1984). The new neurons will permit a continuous stimulation of the respiratory centers in the CNS and hence the maintenance of the respiratory drive during hypoxia. This cellular plasticity, remarkable for an adult neural tissue, was poorly understood until our characterization of the cellular events taking place in the hypoxic organ (Pardal et al., 2010). We have shown that the carotid body constitutes a captivating germinal niche in the adult PNS, containing a heterogeneous population of stem and progenitor cells that support physiological adaptation of the organ. Herein, we present and review recent data deciphering carotid body cellular heterogeneity, providing markers and presenting molecular mechanisms involved in carotid body niche functioning. We will also comment on the potential tools our work might offer to control carotid body functioning and the clinical relevance of these tools for the treatment of a growing number of disease conditions.

2. CB multipotent stem cells and physiological adaptation to hypoxia

The CB is a paired organ situated at the bifurcation of the carotid artery, and constitutes a specialized ganglion of the sympathetic nervous system. This organ is formed after migration of neural crest-derived progenitors from the adjacent superior cervical ganglion, and is innervated by afferent sensory nerve fibers joining the glossopharyngeal nerve (Kameda, 2002). The CB is also composed by a profuse network of blood vessels necessary for its chemoreceptor activity (Kameda, 2005; McDonald and Mitchell, 1975). The neural parenchyma in this organ is organized in glomeruli of chemoreceptor type I cells (also termed as glomus cells). These are specialized neuronal cells able to detect different chemical stimuli arriving in the blood, such as hypoxia, hypercapnia, acidosis, or hypoglycemia, and to communicate the situation to sensory nerves through chemical synapsis (Pardal and López-Barneo, 2002; Urena et al., 1994). The molecular mechanism by which these cells detect the main stimulus, hypoxia, is only recently being thoroughly elucidated (Arias-Mayenco et al., 2018; Fernandez-Aguera et al., 2015). Neuronal glomeruli are surrounded by sustentacular type II cells. These cells express the glial fibrillary acidic protein (GFAP) and were thought to serve as glial cells supporting function and maintenance of neuronal cells (Kameda, 1996). However, we showed a decade ago that these cells function as multipotent neural crest-derived stem cells, being able to activate proliferation in hypoxia and to differentiate into new neuronal cells that will contribute to physiological adaptation of the tissue to the hypoxemic situation (Pardal et al., 2007). We also showed that these cells do not respond to hypoxia themselves, but need communication of the hypoxic stimulus from neuronal cells (Platero-Luengo et al., 2014), similar to other nervous system germinal niches where stem cells are also regulated by neuronal activity (Pardal and Lopez Barneo, 2016). Moreover, we exhibited the capacity of CB type II cells to function as multipotent neural stem cells in vitro, being

able to grow as spherical colonies, called neurospheres, that contained both differentiated and undifferentiated cells (Pardal et al., 2007).

Two crucial cellular processes are executed in the organ under chronic hypoxia as part of the adaptation program. One is the already mentioned neurogenesis, or production of new glomus cells, to permit physiological adaptation by increasing firing rate to CNS and maintaining respiratory drive. The other process is a profound angiogenesis, or production of new blood vessels in hypoxia, to optimize vascularization of the growing neural parenchyma (Chen et al., 2007). We have shown by cell fate mapping that GFAP+ multipotent stem cells participate in both neurogenesis (Pardal et al., 2007) and angiogenesis (Annese et al., 2017; Navarro-Guerrero et al., 2016). Furthermore, by using neurosphere-forming assays in vitro, we have shown that CB stem cells (CBSCs) have the multipotent capacity to differentiate into neuronal cells (Pardal et al., 2007) and vascular cells, including smooth muscle, perycytes, and even endothelial cells (Annese et al., 2017). To the best of our knowledge, our data exposes the only example described of an adult population of PNS stem cells able to convert into both neuronal and mesectodermal derivatives in response to physiological stimuli.

In a normoxic resting situation, GFAP+ CBSCs present cellular protrusions surrounding neuronal cells, a quiescent conformation that optimizes the detection of stimuli coming from glomus cells (Platero-Luengo et al., 2014). However, once activated by hypoxia, these stem cells change their phenotype, switch their filament protein expression from GFAP to nestin, become rounded, and start proliferating (Pardal et al., 2007) (Fig. 1a). These nestin+ proliferative stem cells give rise to specified progenitor cells that differentiate into one of the two cellular lineages, neuronal or mesectodermal, to participate either in neurogenesis or in angiogenesis respectively. Our data suggest that a fair amount of these nestin+ cells remain multipotent and are able to go back to the

quiescent GFAP+ phenotype upon stimulus cessation (Pardal et al., 2007), which probably minimizes stem cell depletion.

An interesting aspect of the behavior of CB nestin+ proliferative progenitors is their migration capability. By combining electron microscopy with immunodetection of GFAP and nestin, using gold particle-associated antibodies, we can study the shape and position of these cells within the CB parenchyma (Fig. 1). We have used different developmental times during silver enhancement procedure, in order to obtain a different gold particle diameter for the detection of GFAP or nestin with ultra-small gold particle-conjugated antibodies (4.67±0.45 nm for GFAP, n=18 particles; 8.3±1.6 nm for nestin, n=37 particles), allowing us to perform both labeling procedures at the same time (Fig. 1a). The position of nestin+ progenitors, compared to GFAP+ cells, is clearly more detached from neuronal glomeruli and closer to blood vessels (see quantification shown in Fig. 1b), which suggests a movement process. We even find some cells in transition, positive for both GFAP and nestin, getting detached from glomus cells (Fig. 1c). Migration might be part of the specification and differentiation process from CBSCs. In fact, cytokines inducing differentiation into mesectodermal lineage, such as erythropoietin (EPO) (see below), are also potent activators of migration, as evidenced by migration assays performed with nestin+ progenitor cells (Fig. 1d-g). We also have preliminary evidence (data not shown) that new neuronal glomeruli are preferentially formed at the periphery of the organ. All data together suggest that both neurogenesis and angiogenesis processes involve progenitor cell migration.

