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Endothelial and Neural Commitment in Differentiating Embryonic Stem Cells

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Abstract

Mouse embryonic stem (ES) cells are pluripotent cell lines derived from the inner cell mass of the blastocyst. Their properties include the capacity to self renew and to in vitro differentiate into embryo-like structures, Embryoid Bodies (EB), that contain derivatives of the ectodermal, mesodermal and endodermal lineages.

In the sphere of vascular biology studies it has been shown in recent years that several signalling pathways involved in angiogenesis and endothelial cell migration are indeed regulated by molecules whose role was formerly identified in the nervous system. These findings suggest a possible interplay between vascular and nervous system during development. To this regard, by using as surface markers Flk-1 and Nrp-1, the two receptors of VEGF (Vascular Endothelial Growth Factor), we purified from EBs a population of cells which can differentiate into endothelial or neural cells upon appropriate culture conditions.

Since the EB is a heterogeneous structure containing many different cell types, we shifted to a more defined ES culture system that allows the simultaneous differentiation mainly of endothelial cells and neurons. The analysis by real-time PCR and immunofluorescence for the expression of endothelial and neural markers confirmed that this model is suitable to study endothelial and neural cell interactions. Moreover, our culture system can be viewed as a microenvironment similar to vascular niche, as many of its components develop upon appropriate stimuli.

Preface

Embryonic stem (ES) cells are pluripotent cells derived from the inner cell mass of blastocyst-stage embryos. Their importance to modern biology and medicine derives from two unique characteristics that distinguish them from all other cells identified to date. First, they have the capacity to self-renew, meaning that they can be expanded and maintained in culture as pure populations of undifferentiated cells for extended periods of time. Second, they are pluripotent, possessing the capacity to generate every cell type in the body under appropriate conditions or stimuli. The pluripotent nature of mouse ES cells was formally demonstrated by their ability to contribute to all tissues of adult mice, including the germline, following their injection into host blastocysts (Bradley et al., 1984). Therefore, ES cells are used as experimental model in order to obtain specific precursor cells and to analyze early differentiation events that are difficult to study in the mouse embryo and inaccessible in the humans. Moreover, the ES cell differentiation system can be viewed as a novel and unlimited source of cells and tissues for transplantation in support of the treatment of a broad spectrum of diseases.

The first part of this work was aimed to assess the commitment of embryoid bodies derived cells toward endothelial or neural fate. We showed that the $Nrp1^{+}/Flk1^{+}$ cell population can adopt either neural or endothelial phenotype, depending on appropriate stimuli coming from the microenvironment, both in vitro and in vivo. These data led to the publication **Microenvironment drives the endothelial or neural fate of differentiating embryonic stem cells coexpressing neuropilin-1 and Flk-1, FASEB Journal (2008).**

Introduction

1. Generation of embryonic stem cell lines

In the early 80s, pluripotent cells called embryonic stem cells were isolated from the inner cell mass (ICM) of developing murine blastocysts (Evans and Kaufman, 1981; Martin, 1981). In order to derive an ES cell line, embryos of early pre-implantation stage (3.5 days after fertilization) are required. At this developmental time the outer layer of the blastocyst is surrounded by the cells of the trophoblast, which will give rise to the embryonic annexes. Within the blastocyst is present a cavity, the blastocoele, and one side is occupied by a cellular mass, the ICM, separated from blastocoele by the cells of the primitive endoderm. The ICM is the source of the epiblast, from which the three embryonic layers will later originate: the definitive endoderm, mesoderm and ectoderm and the germ cells as well (Fig. 1). Hence, the peculiar population of cells that compose the ICM exists in vivo for a very short period of time, and only at this stage is possible to efficiently derive ES cell lines.

The derivation of ES cells from ICM requires a relatively simple protocol. Embryos at the expanded blastocyst stage are plated onto a feeder layer composed by murine embryonic fibroblast. After several days of culture, the epiblast-derived cell mass is disaggregated and replated. Various types of differentiated colonies arise along with colonies of a characteristic undifferentiated morphology. The latter are individually dissociated and replated, thus establishing the ES cell line. They proliferate rapidly in culture, and clonal populations can be easily initiated from single cell (Smith, 2001).

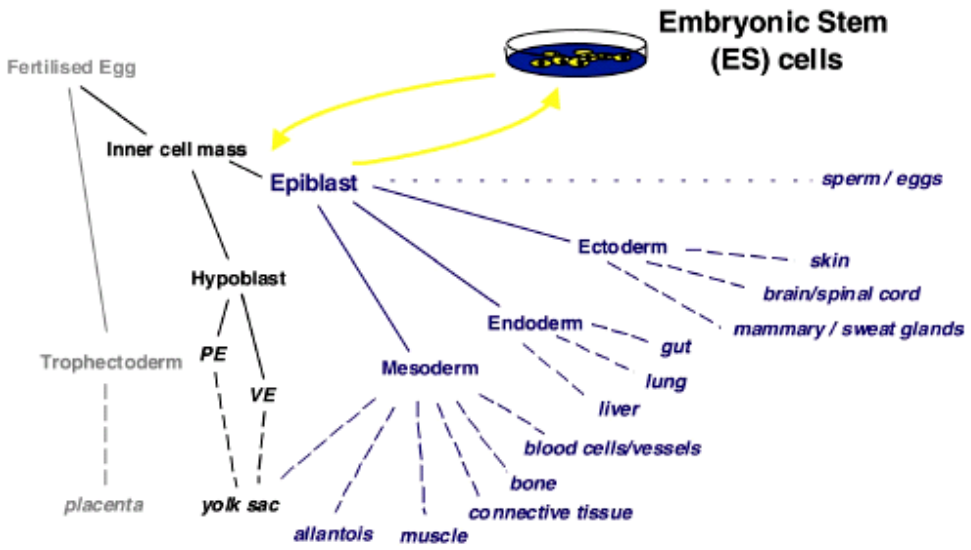

Fig. 1. Lineage diagram of mouse development. Embryonic stem cells are derived from the inner cell mass, thus they are able to enter all the lineages that are generated in vivo by the epiplast (blue lines). ES cells can produce also hypoblast derivatives in vitro but rarely do so in vivo (black lines). Modified from Smith, A.G. (2001). Embryo-derived stem cells: Of Mice and Men. Annu Rev Cell Dev Biol.

Similar techniques were employed in order to derive ES from many species other than mouse, i.e. hamster (Doetschman et al., 1988), rabbit (Graves and Moreadith, 1993), mink (Sukoyan et al., 1993), chicken (Pain et al., 1996), rat (Iannaccone et al., 1994), pig (Shim et al., 1997), cow (First et al., 1994), rhesus monkey (Thomson et al., 1995), and also human. In this case, human embryos originally produced by in vitro fertilization for clinical purpose were cultured to the blastocyst stage and the inner cell masses were then isolated (Thomson et al., 1998). Although basically similar, there are some differences between mouse and human ES (hES) cells in their culture requirements, the morphology of the undifferentiated cells in vitro, and the expression of surface antigens (Brivanlou et al., 2003). Furthermore, hES cells have the potential to differentiate into trophoblast in vitro, a property not observed for mouse ES cells (Odorico et al., 2001; Thomson and Odorico, 2000). The generation of hES cell lines has represented a determinant turn in the cell therapy field. The motivations for this interest consist in the possibility to provide a virtually unlimited source of cells for tissue repair, to investigate and manipulate specific gene function in human cells, and to provide large number of phenotypically defined cell

types for drug screening. Nevertheless, because of serious ethical concerns in the derivation and use of hES from embryos the mouse still remains the organism more easily accessible for developmental biology studies.

2. Defining *stemness*: pluripotency and self-renewal

In order to routinely expand ES cells in vitro as an undifferentiated population, the presence of leukemia inhibitory factor (LIF) in the culture medium and/or a feeder layer of murine embryonic fibroblasts (MEF) are required. Upon these conditions, ES cells can be expanded indefinitely in culture as a homogeneous population without a requirement of immortalization. The reproducible production of chimeric embryos by injecting few ES cells into blastocysts combined to the unlimited generation of identical subclones, confirm that ES cells undergo symmetrical self-renewal. In fact, in contrast to other primary cultures, ES cells appear to be immortal and show no evidence of senescence. This behavior correlates to the high activity of the telomerase, a ribonucleoprotein that, by maintaining telomere length, plays an important role in replicative life-span (Counter, 1996). In addition, mouse and human-derived ES cells express several exclusive proteins that can be considered distinctive of the undifferentiated status. These markers include the stage-specific embryonic antigen (SSEA)-1, SSEA-3, SSEA-4, Oct-3/4, CD9 and alkaline phosphatase (Koestenbauer et al., 2006), with some differences between mouse and human ES cells.

Maintenance of the undifferentiated stem cell phenotype is not a cell-autonomous process: at each ES cell division, the alternative outcomes of self-renewal and differentiation are decided by the interplay between intrinsic and extrinsic factors. Media containing all necessary metabolites and nutrients are not sufficient to support either derivation or maintenance of ES cells. Even though co-culture with a feeder layer was originally considered essential to maintain ES cells undifferentiated, it was later discovered that feeders are dispensable when using conditioned media from Buffalo rat liver cells (Smith and Hooper, 1987). This suggested that the crucial requirement is to provide key signals coming from the milieu, without which the ES cells differentiate.

This finding lead to the identification of a diffusible inhibitor of differentiation, the cytokine leukemia inhibitory factor (LIF), that could alone sustain murine ES cell self-renewal in the absence of feeders (Smith et al., 1988). LIF is also produced by feeder cells, and its expression is stimulated by the presence of ES cells (Rathjen et al., 1990). On withdrawal of LIF (or feeders), proliferation continues but differentiation occurs, and stem cells do not persist in the culture beyond a few days. It was demonstrated that the effect of LIF is mediated via heterodimerization of two members of the class I cytokine receptors, the low-affinity LIF receptor (LIF-R) and gp130, the signal transducer of interleukin-6 (Davis et al., 1993; Gearing et al., 1991).

Besides this, ES cells can also be derived and maintained using a combination of interleukin-6 and soluble interleukin-6 receptor (IL-6/sIL-6R). In this case, signalling is initiated via formation of gp130 homodimers without involvement of LIF-R (Nichols et al., 1994; Yoshida et al., 1994). Signals that derive from gp130 are therefore sufficient for self-renewal. The pathway downstream gp130 is initiated by the recruitment of JAK kinases, which consequently leads to activation of STAT3 and to the MAP kinase signaling cascade. In particular, STAT3 is essential to the LIF signalling pathway, and appears to play a central role in the balance between self-renewal and differentiation (Burdon et al., 1999; Niwa et al., 1998). However, STAT3 is not specific of ES cells but is found in a variety of other cell types, and often is associated with differentiation. Nevertheless, the requirement for gp130 activation appears to be facultative in vivo, because early embryogenesis can proceed in the absence of gp130 (Nichols et al., 2001).

The POU-domain transcription factor Oct-3/4 is expressed in totipotent and pluripotent cells, including oocytes, early-cleavage-stages embryos, the ICM of the blastocyst, epiblast, germ cells and cultured ES, but it is absent from all differentiated somatic cell types in vitro and in vivo (Okamoto et al., 1990; Rosner et al., 1990). Oct-3/4-deficient embryos fail to initiate foetal development because the ICM founder cells do not acquire pluripotency and are restricted to the extraembryonic trophectoderm lineage (Nichols et al., 1998). Oct-3/4 is a critical regulator for maintenance and differentiation of pluripotent cells. Indeed, the amount of Oct-3/4 molecule determines three distinct fates of ES cells: optimal Oct-3/4 level is necessary to maintain the pluripotent status, less than twofold increase of the optimal level causes differentiation into primitive endoderm and

mesoderm, while repression induces loss of pluripotency and dedifferentiation to trophectoderm (Niwa et al., 2000). A few target genes activated by Oct-3/4 have been identified to date, among which the most relevant are *Fgf4* and *Zfp42/Rex1* (Niwa, 2001), both involved in maintaining self-renewal. However, Oct-3/4, as other transcription factors belonging to the POU family, can act as both transcriptional activator and repressor, depending on which cofactor(s) is recruited.

As described so far, Oct-3/4 can be considered a molecular rheostat able to switch the cell fate from self-renewal to differentiation. It cooperates with two other transcription factors, namely Nanog and Sox2, creating the regulatory network responsible for the maintenance of the ES cells' undifferentiated status.

Nanog is a homeodomain protein whose enhanced expression confers constitutive self-renewal on ES cells. Nanog mRNA is present in ICM cells of the living embryo and during development its expression is further restricted to the epiblast. By implantation stage, Nanog mRNA is downregulated, but it becomes readily detectable in primordial germ cells. In vitro, Nanog overexpression can relieve completely of LIF-mediated STAT3 activation, unless *Oct-3/4* is deleted. In fact, continued expression of Oct-3/4 is essential for Nanog-mediated self renewal, suggesting cooperation between these two transcription factors (Chambers et al., 2003). Furthermore, in cell fusion experiments Nanog has shown the ability to transfer pluripotency from ES to neural stem cells, thus being able to re-organize a differentiated epigenome (Silva et al., 2006).

Sox2 is a member of the *Sox* (SRY-related HMG box) gene family that encodes transcription factors with a single High-Mobility-Group DNA-binding domain. Sox2, as Oct-3/4 and Nanog, behave as master regulator of pluripotency and is essential for mammalian embryogenesis. *Sox2* expression is associated with uncommitted dividing stem and precursor cells of the developing central nervous system. Indeed Sox2 is one of the earliest known transcription factors expressed in the developing neural tube (Zappone et al., 2000). In early stages of development, *Sox2* marks the pluripotent lineage of the mouse embryo meaning that, like *Oct-3/4*, it is expressed in the ICM, epiblast, and germ cells. However, unlike *Oct-3/4*, *Sox2* is also expressed by the multipotent cells of the extraembryonic ectoderm. In both lineages Sox2 expression is downregulated as soon as differentiation proceeds (Avilion et al., 2003). In vitro, it was demonstrated that Sox2 can

act synergistically with Oct3/4 to activate target genes containing Sox2:Oct-3/4 enhancers, including *Fgf4*, *Nanog*, *Oct-3/4* and *Sox2* itself (Kuroda et al., 2005; Okumura-Nakanishi et al., 2005; Yuan et al., 1995). Moreover, Sox2 is necessary for regulating multiple transcription factors that affect Oct3/4 expression (Masui et al., 2007), and moderate increases in the level of Sox2 protein in ES cells reduces their self-renewal and promotes their differentiation (Kopp et al., 2008; Rizzino, 2008). These observations together indicates that, like Oct-3/4, Sox2 expression must be preserved within narrow limits, and its essential function is to stabilize ES cells in a pluripotent state by maintaining the required level of Oct3/4 expression. All these data, taken together, allowed the drawing of a map depicting the interacting molecular networks responsible of ES self-renewal (Zhou et al., 2007), (Fig. 2).

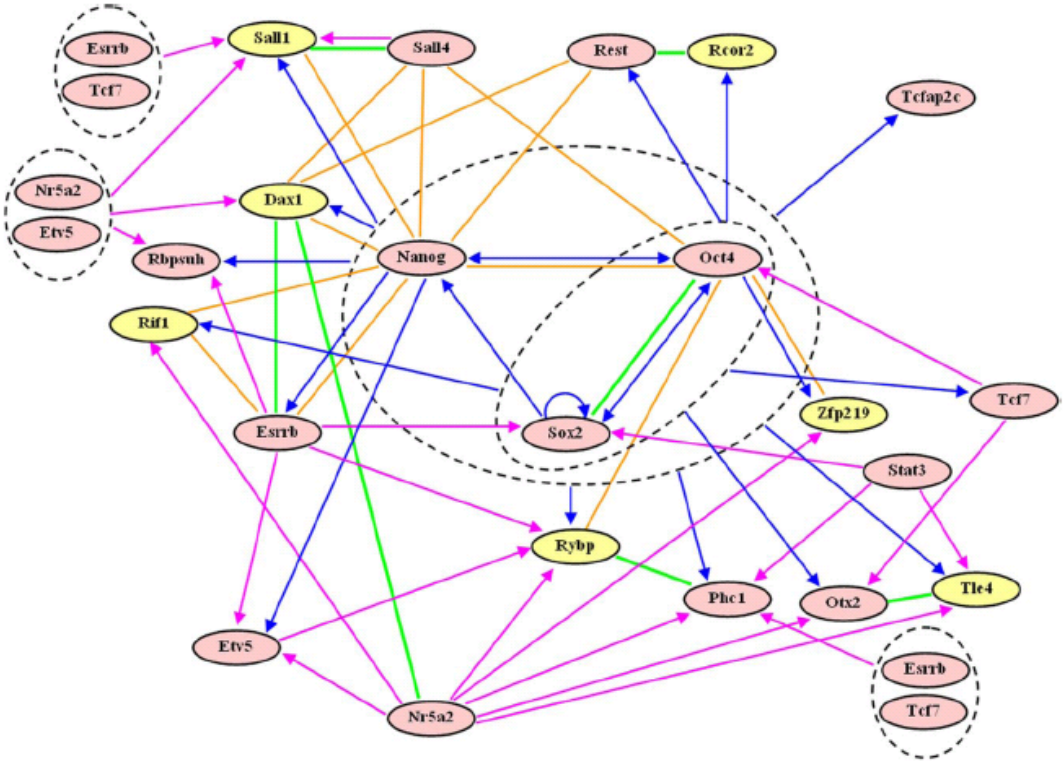

Fig. 2. The regulatory network in mouse ES cells self-renewal. The network is anchored on the master regulators Oct3/4, Sox2 and Nanog, and represents the interactions among the core regulators (pink) and their protein-interaction partners (yellow). Blue and pink arrows indicate regulatory interactions inferred by ChIP-chip analysis. From Zhou et al. (2007). A gene regulatory network in mouse embryonic stem cells. Proc Natl Acad Sci U S A.

