Cell fusion-independent differentiation of neural stem cells to the endothelial lineage

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Somatic stem cells have been claimed to possess an unexpectedly broad differentiation potential (referred to here as plasticity) that could be induced by exposing stem cells to the extracellular developmental signals of other lineages in mixed-cell cultures[1–6]. Recently, this and other experimental evidence supporting the existence of stem-cell plasticity have been refuted because stem cells have been shown to adopt the functional features of other lineages by means of cell-fusion-mediated acquisition of lineage-specific determinants (chromosomal DNA) rather than by signal-mediated differentiation[1,2,5,7,8]. In this study we co-cultured mouse neural stem cells (NSCs), which are committed to become neurons and glial cells[9,10], with human endothelial cells, which form the lining of blood vessels[11]. We show that in the presence of endothelial cells six per cent of the NSC population converted to cells that did not express neuronal or glial markers, but instead showed the stable expression of multiple endothelial markers and the capacity to form capillary networks. This was surprising because NSCs and endothelial cells are believed to develop from the ectoderm and mesoderm, respectively. Experiments in which endothelial cells were killed by fixation before co-culture with live NSCs (to prevent cell fusion) and karyotyping analyses, revealed that NSCs had differentiated into endothelial-like cells independently of cell fusion. We conclude that stem-cell plasticity is a true characteristic of NSCs and that the conversion of NSCs to unanticipated cell types can be accomplished without cell fusion.

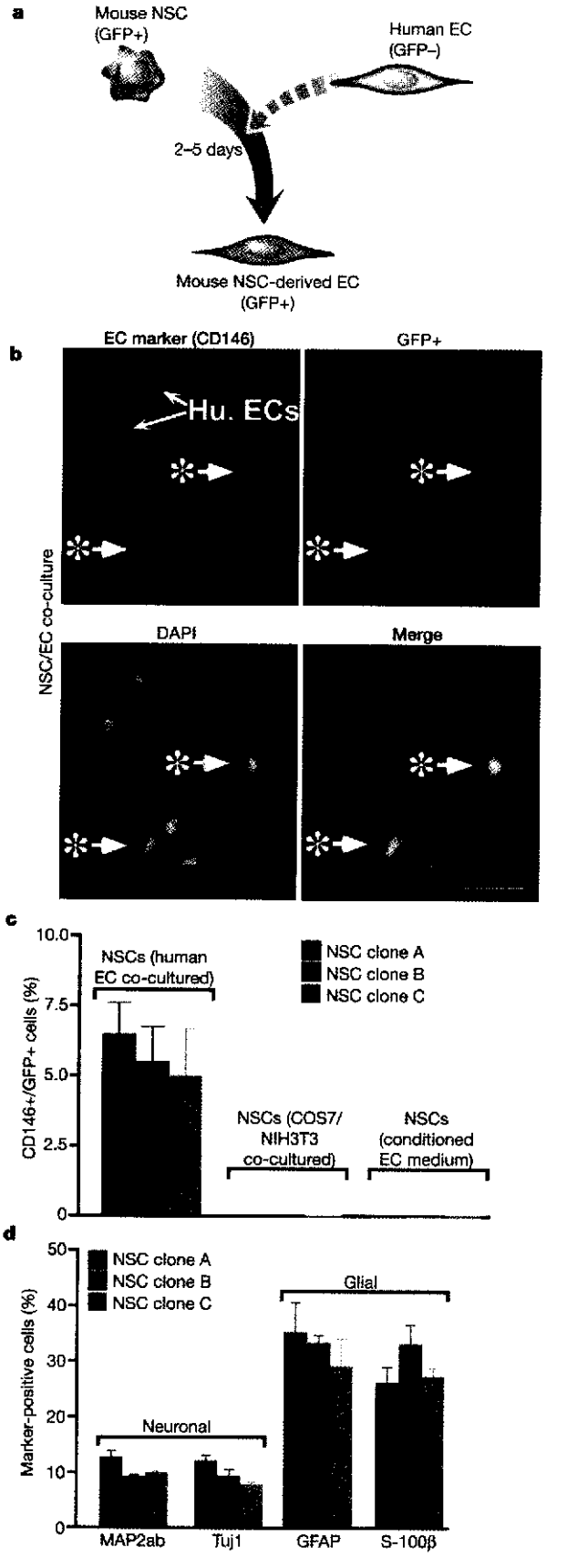
Previous studies have shown that co-cultured stem cells sometimes take on the characteristics of the cell type with which they are cultured[1–6]. Because NSCs are concentrated at interfaces with the vasculature *in vivo*[12], we reproduced this proximity *in vitro* by co-culturing NSCs with endothelial cells, to determine whether endothelial cells could promote the conversion of NSCs to an endothelial-like cell.