In summary, GFAP+ and nestin+ stem cells are the quiescent and proliferative versions respectively of CB multipotent progenitors (CBSCs). They are interchangeable depending on the presence of the hypoxic stimulus, and eventually give rise in hypoxia to more specified progenitor cells that complete differentiation into either neuronal or

mesectodermal cell lineages. This behavior is typically observed in CB neurospheres, which are constituted by a central core full of nestin+ progenitors and mesectodermal cells, and budding blebs in the surface, full of TH+ glomus cells (Navarro-Guerrero et al., 2016; Pardal et al., 2007), resembling the activated situation of the CB niche. Interestingly, in addition to multipotent cells, we have discovered specified progenitors from both lineages, with quiescent phenotype, within the normoxic CB parenchyma in vivo. The existence of these restricted progenitors might confer a clear evolutionary advantage to this niche since these cells are able to convert into differentiated cells under hypoxia much faster than multipotent stem cells. We have found specific markers for these restricted progenitors and have studied their biology and their overlapping marker expression with multipotent cells. Herein, we now expose the main characteristics of restricted progenitors from both cell lineages and discuss the physiological and clinical relevance of their existence within the CB.

3. Neuronal-committed progenitors within the CB parenchyma

During the past century, different morphometric studies distinguished two classes of glomus cells in the CB, classically termed type A and type B glomus cells. This classification was made attending to ultrastructural parameters, such as the diameter of exocytotic vesicles, the number of mitochondria, or the size of the nucleus, among others (Hellström, 1975). Type A glomus cells displayed more abundant dense-core vesicles and with higher diameter. These cells were frequently in contact with nerve terminals and sinusoidal capillaries, exhibiting the expected aspect of chemoreceptor glomus cells. The proportion between both types of cells seemed to be even, but type B cells were usually in the periphery of type A cell glomeruli, and with cytoplasmic extensions surrounding type A cells (Chen and Yates, 1984). We have recently shown that type B glomus cells

seem to be immature neuroblast-like cells, ready to convert into fully mature glomus cells (type A) in response to the hypoxic stimulus (Sobrino et al., 2018).

CB neuroblasts (CBNBs), or type B glomus cells, share multiple features with mature glomus cells, or type A glomus cells, such as the expression of dopaminergic markers like TH or dopamine decarboxylase (DDC). However, they also display proper characteristics of immature cells (Sobrino et al., 2018), such as expression of immature cell markers typical of sympathoadrenal progenitor cells, like HNK-1 (Langley and Grant, 1999) or the transcription factor Ascl1 (Kameda, 2005), and expression of neuroblast markers like Tuj1 or Ncam2 (Bonfanti et al., 1992; Menezes and Luskin, 1994). Moreover, we have shown that CBNBs do not yet contain a mature hypoxia-responsive machinery, since they are not able to respond to acute exposures to hypoxia in the way mature glomus cells do, despite their membrane expression of ion channels and their responsiveness to other chemical stimuli (Sobrino et al., 2018). We have also shown that CBNBs are smaller in size than mature glomus cells, they have less mitochondria and vesicles, smaller vesicle size, and their position in the glomeruli is peripheral (Sobrino et al., 2018). All these ultrastructural characteristics are in consonance with the classical morphometric studies performed in type B glomus cells (Chen and Yates, 1984; Hellström, 1975).

The expression of HNK-1 by CBNBs is particularly interesting, since this cell surface marker allows the prospective isolation of these cells by flow cytometry (Pardal et al., 2007; Sobrino et al., 2018). HNK-1, also known as CD57, constitutes a surface glycoepytope that is usually attached to a glycoprotein related to cell adhesion, such as NCAM, tenascin, or laminin (Langley and Grant, 1999). HNK-1 is typically expressed by migrating neural crest cells during development, where it has been shown to have a role in migration and cell adhesion to the substrate (Bronner-Fraser, 1987; Nagase et al.,

2003). Moreover, HNK-1 has also been involved in synaptic plasticity in the CNS (Kizuka and Oka, 2012). Although the expression of this antigen can vary among species, it has been demonstrated that HNK-1 is expressed in the neural crest of birds, rats, dogs, pigs, and humans, among others, but curiously not in mice (Erickson et al., 1989; Huang et al., 2016; Nagase et al., 2003; Tucker et al., 1988).

An interesting question that arose when characterizing CBNBs was their cellular origin. Numerous studies of carotid body development performed in avian and rat models (Kameda et al., 1994; Le Douarin et al., 1972) demonstrated that glomus cell precursors arriving to the CB from the superior cervical ganglion express Tuj1, PGP9.5, NPY, TH, and HNK-1, together with required expression of transcription factors like Ascl1 (Kameda, 2002, 2005, 2014). We have shown that most of these markers, enzymes, and transcription factors are highly expressed in the adult CB neuroblast population (Sobrino et al., 2018). These similarities between fetal CB neuronal precursors and adult neuroblasts led us to hypothesize that these adult neuroblastic cells might be descendants of those fetal neuronal precursors. However, our in vitro studies have demonstrated the existence of neuroblasts within CB stem cell-derived neurospheres (see response to referees in Sobrino et al., 2018), meaning that CB multipotent stem cells might have the capacity to replenish neuroblasts within the adult tissue. The lack of expression of HNK-1 in the mouse CB has so far impeded a formal cell-fate mapping study in these neuroblastic cells to solve the question of their cellular origin. Nevertheless, we cannot formally discard the possibility of a mixed origin for adult CB neuroblasts, both from multipotent stem cells and directly from fetal glomus cell precursors.