3. Differentiation of embryonic stem cells

Differentiation of ES cells *in vitro* provides a powerful model for addressing questions related to development and lineage commitment, and offers several advantages over comparable approaches in the whole embryo. First, the possibility to generate committed cells provides access to populations otherwise difficult to study *in vivo*. Second, *in vitro* differentiation of ES cells in which both alleles of a specific gene have been disrupted by targeted mutations represents a good tool to investigate the impact of a null mutation, especially if such deletion provokes early embryo death.

Differentiation of ES cells can be achieved in a number of different ways, all based on the removal from contact with feeder cells and from LIF in the culture medium. The principal techniques used to induce ES cells differentiation are the formation of cellular aggregates called embryoid bodies and cultures onto stromal cells or onto extracellular matrix protein-coated dishes, especially if the purpose is to induce specific lineages.

3.1. Differentiation of ES cells via embryoid bodies formation

The principal method to induce differentiation of ES cells is represented by cell aggregation in suspension culture. The culture is initiated by the removal of feeder cells and LIF from the medium and by seeding ES cells in bacterial petri dishes either in liquid or in semisolid medium containing methyl cellulose. Under these conditions, ES cells are unable to adhere to the surface of the culture dish, therefore forming floating spheroid aggregates called embryoid bodies (EBs). Alternatively, the method of culturing ES cells in 'hanging drops' still forces cell aggregation and allows the control on cells number and EBs' dimension. Once formed, the EBs can be transferred to standard tissue cultures in order to complete their development (Keller, 1995). EBs contain ectodermal, mesodermal and endodermal tissues reminding of the embryo at egg-cylinder stage. When kept in suspension, EBs structure consists of an outer layer of endoderm, sometimes surrounding a large cystic yolk sac-like cavity, while the inner part is occupied by ectoderm- and mesoderm-like cells (Abe et al., 1996; Doetschman et al., 1985). EBs, however, differ

from the embryo in the lack of positional cues, thus resulting into a heterogeneous cellular mass often resembling to a teratoma.

Cells within developing EBs can differentiate into a variety of cellular types, including primitive and definitive hematopoietic cells (Doetschman et al., 1985; Nakano et al., 1996; Nishikawa et al., 1998; Wiles and Keller, 1991), endothelial cells (Risau et al., 1988; Wang et al., 1992; Yamashita et al., 2000), cardiomyocites (Maltsev et al., 1993; Miller-Hance et al., 1993), skeletal and smooth muscle cells (Rohwedel et al., 1994; Sanchez et al., 1991; Yamashita et al., 2000), neurons (Bain et al., 1995; Strubing et al., 1995) and glial cells (Brustle et al., 1999; Fraichard et al., 1995).

Therefore, embryoid bodies formation can be used as a powerful tool to study the early phases of embryonic development thanks to their ability to recapitulate the successive maturation steps that occurs in vivo (Rohwedel et al., 1994; Vittet et al., 1996). However, the cellular composition of EBs is very heterogeneous and differentiation take place with the major disadvantage that some EB derivatives are poorly represented, as is the case for neural and muscular cells. This indicates that factors required for the differentiation of these lineages are reduced or almost absent during EB development.

3.2. Direct differentiation of ES cells

The developmental fate of differentiating ES cells depends on genetic programs that are induced by extrinsic factors such as signaling molecules, growth factors, cell-cell contacts and extracellular matrix proteins present in the microenvironment in which ES cells develops. To obtain insights related to this process, methods alternative to EBs formation were developed during time in order to preferentially induce those specific lineages that are difficult to obtain with EBs. One of these methods relies on the use of stromal cell lines that are able to direct, by cell-surface or soluble factors, the differentiation of ES cells. This process is called ‘direct differentiation’ because a suspension of ES cells is seeded upon a layer of stromal cells and differentiation occurs without formation of EBs. For instance, the brain calvaria-derived OP9 stromal cells are reported to be optimal feeders for promoting hematopoietic commitment (Hirashima et

al., 1999), while PA6 stromal cells are useful to induce neuronal differentiation (Kawasaki et al., 2000). It is not well clear however which is the factor(s) produced by these cells promoting the differentiation of one specific lineage at the expense of the others.

The use of extracellular matrix proteins has also been reported to be effective to favor single lineage commitment. ES cells are seeded onto tissue culture dishes coated either with gelatin, collagen or laminin in a defined medium and differentiation occurs within few days. Notably, collagen IV is proficiently used to induce mesodermal subsets like endothelial and hematopoietic precursors (Nishikawa et al., 1998), while neurons arise from ES cells plated onto gelatin-coated dishes when cultured in absence of serum (Ying et al., 2003).

Therefore, the direct differentiation culture system is very useful to obtain precursors or committed cells of the lineage of interest and to understand the molecular mechanisms at the cutting edge of differentiation studies. Furthermore, the possibility to generate large quantities of selected cells is pivotal when the purposes are downstream applications in the transplantation and regenerative medicine field.

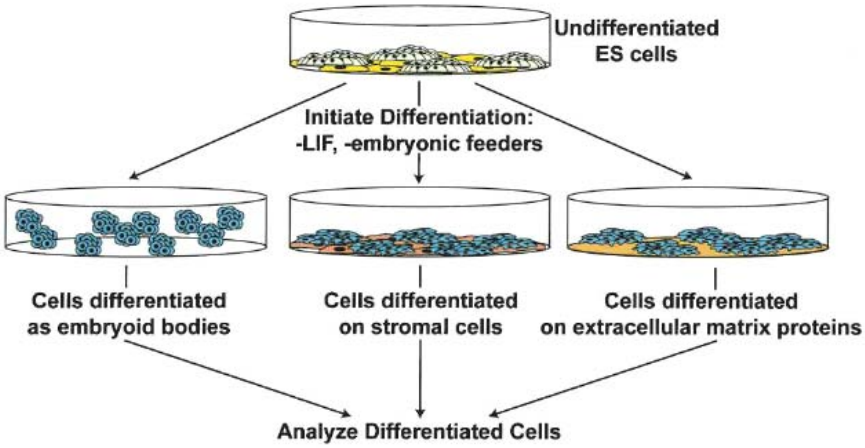

Fig. 3. The three protocols employed to induce ES cells differentiation.

4. Maturation of endothelial cells in the embryo: vasculogenesis and angiogenesis.

During murine embryogenesis, one fundamental process is the formation of the vascular system through the organization of endothelial cells and their angioblastic precursors. Early in the embryo, mesodermic cells from the primitive streak migrate into the extraembryonic yolk sac to form a scattered vascular plexus. Some of these cells are organized in clusters called blood islands, structures composed by an external layer of endothelial cells and an inner part of hematopoietic precursors. At about E7.5, this is the site in charge of the primitive hematopoiesis. In the embryo itself, the primitive embryonic vascular network is formed by the growth of endothelial precursors, called angioblasts, dispersed throughout the mesenchime. Posteriorly, dorsal aorta and cardinal veins develop directly from angioblast aggregation. These early events of *de novo* blood vessels formation constitute a process called vasculogenesis. Later on during embryogenesis, the vascular tree will mature by slow remodeling through successive steps, including sprouting, branching and migration, in a process termed angiogenesis (Risau, 1997) (Fig. 4).

The primary signal that promotes endothelial development is given by the vascular-endothelial growth factor (VEGF). In particular, VEGF-A and its receptors belong to one of the majors signaling pathways active during developmental angiogenesis. VEGF-A displays strong mitogenic activity and promotes migration and sprouting of endothelial cells by binding to its receptors VEGFR-1 (Flt-1) and VEGFR-2 (KDR in human and Flk-1 in mouse) (Ferrara and Kerbel, 2005). Flk-1 is the major mediator of the mitogenic and angiogenic effects of VEGF, while Flt-1 seems to act as a decoy that modulates VEGF activity on the vascular endothelium by preventing its binding to Flk-1 (Park et al., 1994). Embryos lacking Flk-1 die at around E9 of development, showing no evidence of blood vessels or hematopoietic cells formation (Shalaby et al., 1995).

The members of the family of FGFs (fibroblast growth factor) are involved into the growth and differentiation of many tissues during development, including the vasculature. FGF2, in particular, is responsible for mesodermic specification and angioblast differentiation and proliferation. FGF2, acting through the receptor FGFR-1, promotes endothelial cell assembly during vasculogenesis and induces sprouting

angiogenesis. Moreover, FGF2 is synthesized by many tumors and is involved in tumor angiogenesis (Poole et al., 2001).

Other molecules required for vascular development and remodeling comprise angiopoietin1, which binds to its receptor Tie-2, and angiopoietin2, which mainly functions as an antagonist of Tie-2 activation. Tie-2 can heterodimerize with the orphan receptor Tie-1, which is a regulator of the angiopoietin1/Tie2 activity (Yuan et al., 2007).

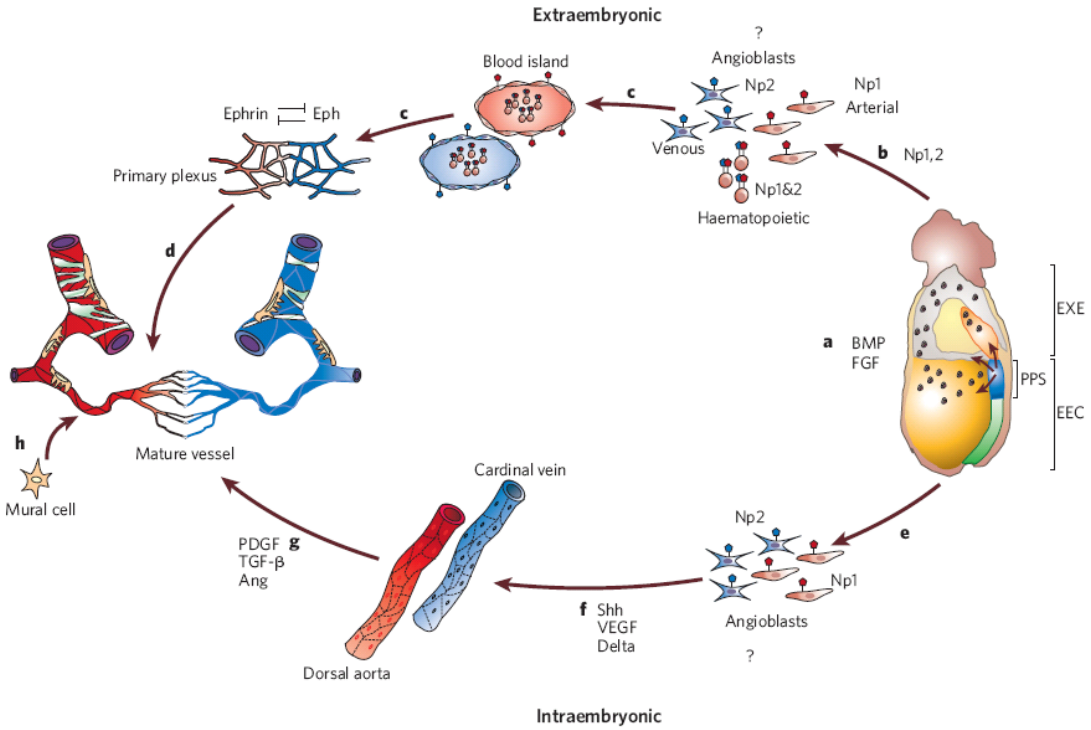

Fig. 4. Formation of a circulatory network from endothelial progenitors. **a**, Vascular progenitors appear in response to bFGF and BMP4 in the posterior primitive streak (PPS) as Flk1+ mesodermal cells. **b**, Flk1+ cells in the primitive streak give rise to haemangioblasts, and **c**, in the yolk sac, these progenitors aggregate into endothelial-lined blood islands that then fuse to generate a primary capillary plexus. **e**, Intra-embryonic angioblasts migrate along distinct pathways before (**f**) aggregating directly into the dorsal aorta or cardinal vein, without a plexus intermediate. **g**, The primary vessels (capillary plexus, dorsal aorta and cardinal vein) then remodel, together with the extraembryonic plexus, to form a mature vasculature. Modified from Coultas et al. (2002). Endothelial cells and VEGF in vascular development. Nature.

4.1. Differentiation of ES cells into endothelium by EBs formation or by 2D cultures

Cell differentiation within the EB is a powerful in vitro model suitable to track the onset of the angioblasts and the subsequent formation of a vascular-like structure. The appearance of endothelial-specific markers like Flk-1, Tie-1, Tie-2, CD31 and VE-cadherin arise at different time during EB differentiation, in a way that recapitulates the in vivo vasculogenesis process (Vittet et al., 1996). Furthermore, the EB vascular plexus is remodeled after six days of differentiation by sprouting angiogenesis and the effect of cytokines as VEGFA or FGF2 can be tested. Treatment with VEGFA induces the formation of a compact peripheral plexus, while FGF2 promotes vessel elongation (Magnusson et al., 2004). Sprouting angiogenesis become particularly evident when EBs are formed in a 3D collagen gel. This environment favors sprouting of structure composed by endothelial cells surrounded by perivascular cells which resemble, for morphology and marker expression, to the pericytes that develop in vivo (Jakobsson et al., 2007).

Other than in embryoid bodies, mesodermal commitment and successive endothelial cells' differentiation can be induced in two-dimensional culture by using collagen IV-coated dishes (Nishikawa et al., 1998). Flk-1, the early differentiation marker for endothelial and blood cells (Shalaby et al., 1997), is induced in about 20% of the cells after few days of culture, thus indicating the onset of mesodermal induction. These cells can subsequently be isolated from the bulk population by fluorescence activated cell sorting (FACS) and replated onto collagen IV matrix to achieve the full endothelial development. The appearance of the specific markers VE-cadherin, CD31 and CD34 indicates that this differentiation pathway is similar to that occurring during mouse embryogenesis (Hirashima et al., 1999). The progeny of the Flk-1+ cells includes also mural cells expressing the alpha-smooth muscle actin (SMA). Flk-1+ population is therefore considered a common progenitor for both endothelial and mural cells. When injected into chicken embryo vasculature, Flk-1+ cells contribute to the developing vessels as both differentiated endothelial and mural cells (Yamashita et al., 2000).

5. Origin of neural cells and neural stem cells in the embryo and in the adult

After gastrulation, the three embryonic germ layers have been formed. The surface layer, or ectoderm, originates skin and nervous system. Signals coming from the notochord, a mesoderm-derived structure that underlies ectoderm, are responsible to induce the formation of a strip of neuroepithelial (NE) cells, called neural plate, which is the origin of the entire nervous system. The edges of the neural plate fold together thus forming the neural tube. Within this structure, NE cells are in contact both with the ventricular surface and the pial surface through radial processes. Initially, they divide symmetrically at the ventricular surface to increase the number of stem cells. Later on, NE cells divide asymmetrically generating a daughter cell that migrates radially to generate both differentiated neurons and a stem cell that remains in the ventricular zone. With the onset of neurogenesis, NE cells are transformed into cells called radial glia, which can be considered as a specialized type of NE cells (Kriegstein and Götz, 2003; Noctor et al., 2002). Like the NE cells proper, radial glial cells divide at the apical surface of the ventricular zone and can generate neurons (Anthony et al., 2004; Malatesta et al., 2000; Noctor et al., 2001). It was found, however, that during telencephalon development a population of proliferating cells that appear as the major source of cortical neurons, arise (Haubensak et al., 2004).

During adulthood, neurogenesis occurs only in two regions of the mammalian brain: the subventricular zone (SVZ) and the subgranular zone (SGZ) of the hyppocampal formation. These specialized structures support self-renewal and differentiation of neural precursors through cell to cell signalling, interaction with extracellular matrix and through basal lamina and vasculature (Doetsch, 2003). Within the adult SVZ, a subset of GFAP-expressing astrocytes divides to give rise to transit-amplifying cells, which in turns generate neuroblasts (Doetsch et al., 1999), (Fig. 5). Moreover, the fact that SVZ stem cells and transit-amplifying cells directly contact blood vessels in sites devoid of astrocytes endfeet, make these structures unique regarded to non-neurogenic regions (Tavazoie et al., 2008).