Three clonal lines of NSCs (clones A, B and C), each originating from a single cell, were isolated from mice engineered to constitutively express green fluorescent protein (GFP) in all cell types. Each of our clonally derived, GFP-labelled mouse NSC lines was then co-cultured with a purified population of non-GFP-labelled primary human endothelial cells for 2–5 days (see Methods). All neural and endothelial cells used in these experiments had been passaged only 4–7 times *in vitro*, minimizing the possibility that mutations would occur and affect the outcome of our experiments. To ascertain whether GFP-labelled NSCs could be induced to adopt an endothelial cell phenotype, we probed co-cultures for cells that co-expressed GFP and the endothelial cell adhesion protein, CD146[13,14] (Fig. 1a).

An antibody specific to CD146 recognized human endothelial cells, but not NSCs, neurons or astrocytes (data not shown). In mouse NSC/human endothelial cell co-cultures, non-GFP-expressing human endothelial cells stained for CD146 in a characteristic cell-surface pattern, with the majority of the CD146 signal concentrated at the cell periphery (Fig. 1b). The presence of human endothelial cells induced approximately 6% of the GFP-labelled cells deriving from NSCs to co-label with CD146 in a staining pattern similar to that of human endothelial cells (Fig. 1b). Whereas multi-nucleated cells have previously been observed after cell fusion[5,8], all documented $GFP^+/CD146^+$ cells in our study were uni-nucleate (Fig. 1b).

All of the GFP-labelled NSC clones tested (clones A–C) converted to a CD146-expressing state at a frequency of 5–6.5% after co-culture with human endothelial cells, but not after co-culture with COS7 or NIH3T3 cells under identical conditions (Fig. 1c). The behaviour of these clonal lines was truly representative of the NSC, as a similar percentage (3–9%) of non-clonally purified, low passage (passage 4–7) NSCs expressed CD146 in response to human endothelial cells (data not shown). In the absence of human endothelial cells, mock treatment of NSCs with serum-rich endothelial cell growth medium caused NSC clones A–C to become neurons and astrocytes at frequencies comparable to those previously reported[10] (Fig. 1d). This finding indicates that each of these clonally derived lines behaved as predicted for NSCs and hence that they were free from contamination by non-NSC cell types. Secreted human endothelial cell factors (that is, physical separation of NSCs and human endothelial cells while mixing the respective media)

were also insufficient to bring about GFP$^+$/CD146$^+$ co-labelled cells, suggesting that induction of CD146 expression requires contact of neural cells and human endothelial cells (Fig. 1c; see below).



Clonal lines of GFP$^+$/CD146$^+$ cells were then purified. Following 5 days of co-culture with human endothelial cells, 600 GFP-labelled cells derived from NSCs were subjected to a single-cell-per-well, fluorescence-activated cell sort (FACS) for GFP-positive cells. GFP-positive cells were then clonally expanded in serum-rich endothelial cell growth medium and screened for the expression of CD146. Three clonal lines (clones 1, 2 and 3) were established at a frequency of 0.5%; lower than the 6% incidence of GFP$^+$/CD146$^+$ cells in co-culture. This discrepancy may be due to cell death during the FACS procedure.

Similar to human endothelial cells, 84–88% of cells within each clonal population of NSC-derived endothelial cell candidates expressed CD146, as seen by indirect immunofluorescence, whereas mock-treated NSCs exposed to serum-rich endothelial cell growth medium in the absence of human endothelial cells did not (Fig. 2a, b). Indeed, GFP and CD146 expression were maintained by clones 1–3 of NSC-derived endothelial cell candidates even at late passage (passage 22; data not shown), suggesting that GFP$^+$/CD146$^+$ clonal cell lines did not require the continued presence of human endothelial cells to maintain expression of this endothelial cell marker. We found that a significant percentage of the human endothelial cell population labelled with the characterized endothelial cell markers vascular endothelial (VE) cadherin (95%), von Willebrand factor (VWF; 69%), isolectin GS–IB$_4$ (lectin; 80%) and CD31 (85%) (Fig. 2a, b)[15–19]. The percentage of cells within each GFP$^+$/CD146$^+$ clonal line that stained positive for these endothelial markers was comparable: VE cadherin (84–88%), VWF (82–92%), lectin (78–80%) and CD31 (77–86%) (Fig. 2a, b). A second VE cadherin antibody also recognized 28% of human endothelial cells and 56–76% of NSC-derived endothelial cell candidates (data not shown). Detergent did not influence the distribution of VE cadherin, confirming the predominantly cell-surface localization of this cell adhesion protein (data not shown)[15,16]. A high percentage of cells within clonal GFP$^+$/CD146$^+$ cell lines also expressed the endothelial proteins, endoglin (86–92%), connexin 40 (89–93%), endothelial nitric oxide synthetase (80–86%), thrombomodulin (81–91%) and claudin 5 (89–95%; Supplementary Fig. 1a). Additionally, even though each GFP$^+$/CD146$^+$ clone originated from NSCs, none of these cell lines expressed markers associated with NSCs or the lineages known to develop from NSCs, such as nestin (NSC), GFAP (astrocyte), RIP (oligodendrocyte), or Map2ab/Tuj1 (neuron) (Fig. 2a, b). In the absence of human endothelial cells, NSCs mock-treated with serum-rich endothelial cell medium expressed Map2ab, Tuj1 and GFAP (Fig. 2a, b). NSCs maintained under serum-free neural growth conditions were positive for nestin, but did not label with CD146, VE cadherin, VWF, CD31 or lectin (data not shown).