Another interesting aspect of CBNBs, in fact related to their cellular origin, is the overlapping expression of markers with other undifferentiated cells. We have performed immunohistochemical studies to show the lack of co-localization of HNK-1 with GFAP

(Fig. 2a), suggesting that CB type II cells do not express the membrane glycoepitope. However, we found partial co-localization between nestin and HNK-1 (Fig. 2b), indicating an overlapping between the expressions of the intermediate progenitor marker and the neuroblast marker. We have preliminary analyzed this overlap by flow cytometry in the normoxic adult CB (Fig. 2c), and have exposed that about half of HNK-1+/TH+ neuroblasts seem to express nestin. This result might denote the existence of two subtypes of neuroblasts, with different grades of maturation. In any case, these flow cytometry data suggest that neuroblasts are likely derived from nestin+ intermediate progenitors (nestin+/HNK-1-/TH- cells in the plot of Fig. 2c), which are themselves the proliferative version of multipotent stem cells (see above), hence confirming neuroblasts as part of the CBSC lineage.

Finally, we have also studied the neurosphere-forming capacity of CB neuroblasts, compared to other populations of undifferentiated cells in the organ (Fig. 2d-h). CB bulk cells were sorted by flow cytometry into three different groups (Fig. 2d) and plated to form neurospheres: HNK-1 highly positive cells (HNK H group; mainly composed by neuroblasts), cells expressing low levels of HNK-1 (HNK L group; with a mix of neuroblasts and nestin+/HNK-1+ progenitor cells (Fig. 2c)), and HNK-1 negative cells (Negative group). Neurospheres obtained from the Negative population were bigger in size (Fig. 2e and f) and contained almost no differentiation into TH+ glomus cells (Fig. 2g and h). These neurospheres resemble those obtained from mesectodermal-restricted progenitors (see below) (Navarro-Guerrero et al., 2016). On the other hand, neuroblasts (HNK H group) gave rise to smaller neurospheres (Fig. 2e and f) composed by almost only TH+ cells (Fig. 2g and h), confirming their neurosphere-forming capacity and their high neuronal specification. Neurospheres from HNK L group displayed a small average

size and a mix of different capacities for dopaminergic differentiation (Fig.2e-h), corroborating the mix of undifferentiated cells present in this population.

3.1. CB neuroblast proliferation in response to hypoxia

In normoxic resting conditions, neuroblasts remain quiescent within the CB neural parenchyma. However, the hypoxic stimulus provokes these cells to enter the cell cycle, executing one or two cell divisions, and rapidly maturing into glomus cells (Sobrino et al., 2018). This recent description of CBNB proliferation in response to hypoxia contributes to the understanding of CB growth-mediated adaptation to chronic hypoxemia (Arias-Stella and Valcarcel, 1976; McGregor et al., 1984). But it also helps to solve a classical debate about the observation of cell cycle protein expression in CB TH+ dopaminergic cells (Chen et al., 2007; Wang et al., 2008). We have shown by time-lapse microscopy that mature glomus cells are postmitotic, and that neuroblasts are able to divide once or twice rapidly under the hypoxic stimulus (Sobrino et al., 2018). Moreover, we have observed in vivo that neuroblast proliferation takes much less time (3 to 4 days) than the stem cell production of new glomus cells, which takes 7 to 10 days to be completed (Pardal et al., 2007; Sobrino et al., 2018). Hence, the presence of quiescent immature neuronal cells within the CB neural parenchyma may have evolved to permit a faster neurogenesis and hence speedier adaptation to the hypoxic environment. Interestingly, the fact that we find some neuroblasts still expressing nestin, and we also find neuroblasts that divide only once while others divide twice, might indicate the existence of different grades of specification among neuroblasts, which could somehow increase duration and efficiency of this fast neurogenesis.

An interesting question that arose during our studies of CBNB proliferation was if these neuroblastic cells were directly sensitive to hypoxia or they rather need niche signalling to respond to the hypoxic stimulus, similar to the case of multipotent stem cells

in the organ (Platero-Luengo et al., 2014). Our in vitro time-lapse microscopy experiments have demonstrated that the hypoxic stimulus per se is sufficient to trigger neuroblast proliferation (Sobrino et al., 2018). Cellular responses to chronic hypoxia depend on the regulation of hypoxia-inducible factors (HIFs) by prolyl-hydroxylases (PHDs). PHDs are oxygen-sensitive enzymes responsible for tagging HIFs for degradation in normoxia (Kaelin and Ratcliffe, 2008). During the lack of O2, the consequent inhibition of PHDs stabilizes HIFs, which in turn activate the expression of HIF-dependent adaptation genes. We have observed a clear expression of HIF2α in CB neuroblasts, especially increased during hypoxia (Sobrino et al., 2018). In fact, recent works have clearly established a prominent role for HIF2α in CB glomus cell growth and survival during development (Hodson et al., 2016; Macias et al., 2018). Moreover, a new study has shown that inactivation of HIF2α leads to a decrease in hypoxia ventilatory response in adult mice, and to the absence of TH+ cell proliferation in the CB, while inactivation of PHD2 has the opposite effect, leading to CB hyperplasia even in normoxia (Fielding et al., 2018). Although further experiments are necessary, all these data suggest that CB neuroblasts are able to respond directly to hypoxia, by increasing proliferation in a HIF2α-dependent manner. Nevertheless, we cannot discard the existence of supplementary mechanisms, involving niche signaling, that might modulate the division of CB neuroblasts. Interestingly, all these works also confirm the presence of neuroblasts in the mouse CB, despite the lack of HNK-1 expression.

3.2. CB neuroblast maturation in response to hypoxia

As mentioned above, CBNBs are immature cells unable to respond to an acute exposure to hypoxia, despite the presence of exocytotic vesicles and the expression of membrane ion channels. These cells are not ready to function as chemoreceptor cells in the organ to translate the hypoxic stimulus (Sobrino et al., 2018). Mature glomus cells increase

intracellular calcium and mitochondrial production of NADPH in response to acute hypoxia (Arias-Mayenco et al., 2018; Fernandez-Aguera et al., 2015), and those responses are basically absent in HNK-1+ neuroblasts (Sobrino et al., 2018). CBNBs do respond, by rising intracellular calcium, to other chemical stimuli, such as high potassium, hypoglycemia, or neuromodulators like ATP or acetylcholine (ACh), meaning that these cells face the specific lack of the hypoxia-responsive machinery. Interestingly, after a short in vitro exposure to low oxygen levels (48h), CBNBs grow in size, lose HNK-1 expression, and acquire the capacity to respond to acute exposures to hypoxia (Sobrino et al., 2018), confirming their conversion into fully mature glomus cells. The specific molecular machinery, involving mitochondria, necessary to respond to hypoxia is only recently being elucidated (Arias-Mayenco et al., 2018; Fernandez-Aguera et al., 2015). However, we have not yet studied formally the expression of the identified hypoxia-responsive molecular elements in CB immature neuronal cells.