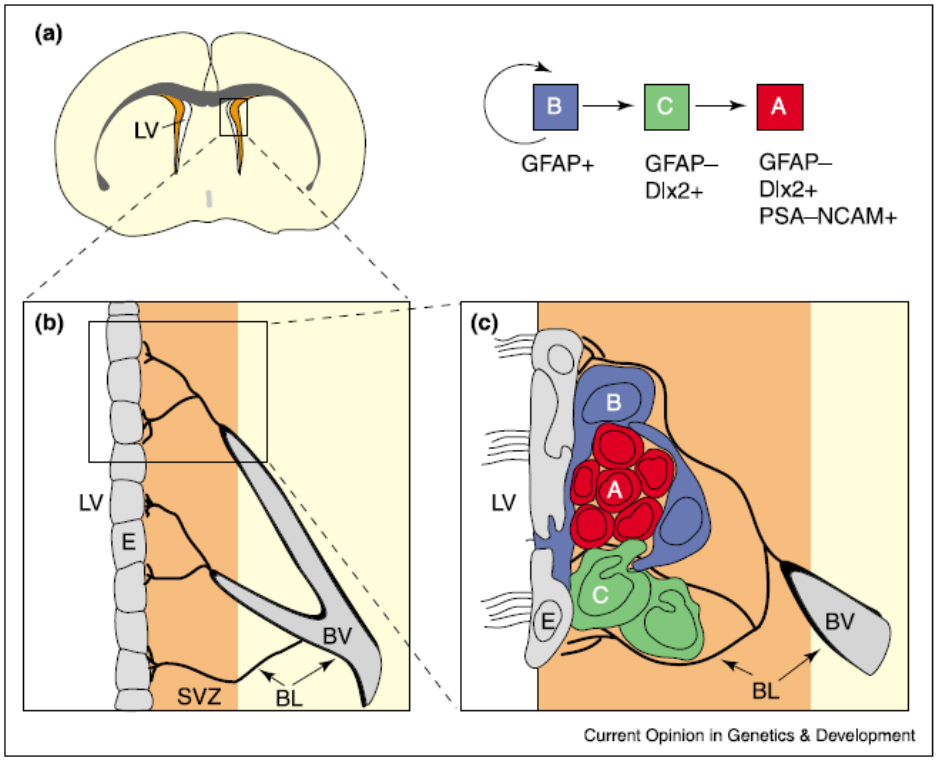

Fig. 5. The SVZ niche. a. The location of the SVZ niche (orange) in the mouse brain is close to the lateral ventricle. b. blood vessels (BV) in the SVZ form a specialized plexus and are likely a source for adult neurogenesis. c. cellular components of the niche: SVZ astrocytes (GFAP+) act as stem cells and divide to generate transit-amplifying Type C cells (GFAP-, Dlx2+), which in turn generate neuroblasts (A) (PSA-NCAM+), that migrate to the olfactory bulb. From Doetsch (2003). A niche for adult neural stem cells. Curr Opin Genet Dev.

5.1. Differentiation of ES cells into neurons by EBs formation or by 2D cultures

Unlike the majority of cell types that develops within embryoid bodies, neural cells can be efficiently generated only after stimulation of cellular aggregates with retinoic acid (Bain et al., 1995), a method that does not reflect any developmental process. However, lowering serum level facilitates the conversion of ES cells into neurons (Gajovic et al., 1997). To date, several protocols useful for neural differentiation have been developed, all based on the deprivation of serum or exogenous stimuli from culture medium. It has been shown that depletion of inductive signals is sufficient to commit neural precursors

in adherent monoculture, but interestingly, intact autocrine FGF signaling is necessary (Ying et al., 2003). Alternatively, the differentiation process can be carried out via the co-culture of ES with a monolayer of bone marrow-derived stromal cells (PA6 cells), in serum free conditions (Kawasaki et al., 2000). The factors that are responsible for the neural induction activity of PA6 cells, however, still remain unknown.

6. Parallelism between endothelial and neural cells differentiation

The vertebrate vascular system consists of a highly organized network of capillaries, arteries and veins that penetrates all the parts of the body, thus allowing oxygen exchange, delivery of nutrients and removal of waste products. The complex sequence of events that direct vascular network formation, including branching, sprouting angiogenesis and vascular patterning, resembles to that occurring during nervous system development. Vessels and nerves ramify throughout all the domains of developing body by following similar pathways of migration (Martin and Lewis, 1989) and, in some instances, they are mutually dependent: in embryonic mouse limb skin sensory nerves are templates for blood vessels branching (Mukouyama et al., 2002). Moreover, as with the growth cone is for neurons the sensory structure that sensitize growing axons to directional information, also nascent capillary sprouts are guided by special endothelial cells called tip cells (Gerhardt et al., 2003).

During the past two decades it has become increasingly evident that families of molecules first described in neuronal patterning also play significant role in the developing vascular system. So far, four families of classical neuronal guidance molecules were found to be also involved in vascular development: Netrins, Ephrins, Slits and Semaphorins. These signals act either as attractive or repelling cues on the tip cells.

Semaphorins are a large family of secreted or cell-anchored proteins with a bifunctional role, attractive or repulsive, depending on the receptor complex to which semaphorins bind and on the crosstalk between receptors (Tamagnone and Comoglio, 2004).

Semaphorins bind to the receptor families neuropilins (Nrp) and plexins. In particular, the secreted semaphorin 3A (Sema3A) controls both vascular patterning and axon guidance through activation of a receptor complex formed by Nrp1 and plexinA, serving as ligand-binding and signal-transducing component respectively (Neufeld and Kessler, 2008). Besides being localized on axons and growth cones where it modulates nerves trajectory during embryonic development (Fujisawa et al., 1995), the glycoprotein Nrp1 possess also the ability to bind the two VEGFA isoforms VEGF165 and VEGF121 in human endothelial cells and to form complexes with VEGFR-2, thus enhancing VEGF-induced signal transduction (Shraga-Heled et al., 2007; Soker et al., 2002). According to that, the embryonic lethality of Nrp1 knock-out mice is mainly due to an impaired neural vascularization, defects in great vessels and in the capillary networks of the yolk sac (Kawasaki et al., 1999; Kitsukawa et al., 1997). Finally, the Semaphorin/Neuropilin system is involved in the development of the cardiovascular and nervous systems as well (Carmeliet and Tessier-Lavigne, 2005; Serini and Bussolino, 2004).

The parallelism between vascular and nervous system is also mirrored at the level of cell commitment and differentiation. *In vitro*, cells purified from bone marrow and cord blood can originate neurons and glia (Buzanska et al., 2002; Goolsby et al., 2003; Reali et al., 2006). On the other hand, mouse neural stem cells (NSCs) acquire an endothelial phenotype when co-cultured with human mature endothelial cells (Wurmser et al., 2004) or when inoculated in collagen gels (Oishi et al., 2004). *In vivo*, bone marrow-derived cells enter the brain and differentiate into neural cells not only in a mouse model but also in patients that underwent bone marrow transplantation (Mezey et al., 2000; Mezey et al., 2003). Moreover, murine NSCs can engraft into the hematopoietic system of irradiated hosts to produce blood cells (Bjornson et al., 1999), and in the quail-chick chimera model the avian cranial neuroectoderm originates smooth muscle cells (Korn et al., 2002). All together these data indicate that there is a plastic correlation between the endothelial and neural commitments.

Results

1. Analysis of Nrp1 expression in differentiating ES cells

Embryonic stem cells (ES) were chosen as *in vitro* model to analyze the role played by Nrp1 during cell differentiation in the embryoid bodies. ES cells were grown for 5 days in hanging drop culture in LIF deprived medium to induce EBs formation, and then allowed to attach to a substrate (day 5) where the EBs continued their expansion (Fig. 6A). To analyze Nrp1 expression in differentiating ES cells, 6 days-old EBs were fixed and immunostained with anti-Nrp1 antibody. As shown in Fig. 6A, Nrp1 was widely expressed in scattered areas throughout the growing EBs. Furthermore, Nrp1 protein was immunoprecipitated from lysates purified from 7 days-old EBs but not from undifferentiated W4-ES cells (Fig. 6B).

To quantify Nrp1 expression in differentiating ES cells, the EBs were analyzed by FACS. As shown in Fig. 6C, Nrp1 expression was absent in undifferentiated ES cells and during the first three days of differentiation; it became significant only at day 4 to finally stabilize at 45-50% of the total EB cells population in the next days.

2. Characterization of EBs-derived $Nrp1^+$ cells

In order to characterize the phenotype of $Nrp1^+$ cells in the EBs, we performed a time-course FACS analysis of EB cells double-labeled for Nrp1 and for markers of either endothelial or neural cell precursors (Fig. 6D). Being the canonical marker of hemangioblasts, Flk1 is expressed from day 4, which is the very first day of appearance of Nrp1 expression. The amount of $Nrp1^+$ cells within the Flk1 component progressively increased over time to almost cover the totality of the Flk1 expressing cells at day 12, time in which the $Flk1^+$ endothelial cells are organized into mature vessels (Fig. 6D,

numbers above bars) (Gualandris et al., 2000). At these late time points, ICAM-2 expression and its $Nrp1^+$ fraction became significant, whereas CD133 expression, which identifies both endothelial (Fons et al., 2004) and neural precursors (Lee et al., 2005), maintained a significant percentage of Nrp1-expressing cells throughout the entire EB growth. The polysialylated embryonic form of the neural molecule NCAM (PSA-NCAM) is characteristic of neural progenitors in the forebrain (Gago et al., 2003), and its expression was shared almost entirely by $Nrp1^+$ cells at all the time points examined. CD31, which is expressed by undifferentiated ES cells (Li et al., 2005), included a significant component of $Nrp1^+$ cells at its peak of expression (day 7) to later stabilize at a lower level at day 12.

3. Characterization of EBs-derived $Nrp1^+/Flk1^+$ and $Nrp1^+/Flk1^-$ cells populations

Because endothelial cells and neurons share the expression of Nrp1 and VEGFR2/Flk1 (Fujisawa et al., 1995; Soker et al., 2002), we focused our attention on the cell populations defined by these two markers, the $Nrp1^+/Flk1^+$, $Nrp1^+/Flk1^-$ and $Nrp1^-/Flk1^+$ cell types (Fig. 7B, in green, red, magenta respectively). The potential of differentiation towards neuronal lineages was investigated by analyzing the expression of the neuronal markers Nestin, Sox1 and PSA-NCAM. The colocalization of nestin with both Flk1 and Nrp1 (arrow and arrowhead respectively in Fig. 7A) was quantified at 50% of the cells of both $Nrp1^+$ populations by intracellular FACS analysis (Fig. 7B). Since the intermediate filament protein nestin is expressed not only by neural precursors but also by the endothelial cells during development (Wiese et al., 2004) the more specific markers sox1 and PSA-NCAM were next investigated. Sox1 is a SRY-related transcription factor whose expression is characteristic of proliferating neural precursors (Bylund et al., 2003; Kiefer, 2007). $Nrp1^+/Flk1^+$ and $Nrp1^+/Flk1^-$ cells that expressed sox1 or PSA-NCAM were detected by immunofluorescence in sparse areas of 7 days-old EBs (Fig. 7A, arrow and arrowhead). The expression of PSA-NCAM was quantified at 42% of $Nrp1^+/Flk1^+$ cells and at 25% of $Nrp1^+/Flk1^-$ cells by FACS analysis (Fig. 7B, green and red). The

lack of PSA-NCAM expression in the $Nrp1^-/Flk1^+$ population thus suggested that neural orientation was not contemplated by the differentiation potential of those cells that did not carry Nrp1 (Fig. 7B, magenta). To summarize, the molecular characterization of the populations defined by Nrp1 and Flk1 revealed an intriguing coexistence of markers of neural precursors with markers of mesoderm/endothelial precursors that made the fate of $Nrp1^+$ cells worth of further investigations.

4. *In vitro* analysis of the potential of differentiation of $Nrp1^+/Flk1^+$ and $Nrp1^+/Flk1^-$ cells

4.1. Endothelial commitment

To better study the differentiation potential of $Nrp1^+$ cells outside the EB environment, $Nrp1^+/Flk1^+$ and $Nrp1^+/Flk1^-$ cells were isolated at 95% of purity from 7 days-old EBs by flow cytometry sorting and then cultured upon different conditions. To support the endothelial differentiation *in vitro,* $Nrp1^+/Flk1^+$ and $Nrp1^+/Flk1^-$ cells were seeded onto confluent monolayers of OP9 stromal cells, described to be optimal feeders for endothelial differentiation (Hirashima et al., 1999), and grown in the presence of EGM2 medium; the maturation into endothelium was evaluated by analyzing the expression of different endothelial markers by RT-PCR at different days (Fig. 8A and 8B). Immediately after sorting (day 0), the $Nrp1^+/Flk1^+$ cells expressed all the endothelial genes tested at levels from two up to eight times higher than those of the $Nrp1^+/Flk1^-$ cells as shown by the quantification data plotted in Fig. 8B. High expression of CD31 messenger at day 0 in both cell types could reflect an inheritance from undifferentiated ES cells (Li et al., 2005). During the next days, the levels of expression of the majority of the endothelial markers in the two cell populations became comparable. These last observations indicate that the co-culture with OP9 feeders could fill-in the initial impairment of the $Nrp1^+$ cells sorted as $Flk1^-$. Immunofluorescent stainings performed on $Nrp1^+/Flk1^+$ cells derived from ES cell expressing the tracer yellow fluorescent protein (EYFP) (Hadjantonakis et

al., 2002) and co-cultured for 2 days with OP9 cells in presence of EGM2 medium proved that CD31 and VE-cadherin mRNAs, previously detected by RT-PCR, were actually translated into the corresponding functional proteins located at cell to cell contacts (Fig. 8C).

4.2. Neural commitment

Previous observations indicated that within the 7 days-old EBs the $Nrp1^+/Flk1^+$ and $Nrp1^+/Flk1^-$ cells are neural committed (Fig. 7A and 7B). To better examine such potential outside the EB environment, purified $Nrp1^+/Flk1^+$ and $Nrp1^+/Flk1^-$ cells were cultured in a neuronal medium and, as control, in endothelial medium. At different days after sorting, $Nrp1^+/Flk1^+$ and $Nrp1^+/Flk1^-$ cells were analyzed by immunofluorescence for the expression of early and late neuronal markers. Sox2, the earliest transcription factor expressed in the neural tube during development (Zappone et al., 2000) and nestin were expressed by 60-80% of the $Nrp1^+/Flk1^+$ population already after 24 hours of growth, not only in neuronal medium, but also in the unfavorable endothelial medium (Fig. 9A and B). At the latest days of growth nestin and sox2 expression started to decline, whereas the level of expression of the mature neuronal marker βIII-tubulin increased, suggesting that neuronal differentiation was progressing. Indeed, as soon as FGF-2 was withdrawn from the neural medium (Materials and Methods) $Nrp1^+$ sorted cells underwent a significant change in morphology characterized by cellular βIII-$tubulin^+$ processes resembling dendrites and axons (Fig. 9C, arrows). Similar results were obtained also with $Nrp1^+/Flk1^-$ cells (data not shown). Indeed, neuronal maturation is fully accomplished only upon the neuron-specific culture conditions as confirmed by the almost absent expression of βIII-tubulin when cells are grown in endothelial medium (Fig. 9B). An analogous analysis was performed on sorted cells to detect the expression of NeuN, a nuclear factor exclusively found in mature neurons (Sarnat et al., 1998): positive nuclei were detected by immunofluorescence when cells were grown only in neuronal medium (Fig. 9E). The results of the quantitative analysis are shown in Fig. 9D. Similar results were obtained with $Nrp1^+/Flk1^-$ cells (data not shown). All together these

data demonstrate that in response to the appropriate exogenous microenvironment the bulk of ES-derived $Nrp1^+$ cell population gives rise to endothelial or to neural cells.

5. *In vivo* analysis of the potential of differentiation of $Nrp1^+/Flk1^+$ and $Nrp1^+/Flk1^-$ cells

5.1. Endothelial commitment

Even with the limits of an *in vitro* culture system, the data shown so far represented a supporting indication of the double endothelial and neuronal commitment of the ES-derived $Nrp1^+$ cells. We next wondered if a more physiological environment, such as a living embryo, could allow the $Nrp1^+$ cells to fully accomplish their commitments. On these basis, $Nrp1^+/Flk1^+$ and $Nrp1^+/Flk1^-$ cells were purified from 7 days-old YFP^+-EBs by cell sorting and microinjected into chicken embryos at stage HH19 of development. To drive the $Nrp1^+/Flk1^+$ population towards the developing vascular system the microinjection was performed in the beating heart of the embryos. Three days after injection, the host vessels were visualized in embryo tissue sections by using Sambucus Nigra Agglutinin (SNA). Fig. 10A shows that the $Nrp1^+/Flk1^+$ injected cells integrated within the new forming vessels as indicated by the alignment of the YFP signal with the signal given by the chicken endothelial specific SNA (Nanka et al., 2001). Identical results were obtained by using the W4 ES cell line (data not shown). Out of 79 embryos that survived the injection, 34 embryos had integrated the injected YFP^+ cells within the vasculature (43% of engraftment). Furthermore, the integrated cells also differentiated into mature endothelium expressing CD31 and VE-Cadherin as demonstrated by immunofluorescent staining of the tissue sections performed with antibodies specific for the murine antigens (Fig. 10B). Similar results were obtained with the $Nrp1^+/Flk1^-$ cells with the exception that the vessels-integrated cells did expressed CD31 but not the more mature marker VE-Cadherin (Fig. 10C), thus confirming the slight delay in pursuing

endothelial maturation by the Nrp-1$^+$ cells sorted as Flk-1$^-$ previously suggested by the *in vitro* data (Fig. 8B).