In addition to the direct effect of hypoxia on neuroblast maturation, we have observed that these cells also mature in response to different niche signaling (Sobrino et al., 2018). Incubation of CBNBs with purinergic molecules (ATP or UTP) or with ACh for 48h induces a very similar maturation process than exposure to hypoxia. Moreover, we have shown expression of purinergic receptors by neuroblasts (Sobrino et al., 2018), and classical morphometric studies indicated the presence of nicotinic receptors in the membrane of type B glomus cells (neuroblasts) (Chen and Yates, 1984). All these data together confirm that CBNBs have the capacity to mature into fully responsive glomus cells in response to purinergic and cholinergic signals being released mainly by neuronal cells. The role of ATP is particularly interesting since it constitutes the main excitatory neurotransmitter released in the CB during hypoxia. ATP has been involved not only in the chemoreceptor synapse of glomus cells with afferent fibers (Zhang et al., 2000), but

also in paracrine communications between type I glomus cells and type II sustentacular cells (multipotent stem cells) (Murali and Nurse, 2015; Piskuric and Nurse, 2013; Tse et al., 2012). We have shown that neuroblasts are a relevant component of the glomerulus and are also participating in this ATP-mediated communication within the niche. Fig. 4a summarizes the progress of neuronal cell differentiation from multipotent GFAP+ CBSCs, indicating the expression of the different markers and the potential intermediate cell types.

4. Participation of CBSCs in hypoxia-induced physiological angiogenesis and the existence of mesectodermal-restricted progenitors

As stated above, CB acclimatization to chronic hypoxia involves a profound angiogenic process, to provide new vessels that will facilitate irrigation of the growing neural parenchyma (Chen et al., 2007; Wang and Bisgard, 2002). The CB is placed in a region where NCSCs display a clear mesectodermal and vascular potency during development. In fact, the whole wall of carotid arteries and of aorta at the level of the aortic arch is derived from the neural crest, as revealed by cell fate mapping studies using Wnt1-cre transgenic mice (Jiang et al., 2000; Kameda, 2014). Therefore, it was not irrational to hypothesize that CBSCs might be able to participate in both neurogenesis and angiogenesis in response to the hypoxic stimulus. Hence, we decided to take the risk and test for the ability of adult CBSCs to convert into vascular cell types during hypoxia. To that end, we performed the same cell fate mapping approach that was previously used to prove the role of CBSCs in neurogenesis (Pardal et al., 2007). By using GFAP-cre/R26R transgenic mice, we showed that an important amount of endothelial cells within the hypoxic organ is derived from GFAP+ CB multipotent stem cells (Annese et al., 2017). Our quantification of this stem cell-dependent angiogenic process revealed that approximately one every three new endothelial cells being produced in hypoxia is derived

from CBSCs (Annese et al., 2017). The other two cells might appear from proliferation of preexisting endothelial cells or from recruitment of circulating endothelial progenitors. Our data also indicated that other vascular cell types, such as smooth muscle cells or perycytes, can also derive from CBSCs (Annese et al., 2017). Finally, in vitro studies suggested that vascular differentiation from CBSCs might be potentiated by hypoxia itself (via HIF2α) and by the release of pro-angiogenic cytokines during the hypoxic stimulus (Annese et al., 2017). Although the classical source for these cytokines is the vessels, in the case of CB some of these cytokines such as EPO or endothelin-1 (ET-1) have been shown to be released by neuronal cells (Lam et al., 2009; Platero-Luengo et al., 2014), constituting a nice example of neuronal activity-dependent instruction of multipotent stem cells. The role of ET-1 is particularly interesting since we showed that this cytokine activates stem cell proliferation after being released by neuronal cells (Platero-Luengo et al., 2014), and it also instructs proliferative progenitors to differentiate into the vascular lineage (Annese et al., 2017; Navarro-Guerrero et al., 2016). Taken together, these results indicate that glomus cells promote angiogenesis through instruction of multipotent stem cells into the vascular lineage, while neurogenesis is probably more dependent on the activity of restricted neuroblasts. After revealing the stunning plasticity displayed by CBSCs during hypoxia, we tried to clarify the cellular mechanisms involved in their participation in angiogenesis. Specifically, we explored the possibility of finding intermediate restricted progenitors belonging to the vascular lineage of CBSCs within the CB parenchyma.

4.1. Mesectodermal-restricted progenitors within the adult CB

When analyzing a microarray of gene expression comparing CB neurospheres with different amounts of neuronal differentiation (Navarro-Guerrero et al., 2016), we identified CD10 (also known as neprilysin) as a cell surface marker highly expressed in

non-neuronal cells. We first discarded CD10 as a marker for CB multipotent cells. By performing neurosphere assays with CD10+ and CD10- cells, we found that the marker was labeling a subtype of progenitor cells that had lost the ability to convert into TH+ glomus cells (Navarro-Guerrero et al., 2016). Typical neurospheres obtained from CD10+ progenitors were larger than normal and had no signs of dopaminergic differentiation (absence of TH+ blebs). Nevertheless, we demonstrated, by cell fate mapping with GFAP-cre/R26R transgenic mice, that these CD10+ progenitors belong to the CBSC lineage, hence they are not just some type of vascular progenitors being recruited from circulation (Navarro-Guerrero et al., 2016).