5.2. Neural commitment

When the purpose was to address the injected cells towards a neural fate, the microinjection was performed into the mesencephalic cavity of embryos. Once injected, cells tend to aggregate into spherical clusters that initially make contacts with the inner face of the developing cerebral tissues. From this time on, cells leave the clusters and start to invade the host tissues (Fig. 11A). Fig 11B shows the location of injected cells five days after injection: the YFP$^+$ Nrp1$^+$/Flk1$^-$ cells have moved from the inner cavity, entered the neurogenesis area of the developing brain, and started to express MAP2 antigen along with the surrounding MAP2$^+$ chicken neurons thus becoming part of the developing cerebral tissue. Analogous results were obtained with Nrp1$^+$/Flk1$^+$ cells (Fig. 11C). Notably, in some cases, even if injected in the encephalic cavity, the Nrp1$^+$/Flk1$^+$ cells were also found fully integrated within the cerebral vessels (data not shown), suggesting that the bi-potentiality of commitment is an intrinsic characteristic of the Nrp1$^+$ cell populations ready to be driven towards endothelial or neural phenotype whenever the exogenous conditions are permissive.

6. Generation of an in vitro model specific for the differentiation of both endothelial and neural cells

Embryoid bodies, as illustrated before, are complicated 3D structures in which many different cell types arise altogether. This property can be useful for a variety of studies, but could be also a drawback when the purpose is to assess the differentiation potential of a cell population that is not well represented in the EB, e. g., neurons. To overcome this problem, several differentiation protocols were developed in order to selectively induce the specific lineage of interest. In particular, we took advantage from neural

differentiation protocols to generate a culture system able to specifically induce the endothelial and neural lineages at the same time.

6.1. Direct differentiation of endothelial and neural cells from ES cells

The differentiation of neural cells takes place when ES cells grow in absence of serum or cytokines (Ying et al., 2003). Furthermore, it has been shown that several stromal cell lines, such as PA6 cells, are able to promote neural differentiation when used as feeders (Kawasaki et al., 2000). In order to induce neural commitment, YFP expressing-ES cells were seeded at low density onto a PA6 cells monolayer in a serum free medium. After few days of culture, some neurons started to appear, as indicated by the acquirement of the neural specific marker β–III tubulin. The differentiation process was accomplished at around day 7, when colonies of neurons developed nerve projection (Fig. 12A). Cells within colonies were also positive for neurofilament H and for the synaptic marker SV2 (Fig. 12, C and D). The expression of YFP by the ES-derived progeny demonstrates that the immunofluorescent staining is not present on PA6 cells, but it is confined to the YFP^+ cells. Cells within the neural colonies also expressed the neural precursor marker Sox2 (Fig. 13A). Moreover, ramified cells resembling to radial glia were identified by the antibody raised against the radial glia marker RC2 (Fig. 13B). These cells could represent neural progenitors (Anthony et al., 2004). Endothelial cell differentiation is not comprised in these culture conditions, as indicated by the complete absence of the endothelial specific marker VE-cadherin (Fig. 12A). Conversely, by adding a cocktail of cytokines containing FGF and EGF at the initiation of the culture, endothelial cells developed along with neurons, as shown by the appearance of a network of cells detectable by using an anti-VE-cadherin antibody (Fig. 12B and 14A). These cells expressed a set of canonical endothelial markers, such as Flk1, ICAM2, and CD31 (Fig. 14 B, C, and D, respectively) though the latter was also found in colonies of cells with undifferentiated morphology (Fig 14E, arrow). CD31 is indeed present in ES cells as cell adhesion molecule; its expression is down-regulated during differentiation, then restored in mature endothelial cells (Li et al., 2005). In order to understand if growth factors

induced different cell types other then endothelial and neural cells, we subsequently performed a screening with several antibodies raised against markers of differentiated cells. We were not able to detect any cell labeled with anti-keratins or GFAP antibodies (data not shown). On the contrary, α-smooth muscle actin (α-SMA) was expressed in PA6 cells especially when the differentiation process occurred in absence of growth factors; indeed, FGF and EGF inhibited α-SMA expression in feeder cells (Fig. 15). Beside this, very few ES-derived cells expressed α-SMA regardless of the presence of FGF and EGF (data not shown). These data, taken together, indicate that the combination of the stromal PA6 cells with a serum-free medium and the addition of growth factors represents an efficient neuro-endothelial model to in vitro differentiate ES cells into endothelium and neurons at the same time.

6.2. Effect of cytokines on the acquirement of endothelial and neural phenotypes

As general effect, the addition of FGF and EGF together stimulated cell proliferation, thus resulting in increased size of colonies. In order to better understand the role of cytokines in the induction of endothelial cell commitment, we stimulated ES cells with FGF, EGF, or VEGF singularly at the onset of the culture system. Each growth factor alone was able to promote the generation of endothelial cells, but induced different cellular morphologies. FGF generated a network of elongated cells, while EGF promoted the formation of sheets of cells VE-cadherin positive (Fig. 16 A and B). On the contrary, VEGF was less effective in inducing the endothelial network, although some cells did develop (Fig. 16C). With regard to neurons, the addition of growth factors did not alter the induction of the neural commitment, compared to the condition without growth factors. However, most likely FGF had an effect on the specification of specific subsets of neurons. In particular, dopaminergic neurons were detected only after FGF stimulation, as shown by the presence of tyrosine hydroxylase positive cells (Fig. 17A). These data suggest that each growth factor gives a different contribution to address the fate of the ES cells that are differentiating within this co-culture system.

7. Gene expression regulation by cytokine stimulation in the neuro-endothelial model of ES cells differentiation

To better characterize the neuro-endothelial model of ES cells differentiation with particular regard to the effects caused by the addition of growth factors, a gene expression analysis was performed by using Taqman low density arrays. We studied the expression of 83 genes chosen among markers of mature endothelial or neural cells, glial/neural precursor markers, stem cell markers, factors involved during germ layer specification, muscle, epithelial and pancreatic markers as well. ES cells were cultured in serum-free medium without any growth factor in order to induce neuronal differentiation, or stimulated with FGF or VEGF for the first three days in order to induce endothelial commitment. After 6 days of culture, we compared the gene expression level between the two experimental conditions. We performed two independent experiments and those genes found differentially expressed with a fold increase above 2.5 in both experiments were considered regulated by growth factors (Fig. 18). As expected, many of the endothelial markers were upregulated after stimulation with either FGF or VEGF. In particular, FGF was able to induce the expression of the endothelial/hematopoietic precursors' marker Tal1 (Scl-1), along with Pecam1 (CD31), angiotensin I converting enzyme (Ace), Endoglin and Angiopoietin1. Conversely, VEGF was more efficient in inducing Cdh5 (Ve-cadherin) and Tie1, compared to FGF (Fig. 19A). Neural differentiation was less prone to be differentially regulated by cytokines in terms of fold change; however, FGF induced in particular the expression of glutammic acid decarboxylase (Gad) and tyrosine hydroxylase (TH), while VEGF showed no effect or even an inhibitory effect on some neural markers. Interestingly, Sema3A and Nrp1, that are two molecules shared by endothelial and neural cells, were upregulated after stimulation with either FGF or VEGF (Fig. 19B). Genes representative for the three embryonic germ layers were also strongly upregulated by FGF but not by VEGF, except the definitive endoderm marker Cxcr4 that was downregulated by both growth factors (Fig. 19C). The stem cell markers Zfp42 (Rex1) and Pou5f1 (Oct3/4) were strongly upregulated after FGF stimulation, thus indicating a possible proliferation within the stem

cell compartment. On the other hand, genes representative for smooth muscle, epidermis, liver and pancreas were not regulated or not detected at all, with the exception of myogenin and keratin 14, which were upregulated by FGF and downregulated by VEGF respectively (Fig. 19D). A control experiment performed with PA6 cells alone, stimulated or not with FGF, showed that more then a half of the genes taken into account were not detected (gray boxes in Fig. 18), and that among the remaining genes just few were slightly upregulated or downregulated. Along with the fact that mRNA content coming from feeder cells represents only about one tenth of the total mRNA extracted from the coculture with ES-derived cells, we consider negligible the presence of feeders mRNA in our analysis.

Taken together, these data reinforced the observations obtained with immunofluorescent analysis, and indicates that the emergence of endothelial cells is dependent on cytokines stimulation. Moreover, this coculture system seems to be specific for the induction of endothelial and neural cells only, as demonstrated by the lack of markers of other cell types.

8. Endothelial and neural differentiation in the vascular niche context

As recently well documented, the microenvironment of the vascular niche comprises neural cells, proliferating neural precursors and specialized endothelial cells (Tavazoie et al., 2008). In our model of neuro-endothelial differentiation, endothelial cells and neurons develop simultaneously form ES cells in a multi-step process. In fact, cells expressing the neural precursor marker nestin were found in proximity with mature neurons. Nestin was expressed also by some endothelial cells, as this marker is shared by a variety of precursor cells (Fig. 20A). The pluripotent stem cell marker CD133 was also expressed by some cells within colonies (Fig. 20B). Therefore, despite the absence of GFAP+ cells, the presence of endothelial cells, neurons and neural precursors, along with the supporting mesenchymal cells, suggests that many of the elements of the vascular niche are included.

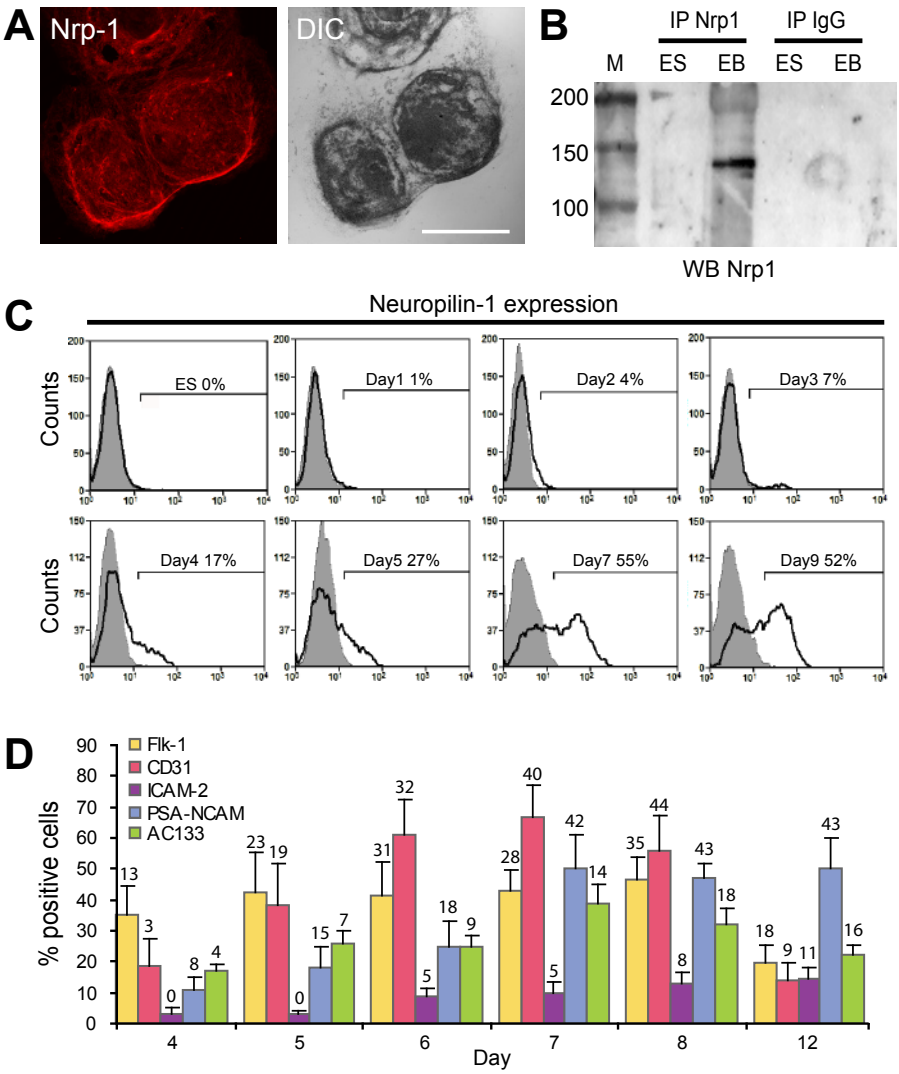

Figure 6. Nrp1 expression in differentiating ES cells. A) 6 day-old EBs (DIC, differential interference contrast) were analyzed by immunofluorescence with an anti-Nrp1 antibody and by using a Leica DM IRB inverted microscope. Scale bar = 1 mm. B) Lysates from undifferentiated ES cells and 7-day-old EBs were precleared with irrelevant IgG (IP IgG) before Nrp1 immunoprecipitation (IP Nrp1) and Western blot analysis (WB Nrp1). Immunocomplexes were detected only in lysates purified from EBs. C) Single-cell suspensions were prepared from both undifferentiated ES cells and EBs and analyzed by FACS using the anti-Nrp1 antibody or its isotype IgG (gray). Nrp1 is not expressed by ES cells and by the EBs until day 4. The experiment shown is representative of 5 experiments. D) FACS analysis of EB-derived cells immunolabeled with anti-Nrp1 antibody in association with anti-Flk1, CD31, ICAM2, PSA-NCAM, or CD133 antibodies. Each colored bar represents the cells positive for the corresponding marker, whereas the number placed on the top is the percentage value of double-labeled $Nrp1^+$ cells. Data are averages ± SD of 5 different experiments.

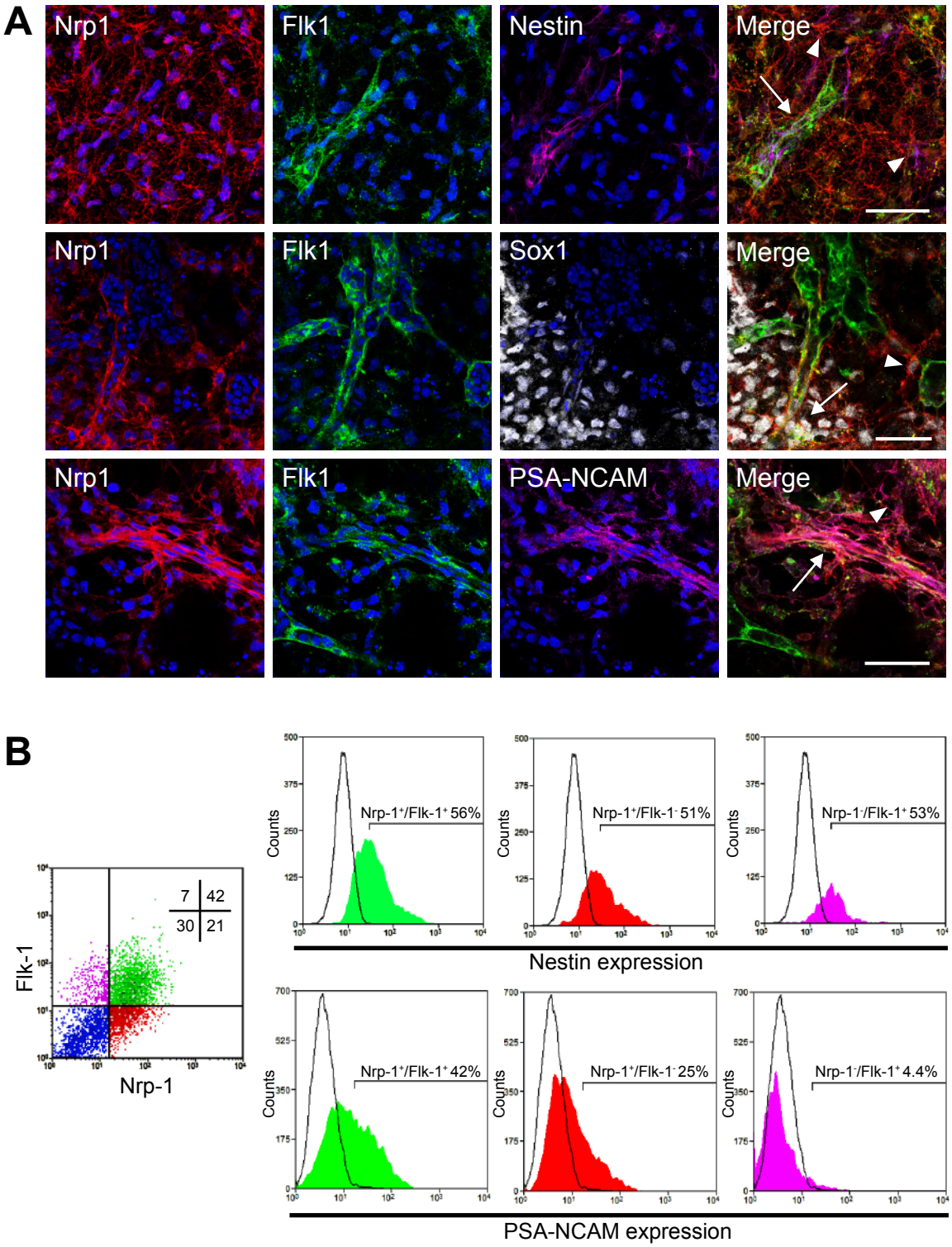

Figure 7. Characterization of EB-derived Nrp1⁺ cells. A) Confocal microscopy analysis of 7-day-old EBs immunostained with the anti-Nrp1, anti-Flk1, and anti-nestin or anti-sox1 or anti-PSA-NCAM antibodies. Nrp1⁺/Flk1⁺ cells (arrow) and Nrp1⁺/Flk1⁺ cells (arrowhead) expressing nestin, sox1, and PSA-NCAM are indicated. Scale bars = 50 μm. B) Quantification by FACS analysis of nestin and PSA-NCAM expression by the Nrp1⁺/Flk1⁺ cells (green), Nrp1⁺/Flk1⁺ cells (red) and Nrp1⁺/Flk1⁺ cells (magenta) vs. control (white). Percentage values of cells belonging to each of the four detected populations are indicated (dot plot, top right).