CD10 is a membrane zinc-dependent metallo-endopeptidase (Turner and Tanzawa, 1997), that has been shown to cleave signaling peptides in CNS synapses (Roques et al., 1993) and in the CB (Kumar et al., 2000). We have shown that CB mesectodermal-restricted progenitors (CD10+) are present in the normoxic parenchyma, probably preserved in a quiescent state thanks to the cleavage of pro-angiogenic cytokines by CD10. During the hypoxic stimulus, the expression of CD10 in these cells is downregulated (Navarro-Guerrero et al., 2016), increasing their sensitivity to pro-angiogenic cytokines being released under hypoxia, such as EPO or ET-1. The presence of these restricted progenitors within the resting CB parenchyma might have evolved to facilitate fast angiogenesis in response to the hypoxic stimulus.

We also searched for potential markers that might be more commonly expressed in the whole vascular lineage of CBSCs. In this regard we found CD34 as another typical marker for the vascular lineage within the CB, but less exclusive than CD10 (Fig. 3). CD34 has been described as a marker for endothelial progenitors and endothelial cells (Shi et al., 1998), but also as a marker for mesectodermal progenitor cells of neural crest origin in different tissues and organs (Diaz-Flores et al., 2014; Sowa et al., 2013). CD34+

cells in the CB belong to the neural crest lineage and are derived from CBSCs, as evidenced by cell fate mapping (Fig. 3a and b). We found overlapping expression of CD34 with the differentiated endothelial marker CD31 and with CB vascular progenitor marker CD10 (Fig. 3c), confirming that CD34 is a marker for the vascular lineage of CBSCs. However, CD34 seems to start being expressed very early in the specification process, as we find co-expression with GFAP or nestin in some cells (Fig. 4d). In fact, the data suggest the existence of some CD34+ cells that have not completely lost their capacity for neuronal differentiation (Fig. 3e-h), meaning that CD34 expression starts before multipotency has been completely switched off. The diagram showed in Fig. 4b summarizes the progress of endothelial cell differentiation from multipotent GFAP+ CBSCs, indicating the overlapping of the different marker expressions and the potential intermediate cell types that might be having a role in this progression.

5. Clinical implications and concluding remarks

The carotid body has been implicated in the pathophysiology of multiple diseases that course with sympathetic over activation, generally affecting the cardiorespiratory system. In some of these pathologies, like in hypertension, sleep apnea, chronic heart failure, or some forms of chronic kidney disease, an over activation of the CB has been proven (Gao et al., 2014; Paton et al., 2013). In some others, like in obesity, obstructive pulmonary disease, asthma, metabolic syndrome, and diabetes mellitus, the CB is in the spotlight because of having some non-clarified role (Cramer et al., 2014; Gao et al., 2014; López-Barneo et al., 2016; McBryde et al., 2013; Paton et al., 2013). In the majority of these illnesses, an increase in the size of the CB has been reported (Cramer et al., 2014), very likely associated to its over activation and to disease progression. Nevertheless, in some cases over activation does not necessarily implies parenchyma growth, but probably just maturation of neuroblasts without proliferation (Sobrino et al., 2018). Nowadays, the CB

constitutes a principal target during the treatment of most of these diseases. In fact, in the case of chronic heart failure and hypertension, resection and denervation of the CB is being tested to try to ameliorate the symptoms (Del Rio et al., 2013; Narkiewicz et al., 2016; Ribeiro et al., 2013). However, clinical research in these pathologies is lately focusing on trying to find drugs that would ameliorate over activation of the CB, in order to avoid direct surgical resection of the organ (McBryde et al., 2013; Pijacka et al., 2016). Our work offers multiple options to try to halt this over activation of the organ. We have characterized the presence of diverse multipotent and restricted progenitor cells within the CB parenchyma, and have clarified the mechanisms by which these cells proliferate and differentiate to contribute to tissue growing. By pharmacologically blocking these processes we should be able to avoid CB growth and hence prevent CB over activation-dependent symptoms.

A malformation of the CB has also been shown in another series of pathologies related to an impaired development, such as Sudden Infant Death Syndrome (SIDS) and Congenital Central Hypoventilation Syndrome (CCHS). These diseases have been revealed to course with a reduction in the number of glomus cells and in the number of exocytotic vesicles per glomus cell (Cutz et al., 1997; Porzionato et al., 2013), which may cause malfunction of the organ and the appearance of serious apneas leading to death. Our results on the study of the biology of CB neuroblasts might shed light on the pathophysiology of these diseases.

Regarding a very different type of pathology, an interesting question is whether the proliferative potential of the CB stem cell niche is related to the appearance of paragangliomas in the organ. These tumors are usually benign and resemble the CB of individuals exposed to chronic hypoxemia (Arias-Stella and Valcarcel, 1976; Kliewer et al., 1989). Moreover, the incidence of CB paragangliomas increases in high-altitude

dwellers (Arias-Stella and Bustos, 1976; Astrom et al., 2003; Saldana et al., 1973). However, it has not been established whether there is a relationship between CB germinal niche and tumorigenesis within the organ. Mitochondrial mutations described as the most frequent cause of congenital paraganglioma (Baysal, 2008; Rustin et al., 2002) do not give rise to any type of growth when studied in animal models (Diaz-Castro et al., 2012; Piruat et al., 2004). On the other hand, a recent work has demonstrated that inactivation of PHD2 in TH+ dopaminergic cells induces paraganglioma-like growth in the mouse CB (Fielding et al., 2018). Stabilization of HIF2α in dopaminergic cells seems to promote massive proliferation of TH+ cells, accompanied by strong vascularization. These data suggest that there could be a relationship between the cellular mechanisms for CB hypertrophy and the appearance of paragangliomas. In any case, our understanding of CB niche functioning will very likely improve our capacity to treat paraganglioma tumors.

Finally, another clinical aspect that might benefit from our studies on the CB niche is the use of CBSCs in cell therapy against Parkinson disease. CB cell aggregates have been successfully transplanted into the brain for the amelioration of Parkinson symptoms in animal models, due to the release of dopaminotrophic factors by neuronal type I cells (Villadiego et al., 2005). CBSC cultures have been proposed as a strategy to optimize glomus cell transplantation (Pardal and Lopez-Barneo, 2012). Our results might help to obtain better yields of glomus cell production in vitro and hence to increase the efficiency of this type of transplants against Parkinson disease.

In summary, the CB has evolved as a remarkable oxygen detector in mammals, containing a stunning germinal niche within the adult PNS, necessary for a correct physiological adaptation to a changing environment. Our recent data on the characterization of diverse stem and progenitor cells present within the CB parenchyma is increasing our understanding of the organ physiology and pathology, will very likely

improve the treatment of a variety of different diseases related to CB malfunction, and will probably influence the use of the CB niche for cell therapy against neurodegenerative disorders.

6. Bibliography

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Figure Legends

Fig. 1 Migratory movements of nestin+ progenitors within the CB parenchyma. (**a**) Composite of two electron micrographs displaying the detection of GFAP+ (blue pseudo-colored) and nestin+ (green pseudo-colored) cells, using gold particle-associated antibodies, within the parenchyma of a normoxic rat CB. Different developmental times gave rise to different gold particle sizes for both stainings (panels **1** and **2**). Scale bars: 2μm in (a) and 200nm in (a1 and a2). (**b**) Quantification of the distance between GFAP+ or nestin+ cells and the closest TH+ cell (left graph) or the closest endothelial cell (right graph), using confocal microscopy (pictures not shown). (**c**) Electron micrograph showing an example of a GFAP/nestin double positive cell. EC: endothelial cell. Scale bars: 2μm and 200nm in inset (3). (**d**) Transwell migration assay with CB progenitors. Neurosphere-dissociated cells are plated on one side of the porous filter and photographed on the other side after 72h and Crystal Violet staining. EPO: erythropoietin at 7IU/mL. PD: 50μM of PD98059 (Sigma), inhibitor of EPO signaling. Ab: EPO-neutralizing antibody (1:20; Santa Cruz). (**e**) Migratory index measured in the different conditions shown in (d). (**f**) Time-lapse microscopy measurements of nestin+ progenitor cell movement in flat substrate, without (left) or with (right) EPO at the indicated concentration in the culture medium. Trajectories are delineated with ImageJ software. (**g**) Quantification of cumulated distance of the cellular movement shown in (f), but in the indicated conditions. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, Student's t test.

Fig. 2 Marker expression in CB neuroblasts. (**a**) Immunohistological detection of HNK-1 (green) and GFAP (red) in a section of a normoxic rat CB. The inset (1) further shows the lack of co-localization. Scale bars: 10μm. (**b**) Immunostaining for HNK-1 (green) and nestin (red) in a normoxic rat CB slice showing HNK-1+ cells (blue arrowheads), nestin+ cells (white arrowheads), and HNK-1/nestin double positive cells (yellow arrowheads). Scale bar: 10μm. (**c**) Flow cytometry plot showing CB bulk cells stained with antibodies against HNK-1, nestin, and TH. Nestin+ cells have been previously gated in blue in order to be visible in the TH vs. HNK-1 plot. (**d**) Cytometric plot showing the staining of CB bulk cells with anti-HNK-1 antibodies. Three different populations (High, Low, and Negative), regarding expression of HNK-1, were sorted to form neurospheres. (**e**) Bright field pictures of neurospheres obtained from the cell populations shown in (d). Scale bar: 200μm. (**f**) Quantification of diameters of the neurospheres shown in (e). (**g**) Examples of neurospheres stained with nestin (green) and TH (red), from the three groups shown in (d) and (e). Scale bar: 100μm. (**h**) Quantification of dopaminergic differentiation present in neurosphere sections from the study shown in (d-g). Cell nuclei were counterstained with DAPI. * $p<0.05$, *** $p<0.001$, Student's t test.

Fig. 3 Marker expression in CB mesectodermal-restricted progenitors. (**a**) Example of a CD34+/X-Gal+ cell obtained after cell dispersion of a normoxic CB from a Wnt1-cre/R26R mouse, corroborating the neural crest origin of this type of cells in the CB. Scale bar: 10μm. (**b**) Example of a CD34+/X-Gal+ cell obtained from the CB of a GFAP-cre/R26R mouse, indicating that this type of cells can derive from GFAP+ CB stem cells. Scale bar: 10μm. (**c**) Flow cytometry plots showing co-expressions of the vascular markers CD31, CD34, and CD10 in normoxic CB cells. (**d**) Immunocytochemical

examples of co-expression of GFAP with CD34, or nestin with CD34, in normoxic CB dispersed cells. Scale bar: 10μm. (**e**) Neurospheres, obtained from culturing the indicated sorted cells, were plated onto adherent and stained for endothelial cell-specific lectin (GSA I; green), TH (red), and DAPI (blue), to study multipotentiality of the sorted cells. Scale bar: 100μm. (**f**) Quantification of neurosphere formation from the experiment shown in (e). (**g** and **h**) Quantification of the presence of differentiated cells in the cultures shown in (e). * $p<0.05$, *$p<0.01$, Student's t test.

Fig. 4 CBSC lineage progression. (**a**) Progression of the neuronal lineage of CBSCs, showing marker expression and potential cell types present in the niche. (**b**) Progression of the endothelial lineage of CBSCs, showing marker expression and potential cell types present in the niche. Curved arrows symbolize proliferation capacity.