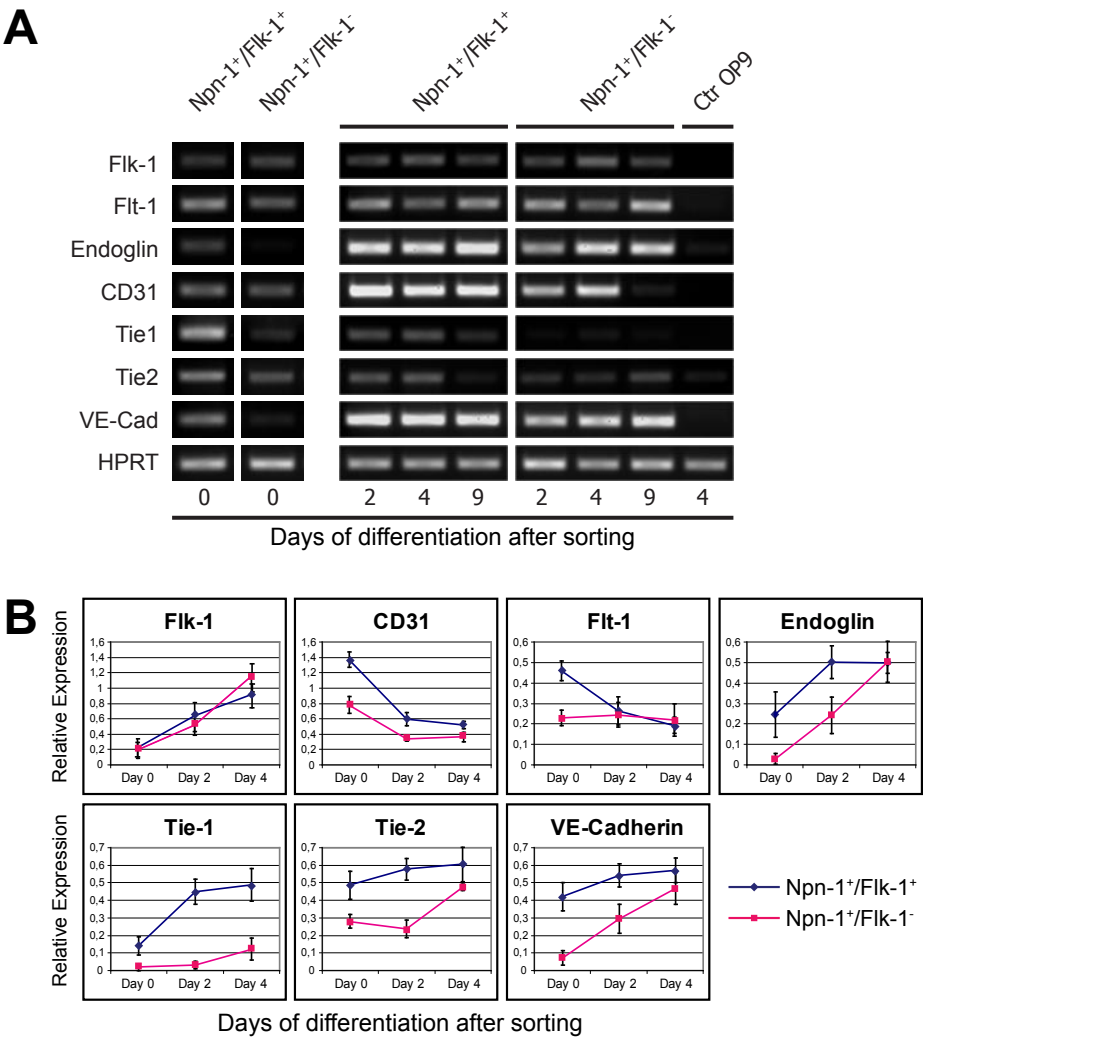

Figure 8. In vitro differentiation of ES-derived Nrp1$^+$ cells toward endothelium. A) Nrp1$^+$/Flk1$^+$ and Nrp1$^+$/Flk1$^+$ cells purified by cell sorting from 7-day-old EBs were cultured onto OP9 feeders for 4 days. At different time points, total mRNA was extracted and a semi-quantitative RT-PCR was performed in order to evaluate the presence of Flk1, Flt1, Endoglin, CD31, Tie1, Tie2, and VE-cadherin mRNA. OP9 cells maintained in the endothelial medium for 4 days were used as control. B) Images of ethidium bromide-stained gels were acquired with a Molecular Imager Chemidoc XRS and signals of the genes of interest were normalized to the signal given by the housekeeping gene HPRT. After an initial impairment of the Nrp1$^+$/Flk1$^+$ cells vs. Nrp1$^+$/Flk1$^+$, the levels of expression of all the endothelial markers, with the exception of Tie1, equalized over time. C) Immunofluorescent staining of Nrp1$^+$/Flk1$^+$ cells derived from YFP$^+$-ES cells revealed the presence of CD31 and VE-cadherin proteins in the membranes at cell-to-cell contacts. Scale bar = 30 μm.

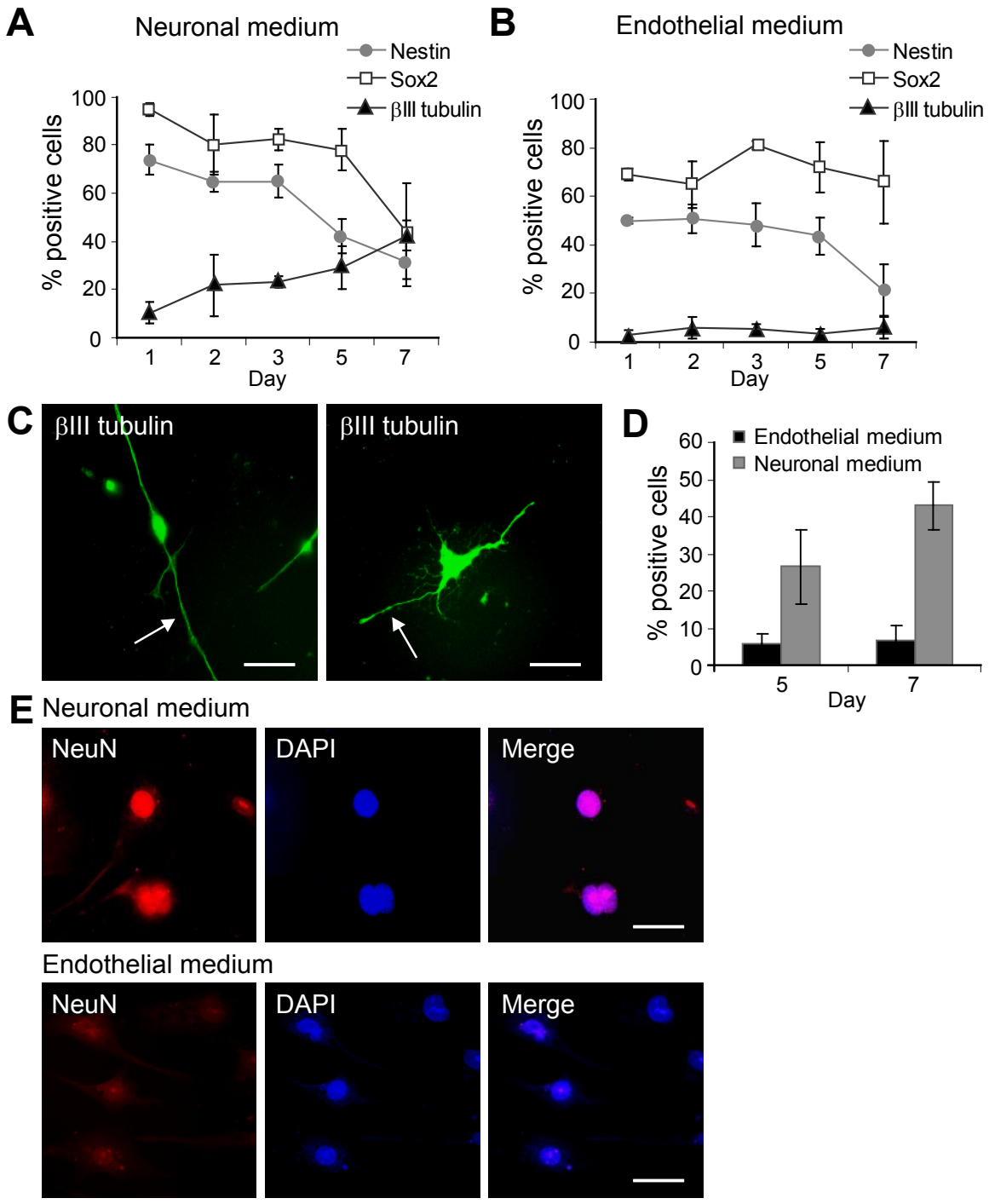

Figure 9. In vitro differentiation of ES-derived Nrp1⁺ cells toward neurons. A, B) Nrp1⁺/Flk1⁺ cells purified by cell sorting from 7-day-old EBs were cultured for 7 days in a neuronal medium to favor neural commitment (A) and, as control, in an endothelial-suited medium (B). At different days the expression of nestin, sox2 and of βIII-tubulin was examined by immunofluorescent staining. Positive cells were counted in 5 different fields randomly chosen from the coverslips of 4 different experiments. The number of positive cells was expressed as percentage value of the total cells counted ± SD. C) Expression of βIII-tubulin by Nrp1⁺/Flk1⁺ sorted cells. Note the branched cell morphology (arrows). D) Nrp1⁺/Flk1⁺ cells were cultured for 7 days in neuronal or in endothelial medium. At different days, the expression of NeuN was examined by immunofluorescent staining. Positive cells were counted over the total cells present in 5 different fields randomly chosen from the coverslips of 4 different experiments. The number of the positive cells was expressed as a percentage value of the total cells counted ± SD. E) Expression of NeuN by Nrp1⁺/Flk1⁺ cells cultured in neuronal or endothelial medium. Scale bars = 25 μm (C, E).

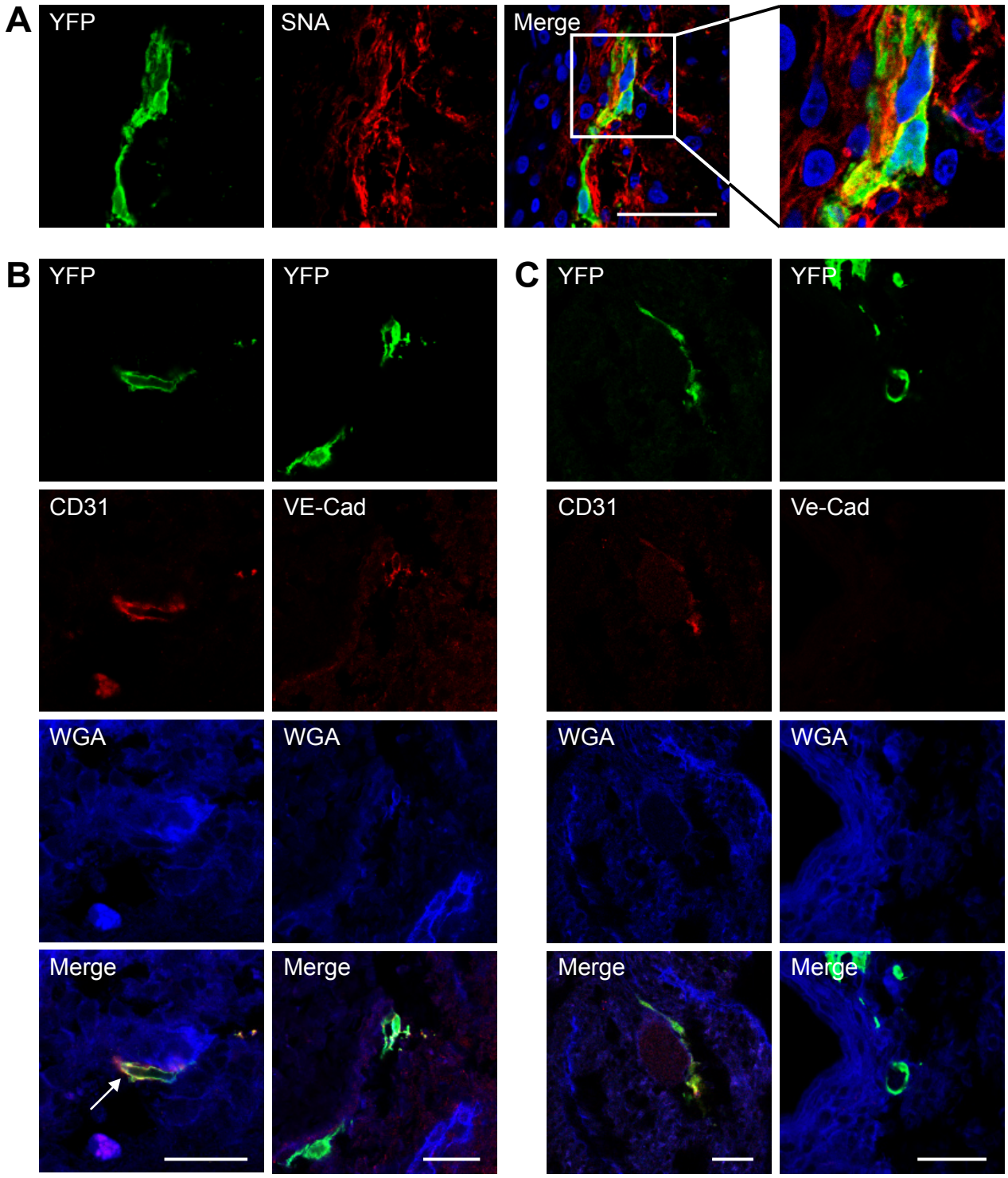

Figure 10. In vivo differentiation of ES-derived Nrp1$^+$ cells into endothelial cells. A) Nrp1$^+$/Flk1$^+$ cells purified from 7-day-old YFP$^+$ EBs were injected into the heart of HH19-stage chicken embryos; 3 days after injection, tissue sections were analyzed by confocal microscopy. The embryo vasculature was visualized with SNA. Inset: injected cells that integrated within the labeled host vessels. B) Confocal images of tissue sections obtained from chicken embryos injected with YFP$^+$-ES-derived Nrp1$^+$/Flk1$^+$ cells whose vessels were visualized by Alexa-Fluor 405-conjugated wheat germ agglutinin (WGA). CD31 and VE-cadherin were detected by immunofluorescent staining with murine specific anti-CD31 and anti-VE-cadherin antibodies. Note how the injected cells integrate and differentiate within the host vessels to perfectly match the vessel lumen border (arrow). C) Confocal images showing the integration and CD31 expression of Nrp1$^+$/Flk1$^-$ cells within the embryonic vasculature 3 days after injection. Contrary to what observed with the Nrp1$^+$/Flk1$^+$ cells, no immunoreactivity was detected with the anti-VE-cadherin antibody. Scale bars = 30 μm.

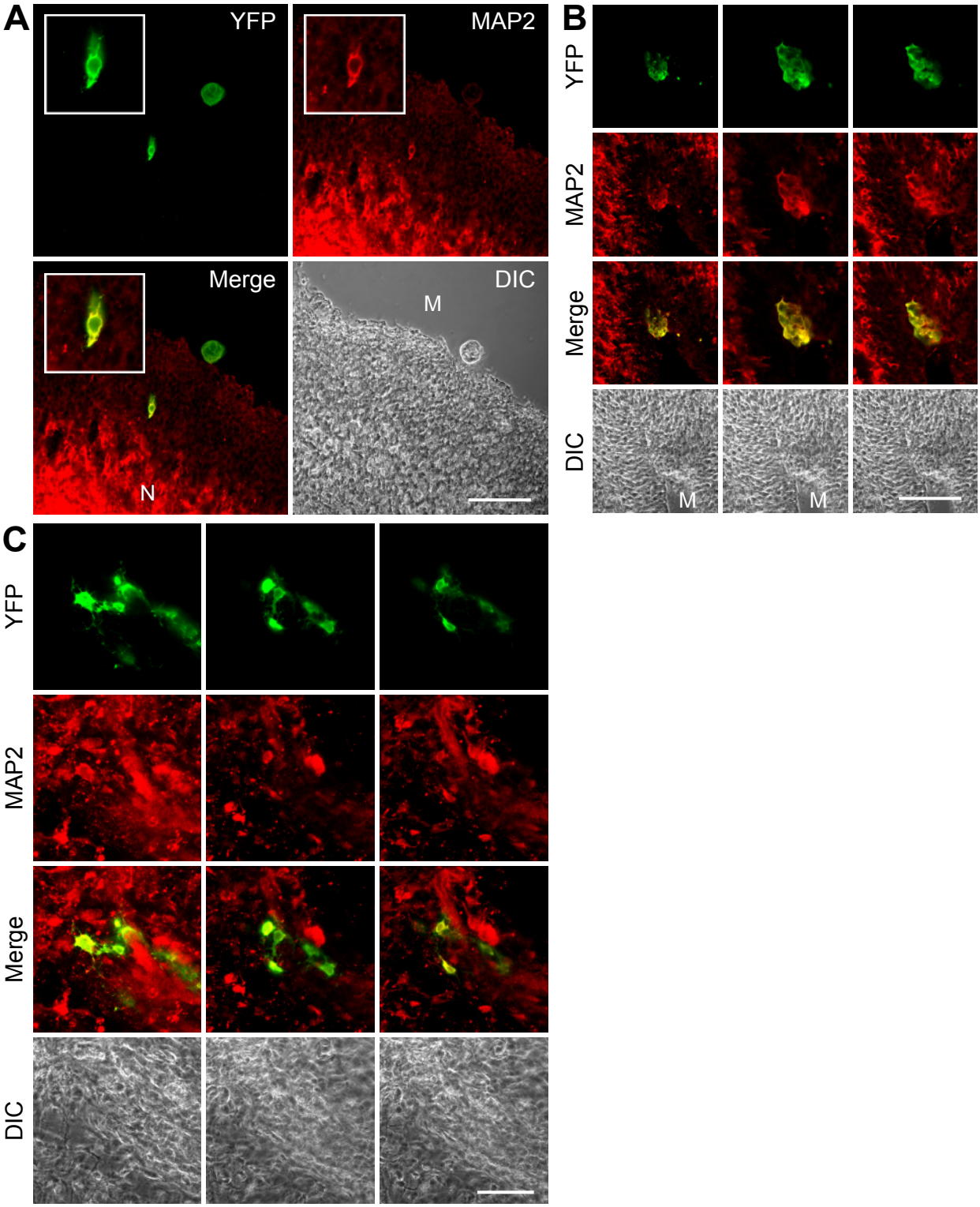

Figure 11. In vivo differentiation of ES-derived Nrp1$^+$ cells into neural cells. A) Nrp1$^+$/Flk1$^-$ cells purified from 7-day-old YFP$^+$ EB cells were injected into the mesencephalic cavity of HH19-stage chicken embryos; 3 days after injection, tissue sections of the developing brain were analyzed by confocal microscopy by using an anti-MAP2 antibody that recognizes both mouse and chicken neurons. Differential interference contrast (DIC) image shows a cluster of injected cells within the mesencephalic cavity (M) that makes contact with the inner face of the developing tissues: inset shows an enlargement of cells entering the MAP2-labeled neurogenesis area of the developing brain (N). Invading YFP$^+$ cells express MAP2 as well. B) Confocal images of serial optical sections of the same field showing a group of Nrp1$^+$/Flk1$^-$ cells 5 days after injection. The YFP$^+$-injected cells also express MAP2 along with the surrounding chicken neurons. C) Confocal images of serial optical sections of the same field, showing Nrp1$^+$/Flk1$^+$ cells 5 days after being injected into the mesencephalic cavity of HH19-stage chicken embryos. Scale bars = 50 µm.

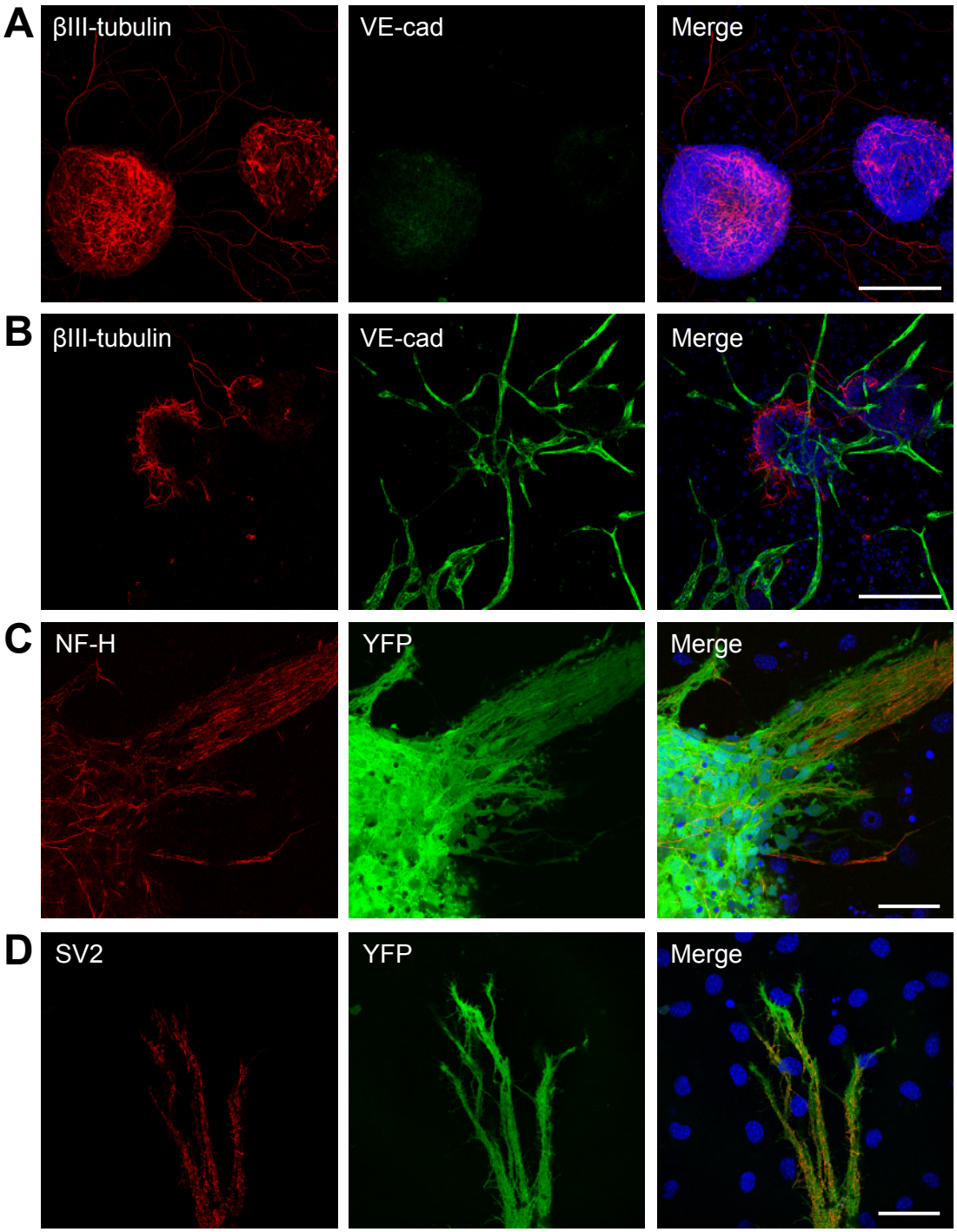

Figure 12. In vitro differentiation of ES cells into neurons and endothelial cells. A) After 7 days of culture in the neural-suited differentiation medium, ES-derived cells form rounded colonies of neurons with outgrowing neural processes. No endothelial cells are detected. B) Addition of FGF and EGF stimulates the differentiation of a network of endothelial cells that can be detected with an anti-VE-cadherin antibody. C, D) Expression of the neural markers neurofilament H and SV2 is confined to the progeny of ES-derived YFP^+ cells. Scale bars = 200 μm (A, B); 50 μm (C, D).

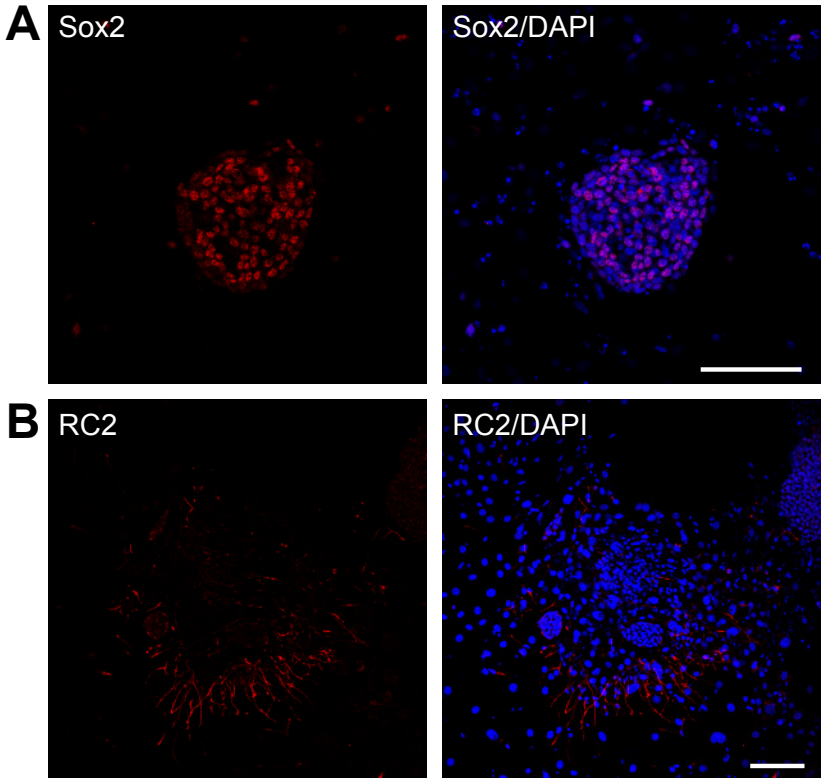

Figure 13. Expression of neural precursor markers during differentiation. A) The neural precursor marker Sox2 is detected in some nuclei of cells within differentiating colonies after 6 days of differentiation. B) Cells with radial glia morphology are labelled with the anti-RC2 antibody. Scale bars = 100 µm.

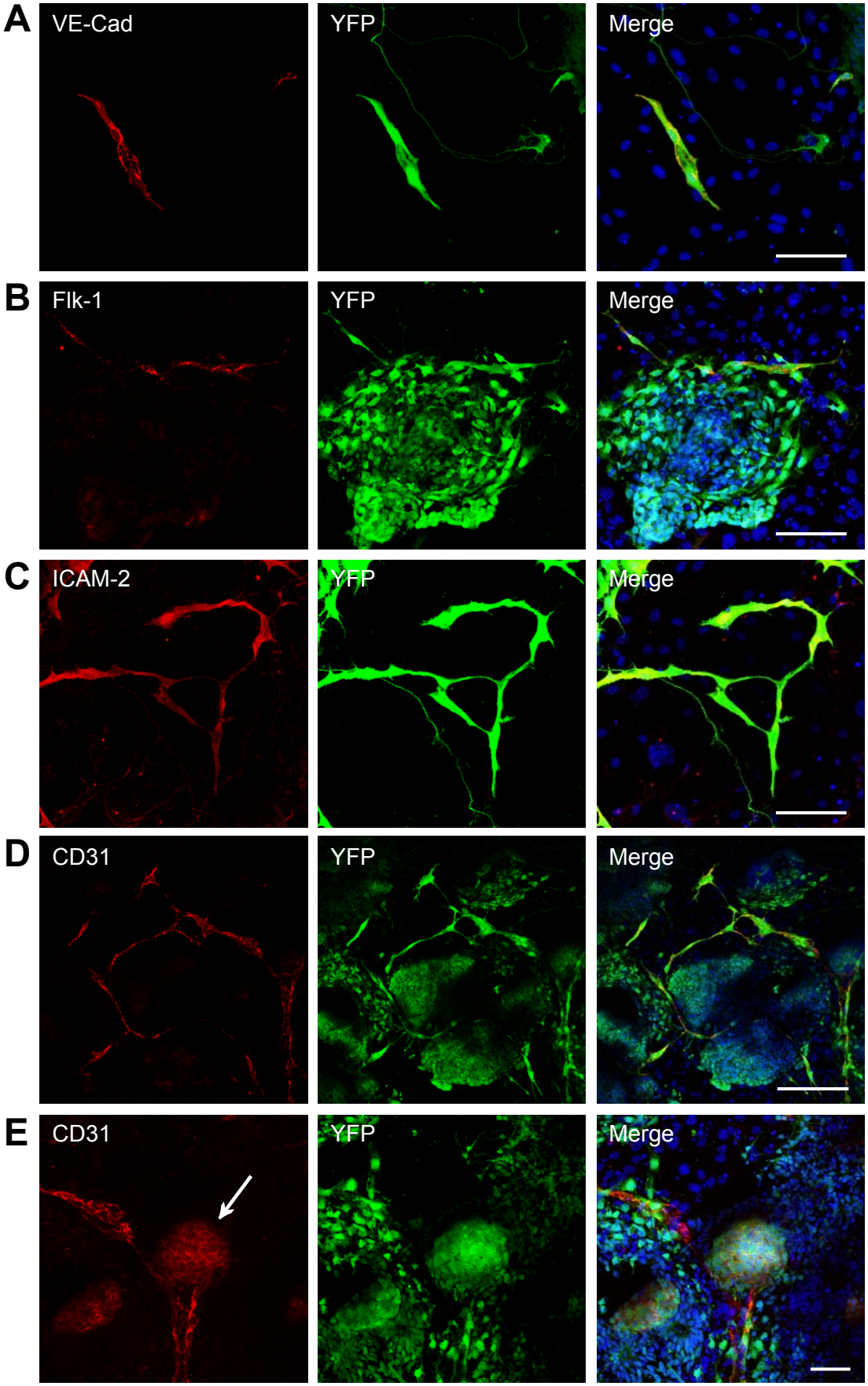

Figure 14. Expression of endothelial markers by ES-derived YFP$^+$ cells. After 6 days of differentiation in medium containing FGF and EGF, endothelial cells can be detected with antibodies against VE-cadherin (A), Flk1 (B), ICAM2 (C) or CD31 (D). CD31 is also expressed within colonies containing undifferentiated cells (E, arrow). Scale bars = 100 µm.

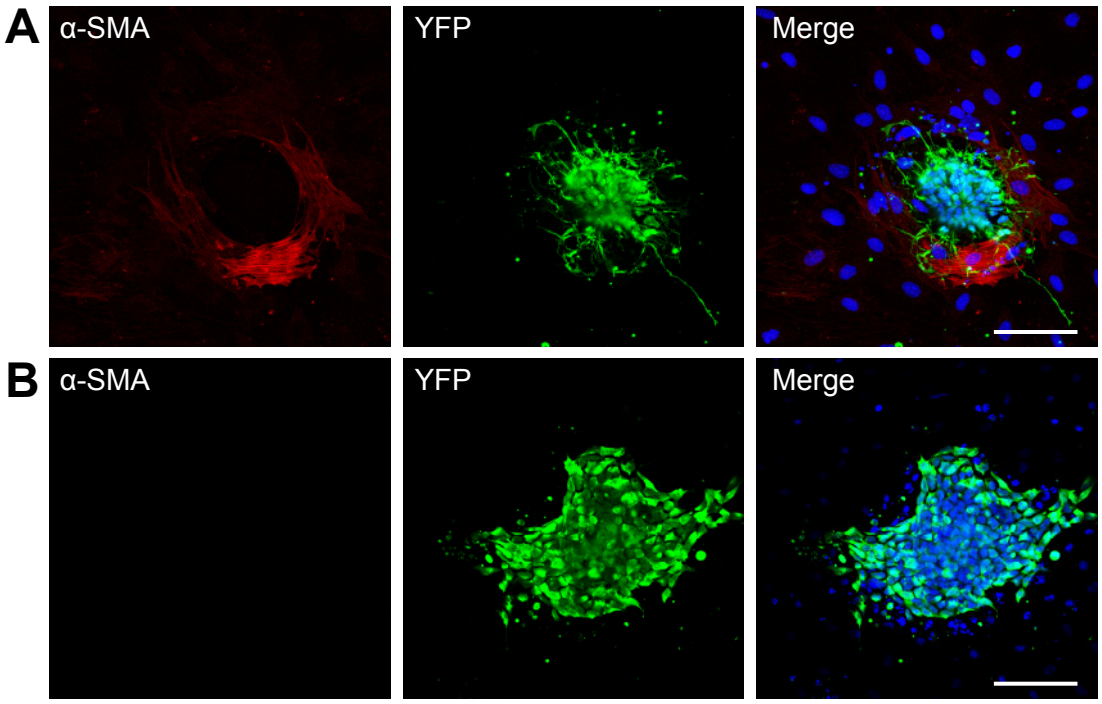

Figure 15. Expression of smooth muscle actin is dependent on culture conditions. A) PA6 cells differentiate into α-SMA expressing cells that surround colonies of ES-derived YFP^+ cells, only if the culture medium is avoided of growth factors. B) Addition of FGF and EGF prevents the expression of α-SMA in PA6 cells. Scale bar = 100 μm.