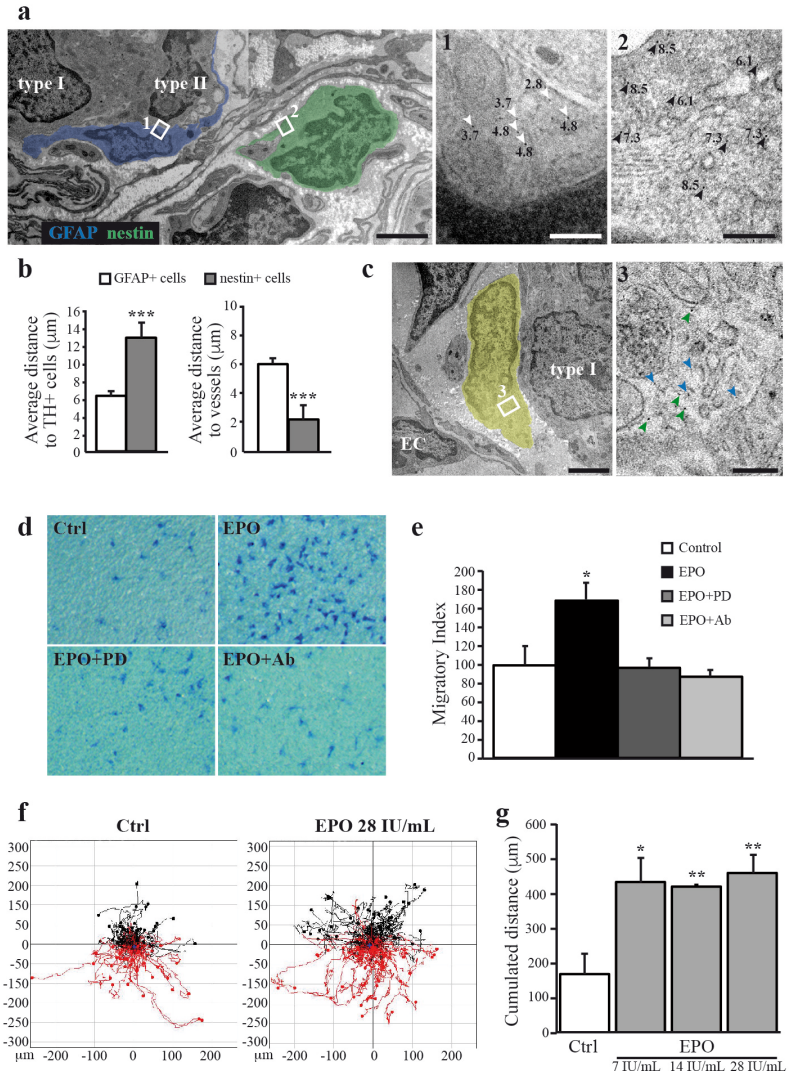
a
type I
type II
1
2
GFAP nestin
2.8
3.7
4.8
3.7
4.8
4.8
8.5
6.1
8.5
6.1
7.3
7.3
7.3
8.5
b
GFAP+ cells
nestin+ cells
Average distance to TH+ cells (µm)
Average distance to vessels (µm)
***
***
c
type I
EC
3
d
Ctrl
EPO
EPO+PD
EPO+Ab
e
Control
EPO
EPO+PD
EPO+Ab
Migratory Index
*
f
Ctrl
EPO 28 IU/mL
µm
g
Cumulated distance (µm)
*
**
**
Ctrl
EPO
7 IU/mL
14 IU/mL
28 IU/mL

Figure 1

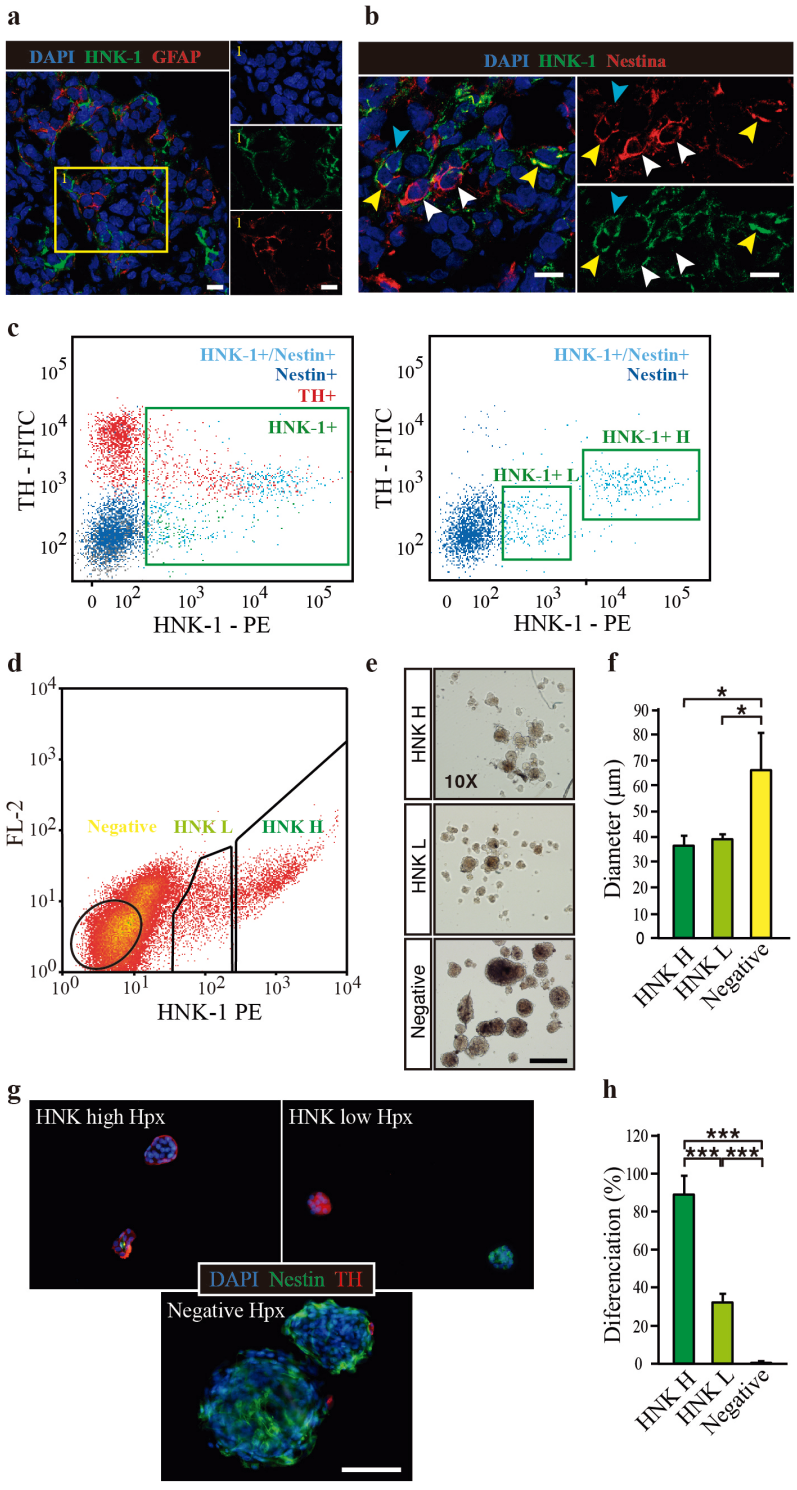
a
DAPI HNK-1 GFAP
b
DAPI HNK-1 Nestina
c
HNK-1+/Nestin+
Nestin+
TH+
HNK-1+
TH - FITC
HNK-1 - PE
HNK-1+ H
HNK-1+ L
d
Negative
HNK L
HNK H
FL-2
HNK-1 PE
e
HNK H
HNK L
Negative
10X
f
Diameter (µm)
HNK H
HNK L
Negative
g
HNK high Hpx
HNK low Hpx
DAPI Nestin TH
Negative Hpx
h
Diferenciation (%)
HNK H
HNK L
Negative