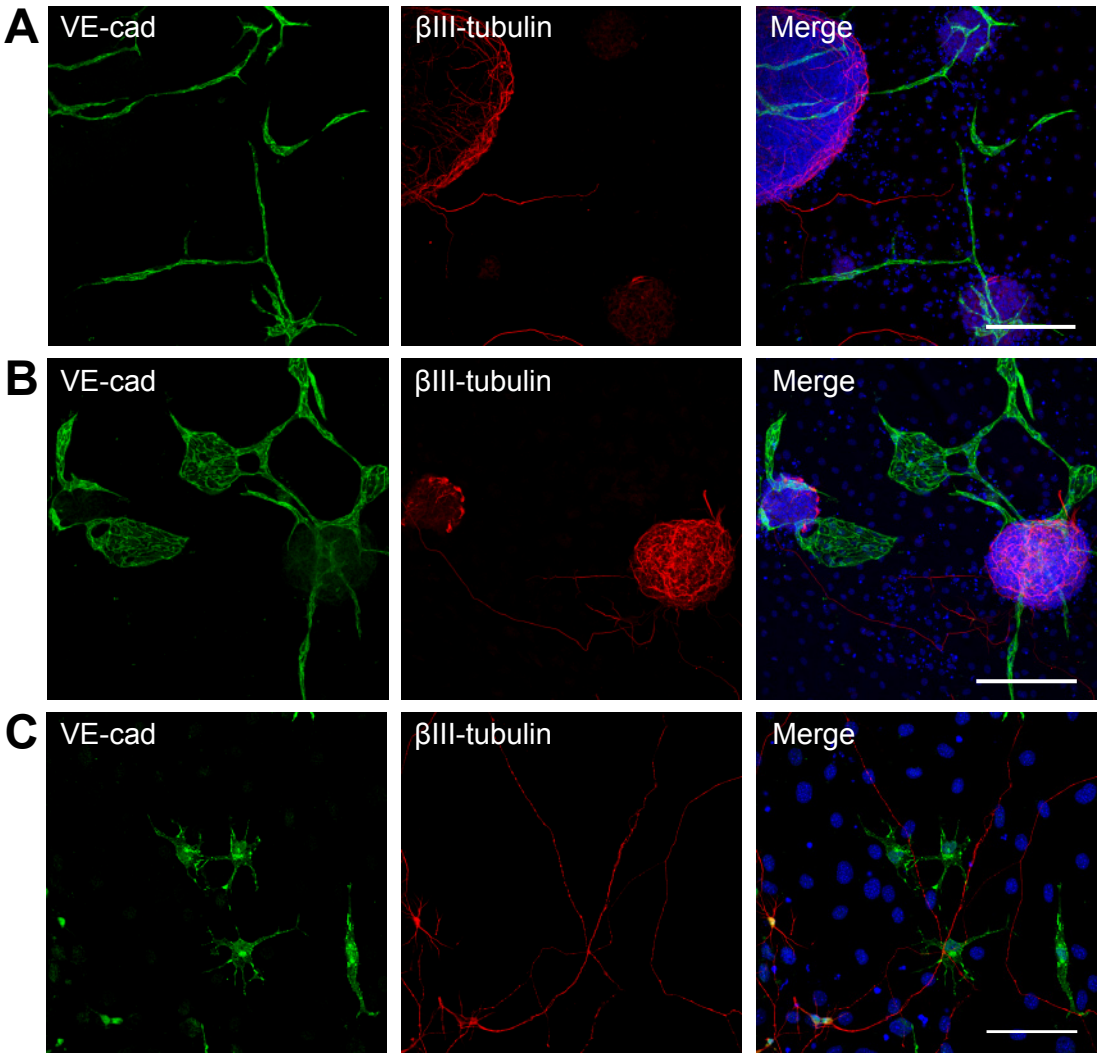

Figure 16. Effect of FGF, EGF, or VEGF on endothelial commitment and cell morphology. Growth factors were added to the culture medium at day 0. A) Endothelial cells generated after FGF stimulation acquire an elongated morphology, while (B) EGF promotes the formation of VE-cadherin sheets. C) Stimulation with VEGF at the onset of the culture is less efficient in the induction of endothelial commitment, although some scattered cells appear. Scale bars = 200 μm (A, B); 100 μm (C).

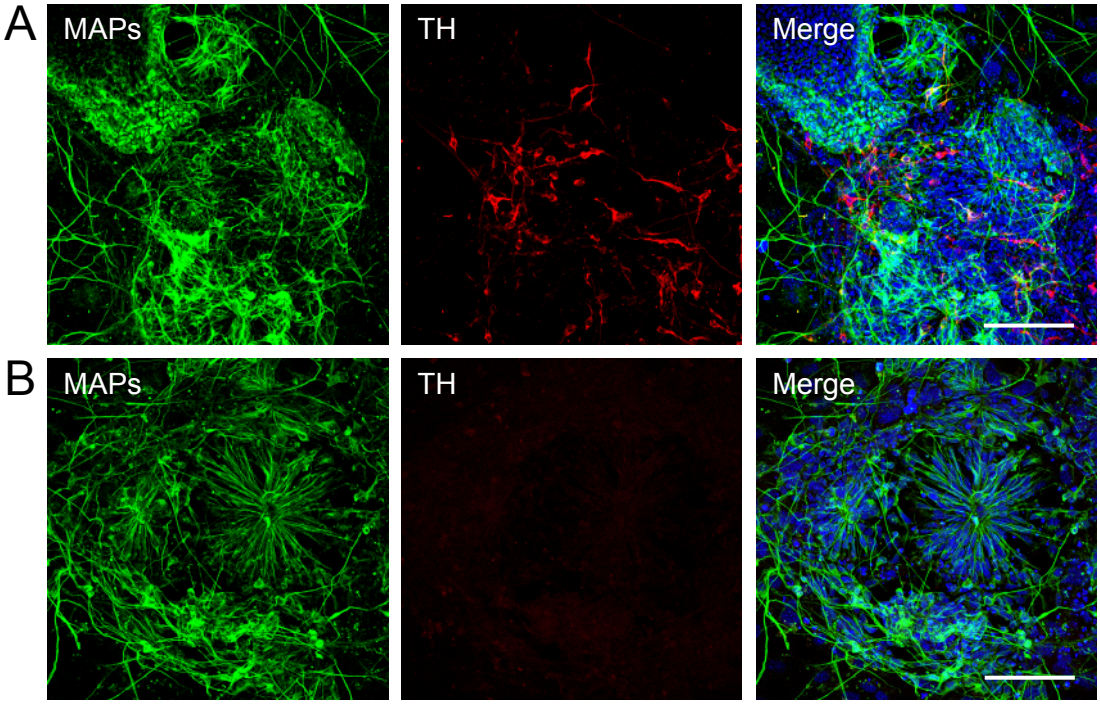

Figure 17. Effect of FGF on neural phenotipe acquisition. A) Clusters of TH^+ cells develop when cells are stimulated with FGF, indicating an effect on the specification of dopaminergic neurons. On the contrary, in the absence of FGF no TH^+ cells were detected (B). Note that neural differentiation is not affected by the presence of FGF, as neurons expressing the microtubulin associated proteins (MAPs) develop in a similar way. Scale bars = 100 μm.

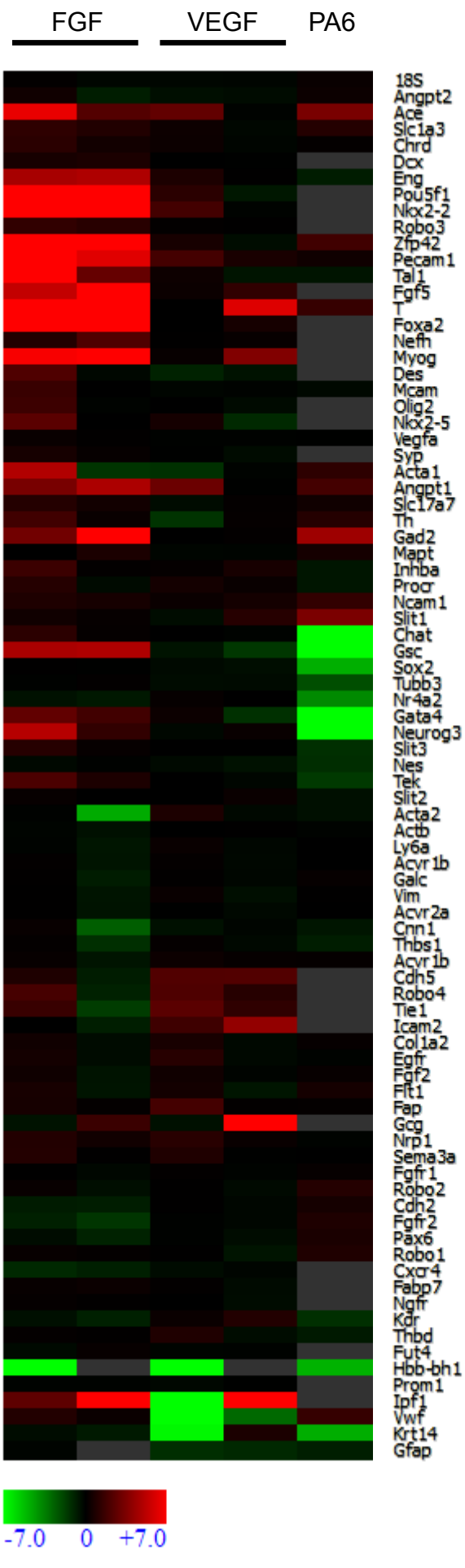

Figure 18. Gene expression analysis of ES-derived cells cultured in presence of citokynes. Gene expression data were analyzed with GEDAS software in order to visualize upregulated (red) or downregulated (green) genes. Cells were stimulated either with FGF or VEGF, and two independent experiments were compared. A hierarchical clustering was performed in order to better visualize groups of up- or downregulated genes. The control experiment performed with PA6 cells alone stimulated with FGF, shows that most of the genes taken into account are not expressed by these cells (gray boxes), and among the expressed ones, only few are regulated by FGF.

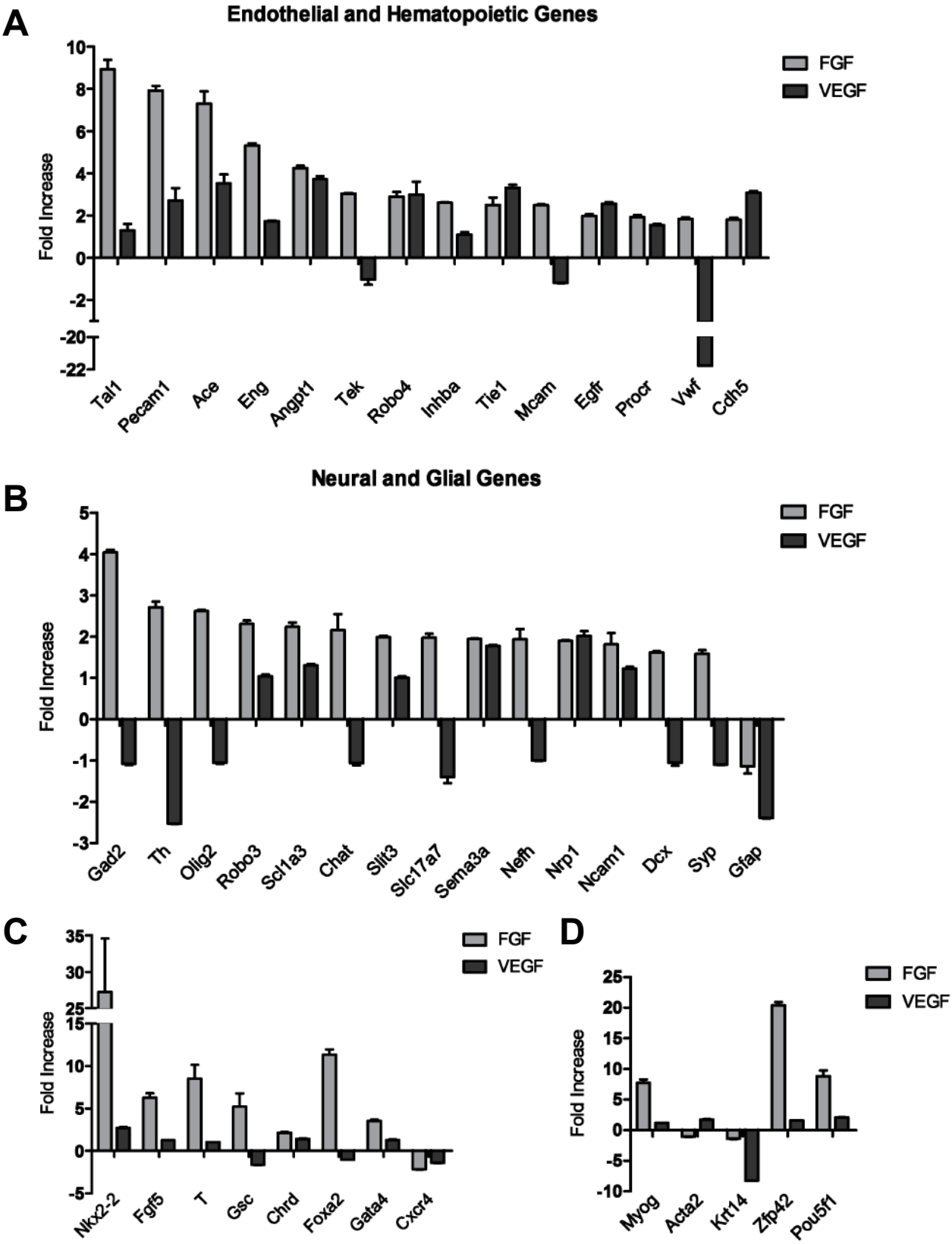

Figure 19. Genes regulated upon FGF or VEGF addition after 6 days of coculture of ES and PA6 cells. A) FGF induces the expression of several endothelial markers, such as Pecam1, Ace, Endoglin, Angiopoietin1 and the endothelial/hematopoietic marker Tal1. VEGF on the contrary is more efficient in inducing the more mature marker Cdh5 (VE-cadherin). B) Induction of neural markers by cytokines is less pronounced compared to endothelial ones; however FGF is able to induce several markers including tyrosine hydroxylase, confirming data from immunofluorescent analysis. Note that VEGF, like FGF, upregulates Sema3a and Nrp1, two genes expressed also by endothelial cells. C) FGF stimulates the expression of markers of the three embryonic germ layers, except the endoderm marker Cxcr4. D) The remaining regulated genes comprise the muscular marker Myogenin and the two stem cell markers Zfp42 and Pou5f1. Error bars indicates SEM of one representative experiment performed in duplicate.

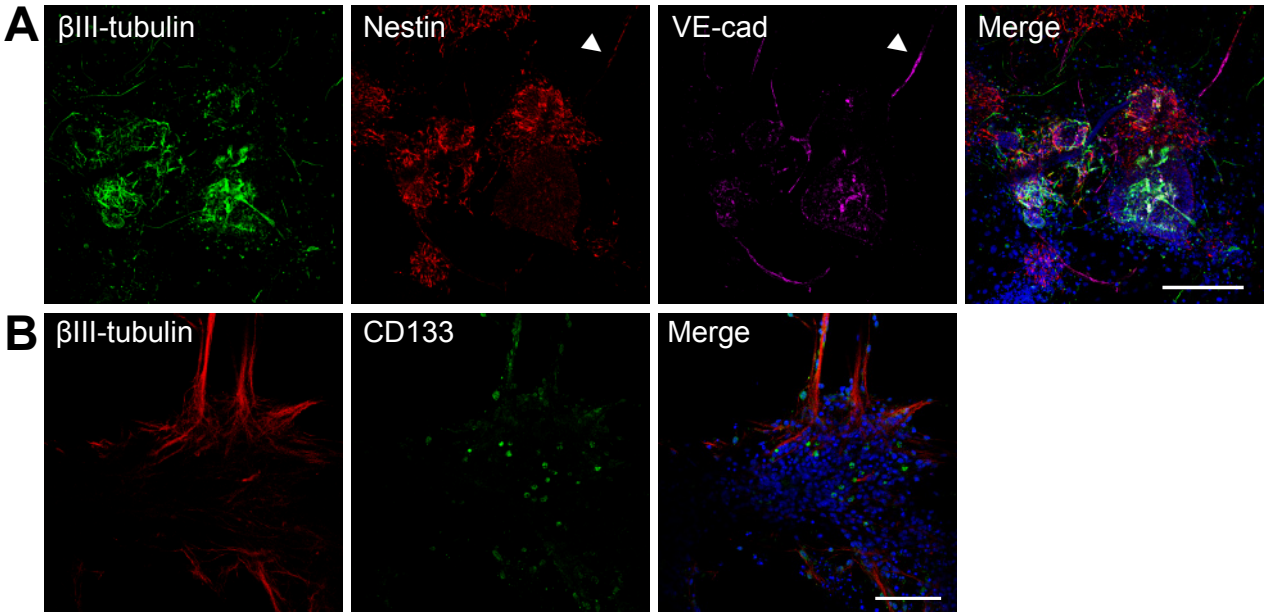

Figure 20. Neural and endothelial differentiation in the vascular niche context. A) Expression of the precursor marker nestin is detected in cells that have not yet acquired the neural marker βIII-tubulin, and in some endothelial cells (arrowhead). B) Some rounded cells expressing the pluripotent stem cell marker CD133 develop within differentiating colonies. Scale bars = 200 μm (A); 100 μm (B).

Discussion

Commitment of Nrp1$^+$ cells derived from embryoid body

In this study we used Nrp1 and Flk1 to identify a discrete, non-clonal population of uncommitted ES-derived cells that can differentiate into endothelial or neuronal cells depending on the suitable environmental conditions, both *in vitro* and *in vivo*. Within the EBs we identified, characterized and isolated the Nrp1$^+$/Flk1$^+$ and Nrp1$^+$/Flk1$^-$ lineages and we analyzed their potential of differentiation in respect to endothelial and neural commitments. Nrp1 was selected for its pivotal role in the vascular and neural systems and for its activity on stem cell differentiation. Indeed, Nrp1 was suggested to be involved in neural commitment of adult NSCs (Maurer et al., 2003) and in VEGF-A mediated endothelial differentiation of bone marrow-derived AC133$^+$ cells (Fons et al., 2004). Flk1 is the canonical marker of multipotent mesodermal progenitors that can differentiate into endothelial, haematopoietic, mural cells and cardiomyocytes (Kattman et al., 2006; Yamashita et al., 2000).

The comparative analysis of Nrp1$^+$/Flk1$^+$ and Nrp1$^+$/Flk1$^-$ cells indicates that the presence of Flk1 favors the endothelial commitment of the Nrp1$^+$ cells both *in vitro* in OP9 co-cultures, and *in vivo,* where we observed that the Nrp1$^+$/Flk1$^-$ cells seem to be delayed in integrating within the embryonic host vessels and in differentiating into mature VE-Cadherin$^+$ endothelium (Fig. 8B and 10C). Thus, Flk1 presence matters in terms of endothelial commitment, as previously well documented. If the presence of Flk1 facilitates the endothelial maturation, conversely the absence of Nrp1 seems to impair the neural differentiation since the Nrp1$^-$/Flk1$^+$ cells did not express neuronal markers as extensively as the two Nrp1$^+$ counterparts both when included in the EB (Fig. 7B) and when isolated as pure cell population (data not shown).

Even though the presence of markers of neural precursors such as nestin, sox1, sox2 and PSA-NCAM was suggestive of a neural potential, only the presence of the proper neuronal environment allowed both $Nrp1^+/Flk1^+$ and $Nrp1^+/Flk1^-$ cells to definitively maturate into βIII-tubulin$^+$ and $NeuN^+$ neurons. *In vivo*, the $Nrp1^+$ sorted cells integrated into the developing brain of chicken embryos where they differentiated into $MAP2^+$ neurons independently of their initial production of Flk1.

The topic of the bi or multi-potentiality of progenitor cells has recently become controversial. Despite the numerous indirect evidences supporting the existence of the hemangioblast (Choi et al., 1998; Huber et al., 2004; Kattman et al., 2006), recent in vivo studies argue against its bipotentiality. Cell-lineage tracing of cells from the primitive streak to the yolk sac failed to reveal a common haematopoietic and endothelial progenitor (Kinder et al., 2001). Moreover, the analysis of tetrachimeras derived from different ES cell lines stably expressing separate fluorescent tracers showed that each blood island has contributions from multiple clonal progenitors (Ueno and Weissman, 2006). Our results are more in line with these studies especially considering that a putative neuron-endothelial precursor, by trespassing the ectoderm-mesoderm boundaries, would represent a concept of bipotent progenitor more challenging than the hemangioblast. The idea of a bipotent progenitor within our $Nrp1^+/Flk1^+$ cells was justified by the model used, the ES cells, and by the presence of Flk1. By using the same model with alternative protocols different groups showed that ES-derived $Flk1^+$ cells could differentiate into multiple mesoderm-derived lineages (Kattman et al., 2006; Wu et al., 2006; Yamashita et al., 2000). Regarding these data, the unique presence of Nrp1 along with other neural markers gives more challenges to our model. It was proposed that Flk1 marks a common mesodermal precursor that segregates to successive subsets of Flk1-expressing or non-expressing cells whose fate is then determined by coexpression of lineage specific transcription factors (Ema and Rossant, 2003). Hence, our $Nrp1^+/Flk1^+$ population could represent a transient intermediate pool of cells in which the committed progenitors arise thanks to the loss or acquisition of specific molecules.

The possibility to purify this pool of cells by using Nrp1 and Flk1, along with the encouraging in vivo transplantation data, opens new perspectives for the treatment of

disorders characterized by defects of both the neural and vascular systems, including stroke, diabetic neuropathy, and Alzheimer's disease (Murry and Keller, 2008).

In vitro simultaneous endothelial and neural differentiation: a model for vascular niche

In vitro differentiation of ES cells is a powerful tool to generate specific precursor cells. Here, we describe a new differentiation system of ES cells that allows the generation of a mixed culture of neurons and endothelial cells, without any other cell types. The analysis of neural markers expression by immunofluorescent stainings showed that neural differentiation in our conditions proceeds in a way similar to that described in other differentiation protocols (Kawasaki et al., 2000; Ying et al., 2003). However, this system is different from others published to date, since thanks to the use of feeder cells and cytokines, endothelial cells arise among neurons (Fig. 12B, 16 and 20A). With particular regard to the induction of endothelial commitment, our data indicate that stimulation with FGF of EGF promotes endothelial differentiation, making this consistent with the well demonstrated role of FGF during early vascular development (Risau and Flamme, 1995). Even though it has been shown that PA6 cells produce VEGF (Hirashima et al., 1999), this is not sufficient to trigger endothelial commitment, as almost no VE-cadherin^{+} cells differentiate in absence of FGF or EGF (Fig. 12B). Moreover, exogenous addition of VEGF at the onset of the culture is less efficient in inducing endothelial differentiation compared to FGF (Fig. 16C). Nonetheless, the presence of feeder cells seems to be crucial to support endothelial differentiation, by factors that at the present remain unknown. Gene expression analysis revealed that endothelial markers increased their expression when cells were stimulated with FGF, and, to a lesser extent, with VEGF, while neural markers were little induced by cytokines. The increased size of the colonies after FGF stimulation reflected its proliferating activity. As a consequence, we found that the stem cell markers Zfp42 (Rex1) and Pou5f1 (Oct3/4) were strongly upregulated, suggesting the presence of a compartment of undifferentiated stem cells. This was confirmed by the labeling of some cell clusters with an anti-SSEA1 antibody, a marker

that specifically recognize undifferentiated stem cells (data not shown). On the contrary, both real time PCR and immunofluorescent analysis demonstrated that markers for cell types other than neurons and endothelial cells were not detected, or even their expression were downregulated, as occurs, for instance, for α-SMA expression (Fig. 17).

The system of differentiation described here display several key properties of a developing vascular niche. As previously described (Doetsch, 2003; Shen et al., 2008), a typical example of vascular niche that is found in SVZ includes a basal lamina, ependymal cells, neuroblasts, a transit amplifying population, and neural precursors with glial phenotype, together with blood vessels. In our model of differentiation, after few days of culture are visible cells at different stages of differentiation. Among neurons expressing β–III tubulin and mature endothelial cells (Fig. 20), are present cells with glial phenotype expressing the radial glia marker RC2 (Fig. 13B), precursor cells expressing the pluripotent cell markers nestin (Wiese et al., 2004) (Fig. 20A) or CD133 (Fons et al., 2004; Lee et al., 2005) (Fig. 20B), and few clusters of SSEA1+ cells (data not shown). Contrarily to what has been demonstrated in vivo, in our culture system we did not observed any $GFAP^{+}$ cells, but this can be explained with the fact that growth factors such as FGF and EGF inhibit the expression of such marker in neural precursors in vitro (Conti et al., 2005). However, radial glia cells expressing RC2 and BLBP (data not shown) develops within four days of culture, that is consistent with their role of neural precursors (Anthony et al., 2004). Finally, the underlying layer of PA6 cells provides physical support and probably instructing signals to differentiating cells.

The characterization of this neuro-endothelial differentiation model shows that neurons and endothelial cells can develop in vitro in the same culture condition and temporal window. Therefore, this model can be viewed as a useful tool to study the mutual interaction between endothelial and neural cells during early phases of development, or in structures such as the vascular niche, the latter receiving increasing consideration as potential clinical target (Aliev et al., 2008).

Materials and Methods

Cell lines

W4 mouse ES cells were kindly provided by Dr. A. Joyner (Auerbach et al., 2000). R1 ES cells expressing the Enhanced Yellow Fluorescent Protein (EYFP) were obtained from Dr A. Nagy (Hadjantonakis et al., 2002). Both ES cells lines were cultured as described (Gualandris et al., 2000). Murine embryonic fibroblasts (MEF) were purchased from ATCC (LGC Standards, Milano, IT). OP9 and PA6 stromal cell lines, kindly provided by Dr T. Schroeder, were cultured as described (Schroeder et al., 2006).

In vitro differentiation of ES cells

EBs were obtained by the "hanging drop" procedure as previously described (Gualandris et al., 2000). The age of the EBs was indicated with progressing numbers starting from the first day of culture in the absence of LIF. For fluorescence immunostaining the EBs were plated onto Lab-Tek II CC2 chamber slides (Nunc, Naperville, IL). For 2D differentiation of ES cells, W4 or R1-YFP cells were seeded onto a confluent monolayer of PA6 cells at a density of 5000 cells/cm^2 in N2B27 medium (Ying et al., 2003), supplemented with insulin 20 μg/ml, BSA 50 μg/ml and ascorbic acid 200 μM, all from Sigma. When needed, FGF, EGF or VEGF (R&D Systems, Minneapolis, MN, USA) were added to the culture medium at the onset of the culture to a final concentration of 20 ng/ml for FGF and EGF, or 50 ng/ml for VEGF. After three days, growth factors were removed, and then medium was changed every 2 days.

Cell staining for FACS analysis and for cell sorting

EBs were dissociated into cell suspension by trypsin treatment (0.05%) for 1 minute and by a mechanic dissociation through 25G needle. Cells were stained with 1 μg/10^6 cells of goat anti-Nrp1 antibody alone or in association with one or two of the following

antibodies: anti-Flk1, CD31, ICAM2, CD133, PSA-NCAM, and PE-anti-Flk1. Antibody brands and dilutions are given in table 1. The secondary antibodies used were: FITC or APC-conjugated-anti-goat to detect Nrp1, PE-conjugated-anti-rat to detect Flk1 and CD133 and FITC–conjugated anti-mouse IgG or IgM to detect nestin and PSA-NCAM (Southern Biotech., Birmingham, AL). To prepare cells for cell sorting the secondary antibodies used were PE-conjugated anti-rat and biotin-conjugated anti-goat followed by an AlexaFluor 647-PE-conjugated streptavidin (Invitrogen, Carlsbad CA). Cell sorting was performed with Beckman Coulter Epics Altra. The accuracy of the sorting procedure was confirmed by analyzing representative samples of the two sorted populations through a Becton Dickinson FACS Calibur purposely reserved for routine analysis only. Such analysis revealed that cell populations were sorted at 95-97% of purity.

Culturing sorted cells

Once sorted, cells were seeded onto OP9 monolayers and cultured in EGM2 medium composed by endothelial cell basal medium-2 and the EGM-2 bullet kit (Bio Whittaker, Walkersville, MD, USA).

For the neural differentiation, sorted cells were seeded onto laminin-1-coated coverslips and grown for the first 2 days in a serum-free DMEM/F12 medium supplemented with N2 supplement (Invitrogen), laminin-1 1 μg/ml (Sigma, St. Louis, MO, USA) and FGF-2 10 ng/ml (R&D Systems). FGF-2 was withdrawn from the medium for the following 48 hr after that medium was replaced with Neurobasal medium plus B27 supplement, 2% horse serum, NGF 50 ng/ml (Roche, Mannheim, DE) and BDNF 10 ng/ml (R&D Systems).

RNA extraction and RT-PCR

For the semi-quantitative RT-PCR, total RNAs from the co-cultures of YFP sorted cells and OP9 cells, or OP9 cells alone were purified at the times indicated using Trizol reagent (Invitrogen) and DNA-free DNase I (DNA-free^{TM}, Ambion, Austin, TX, USA). RNAs were reverse transcribed using SuperscrptII cDNA synthesis kit (Invitrogen). Control reactions without reverse transcriptase were performed for each RNA sample. PCR reactions were carried out with 100 ng of first strand cDNA using Platinum Taq

polymerase (Invitrogen) and optimized to allow semi-quantitative comparisons within the log phase of amplification. Comprehensive list of the primer sequences is given in the table 2. Images of ethidium bromide-stained gels were acquired with a Molecular Imager Chemidoc XRS (Biorad, Hercules, CA, USA) and densitometric analysis was performed with Quantity One software (Biorad); results were normalized over the expression of the HPRT housekeeping gene and expressed as relative units.

For the real-time PCR analysis, total RNAs from the co-culture of W4 ES cells and PA6 cells, or PA6 cells alone, were purified after 6 days of differentiation using Trizol reagent, then treated with DNA-free DNase I. RNAs were reverse transcribed with High-Capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA), and cDNA was loaded on a Taqman Custom Array (Applied Biosystems) following the manufactory instructions. Expression data were analyzed with SDS 2.3 and GEDAS software.

Indirect immunofluorescence

The list of the primary antibodies used in these experiments is given in table 1. All the images were captured by using a Leica TCS SP2 AOBS confocal microscope and analyzed with Leica Confocal Software (LCS).

EBs and cultured cells were fixed with PBS, 4% paraformaldehyde. Incubation with the primary antibodies was carried out for 1 hr at 37°C in moist chamber. Chicken frozen sections were incubated in moist chamber overnight at 4°C with primary antibodies and, only when required, they were incubated with biotinylated Sambucus Nigra Agglutinin (Vector, CA, USA) and AlexaFluor-555-streptavidin (Invitrogen).

Immunoprecipitation and western blot analysis

Cell lysates of W4 undifferentiated ES cells and of 7 days-old EBs were obtained on cold with 10 mM Tris/HCL pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Triton-X-100, 0.1 mM $ZnCl_2$ supplemented with protease inhibitors cocktail (Sigma). Protein content was valuated by BCA assay (Pierce, Rockford, IL, USA). Nrp1 protein was immunoprecipitated with 1 μg of rabbit polyclonal anti-Nrp1 antibody (Santa Cruz, CA, USA) and immunocomplexes were loaded onto 7.5% SDS-PAGE and transferred to

PVDF membrane (Millipore Co., Bedford, MA, USA). The filter was decorated with the goat polyclonal anti-Nrp1 antibody C19 (Santa Cruz). Immunocomplexes were then visualized by ECL system (Pharmacia-Amersham Biotech., UK).

Microinjection experiments of chick embryos

Purified $Nrp1^{+}/Flk1^{+}$ or $Nrp1^{+}/Flk1^{-}$ cells were microinjected or in the beating heart or in the mesencephalic cavity of HH19 chicken embryos by using a manual micro-injector (CellTram Oil, Eppendorf, Hamburg, DE). At different days after injection, the embryo vasculature was labeled with AlexaFluor 405-conjugated wheat germ agglutinin that was injected into an artery of the vitelline sac. Immediately after, embryos were sacrificed, fixed with zinc saline formalin (Bio-Optica, Milano, IT) for 48 h at 4°C or with PBS 4% paraformaldehyde for 24 hours at 4°C and cryoprotected in 15% sucrose for 8 hours and then in 30% sucrose overnight at 4°C. After embedding and freezing them in Killik compound (Bio-Optica), 12-20 μm sections were cut using a Leica CM3050 S cryostat.

Table 1. Antibodies used for immunofluorescence and FACS analysis

Primary Antibodies	Host	Dilution for IF
CD133 clone 13A4 (eBioscience)	Mouse monoclonal	
CD31 clone Mec13.3 (BD Pharmingen)	Rat monoclonal	1:100
Flk1 clone Avas 12α1 (BD Pharmingen)	Rat monoclonal	1:100
GFP (Invitrogen)	Rabbit	1:1500
ICAM2 clone 3C4 (BD Pharmingen)	Rat monoclonal	1:100
MAPs (Sigma)	Rabbit policlonal	1:200
Nestin (BD Pharmingen)	Mouse monoclonal	1:100
NeuN (Chemicon)	Mouse monoclonal	1:50
Nrp1 (R&D Systems)	Goat	1:200
PSA-NCAM clone 2-2B (Chemicon)	Mouse monoclonal	
RC2 (Dev. Studies Hybridoma Bank)	Mouse monoclonal	1:20
Sox1 (Abcam)	Rabbit	1:100
Sox2 (Chemicon)	Rabbit	1:100
VE-Cadherin (R&D Systems)	Goat	1:100
βIII-tubulin (Sigma)	Mouse monoclonal	1:1000
Cytokeratin (Dako)	Mouse monoclonal	1:400
TH (Chemicon)	Mouse monoclonal	1:100
Neurofilament H (Affiniti)	Mouse monoclonal	1:100
GFAP (Sigma)	Mouse monoclonal	1:200
SV2 (Dev. Studies Hybridoma Bank)	Mouse monoclonal	1:100
PE-Flk1 (BD Pharmingen)	Rat monoclonal	
APC-Flk1 (BD Pharmingen)	Rat monoclonal	

Table 2. Primers used for RT-PCR analysis

Transcript	Forward	Reverse	Size (bp)
CD31	5'-GTCATGGCCATGGTCGAGTA-3'	5'-CTCCTCGGCATCTTGCTGAA-3'	260
Endoglin	5'-CTTCCAAGGACAGCCAAGAG-3'	5'-GTGGTTGCCATTCAAGTGTG-3'	221
Flk1	5'-TCTGTGGTTCTGCGTGGAGA-3'	5'-GTATCATTTCCAACCACCCT-3'	269
Flt1	5'-CCAAGGCCTCCATGAAGATA-3'	5'-ATACTGTCAGGGGCTGGTTG-3'	248
Tie1	5'-CTCACTGCCCTCCTGACTGG-3'	5'-CGATGTACTTGGATATAGGC-3'	228
Tie2	5'-CCTTCCTACCTGCTA-3'	5'-CCACTACACCTTTCTTTACA-3'	441
VE-Cadherin	5'-TCAGAACCGGATGACCAAGT-3'	5'-AAGTGTCGCTGGAAGACAGG-3'	274
HPRT	5'-GCTGGTGAAAAGGACCTCT-3'	5'-CACAGGACTAGAACACCTGC-3'	248

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