Figure 2

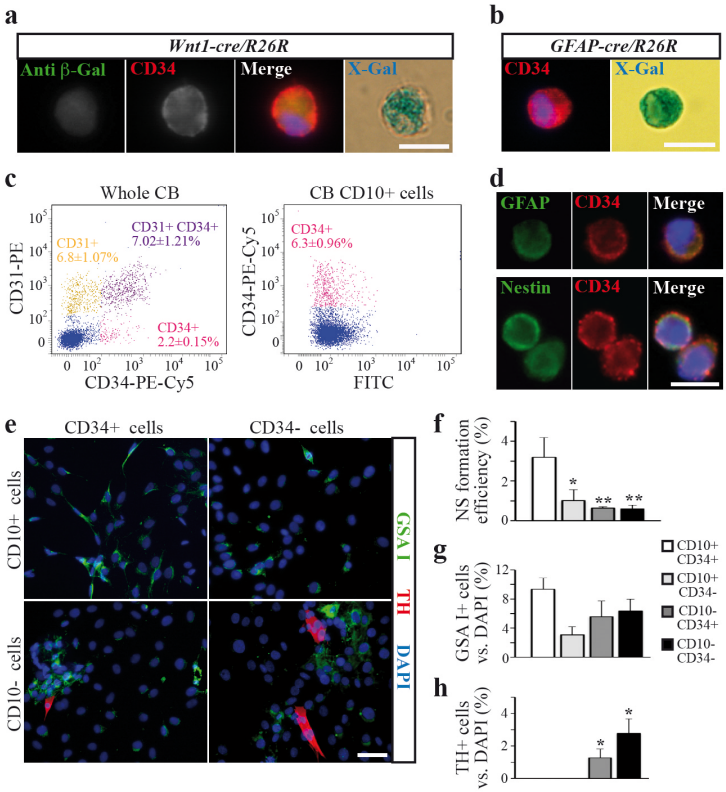
a
Wnt1-cre/R26R
Anti β-Gal
CD34
Merge
X-Gal
b
GFAP-cre/R26R
CD34
X-Gal
c
Whole CB
CB CD10+ cells
CD31-PE
CD34-PE-Cy5
CD31+
6.8±1.07%
CD31+ CD34+
7.02±1.21%
CD34+
2.2±0.15%
CD34+
6.3±0.96%
FITC
d
GFAP
CD34
Merge
Nestin
CD34
Merge
e
CD34+ cells
CD34- cells
CD10+ cells
CD10- cells
GSA I
TH
DAPI
f
NS formation efficiency (%)
g
GSA I+ cells vs. DAPI (%)
CD10+ CD34+
CD10+ CD34-
CD10- CD34+
CD10- CD34-
h
TH+ cells vs. DAPI (%)

Figure 3

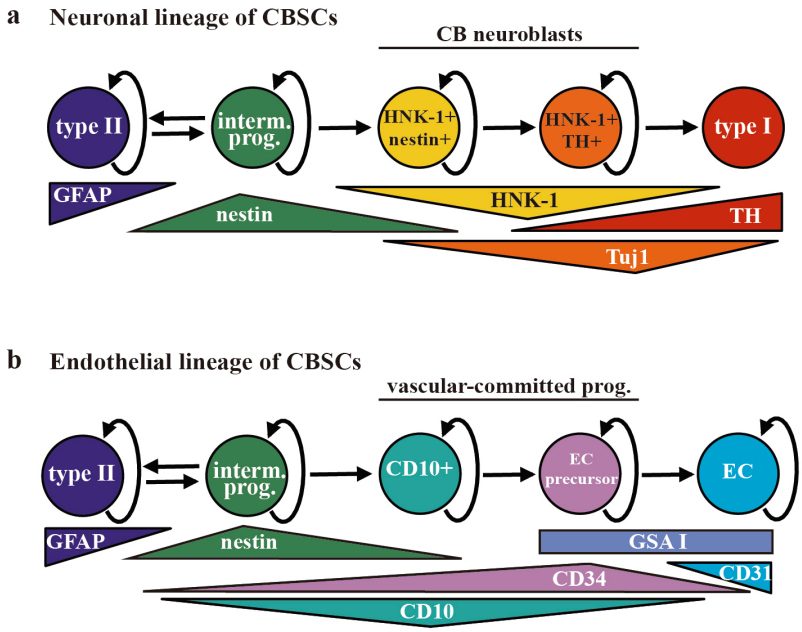
a
Neuronal lineage of CBSCs
CB neuroblasts
type II
interm. prog.
HNK-1+ nestin+
HNK-1+ TH+
type I
GFAP
nestin
HNK-1
TH
Tuj1
b
Endothelial lineage of CBSCs
vascular-committed prog.
type II
interm. prog.
CD10+
EC precursor
EC
GFAP
nestin
GSA I
CD34
CD31
CD10

Figure 4