Reverse transcriptase-polymerase chain reaction confirmed that NSCs that were exposed to neural growth conditions or mock-

Figure 1 NSCs can be induced to express the endothelial marker, CD146. **a**, GFP-labelled mouse NSCs were mixed with non-GFP-labelled human endothelial cells (ECs) and co-cultured under serum-rich endothelial cell growth conditions for 2–5 days. We then screened for NSC-derived endothelial cells, which were predicted to co-express GFP and the endothelial marker, CD146. **b**, Fluorescent images of a single field of co-cultured human endothelial cells and GFP-labelled NSCs. The endothelial cell marker CD146, GFP, nuclear DAPI and a merge of these three images are shown. Human endothelial cells and GFP$^+$/CD146$^-$ cells (*) are indicated. Scale bar, 30 μm. **c**, Quantification of GFP$^-$/CD146$^+$ cells derived from three clonal lines of NSCs (clones A–C) after co-culture with either COS7, NIH3T3 or human endothelial cells, or after treatment of NSCs with human endothelial cell-conditioned medium for 5 days. Mean values ±s.d. are shown. **d**, Mock treatment of three NSC clones (A–C) with serum-rich endothelial cell medium for 5 days induced differentiation to neuronal (Mab2ab or Tuj1 markers) and glial (GFAP or S-100β markers) lineages. Mean values ±s.d. are shown.

treated with endothelial cell medium were enriched for transcripts of the neural lineages (nestin or Map2ab/GFAP, respectively). These transcripts were down-regulated in NSC-derived $GFP^+/CD146^+$ cells (Fig. 2c). Instead, $GFP^+/CD146^+$ cells expressed messages for endothelial VE cadherin, CD31, Flk-1, Tie-2, endoglin, connexin 40, endothelial nitric oxide synthetase and thrombomodulin[16,19,20] (Fig. 2c; Supplementary Fig. 1b).

After determining that these three lines of NSC-derived endothelial cell candidates expressed multiple markers of endothelial cells and lacked expression of neural markers, we investigated the possibility that these cells might also have adopted the functional properties of endothelial cells. One ultra-structural feature of endothelial cells expressing VWF is the presence of a unique class of secretory vesicles, known as Weibel–Palade bodies. Electron microscopy revealed that these vesicles, which are 200–400 nm in diameter and characterized by an electron-dense lumenal matrix[21], were present in human endothelial cells and clones 1–3 of NSC-derived endothelial cell candidates (Fig. 3a). Another functional hallmark of differentiated endothelial cells is the capacity to form capillaries. Although this property is not manifested during growth conditions, it can be induced by plating endothelial cells onto a collagen/laminin-coated substratum (Matrigel)[22]. Therefore, non-human endothelial cell co-cultured NSCs, human endothelial cells and clonally purified populations of NSC-derived endothelial cell marker-positive cells were assayed for Matrigel-dependent vascular cord formation *in vitro*. Time-lapse imaging revealed that each clone of NSC-derived endothelial cell marker-expressing cells and human endothelial cells, initially plated as dissociated cells, began aggregating and elongating within 2 h. Vascular cord formation for each NSC-derived, endothelial-like clone and human endothelial cells proceeded with identical kinetics and was complete by the 12-h time point (Fig. 3b; Supplementary Fig. 2). In contrast, whereas mock-treated NSCs remained motile throughout most of the assay, these cells failed to form the same structures (Fig. 3b). Collectively, this body of evidence suggests that the co-culture of NSCs with human endothelial cells can divert NSC-derived

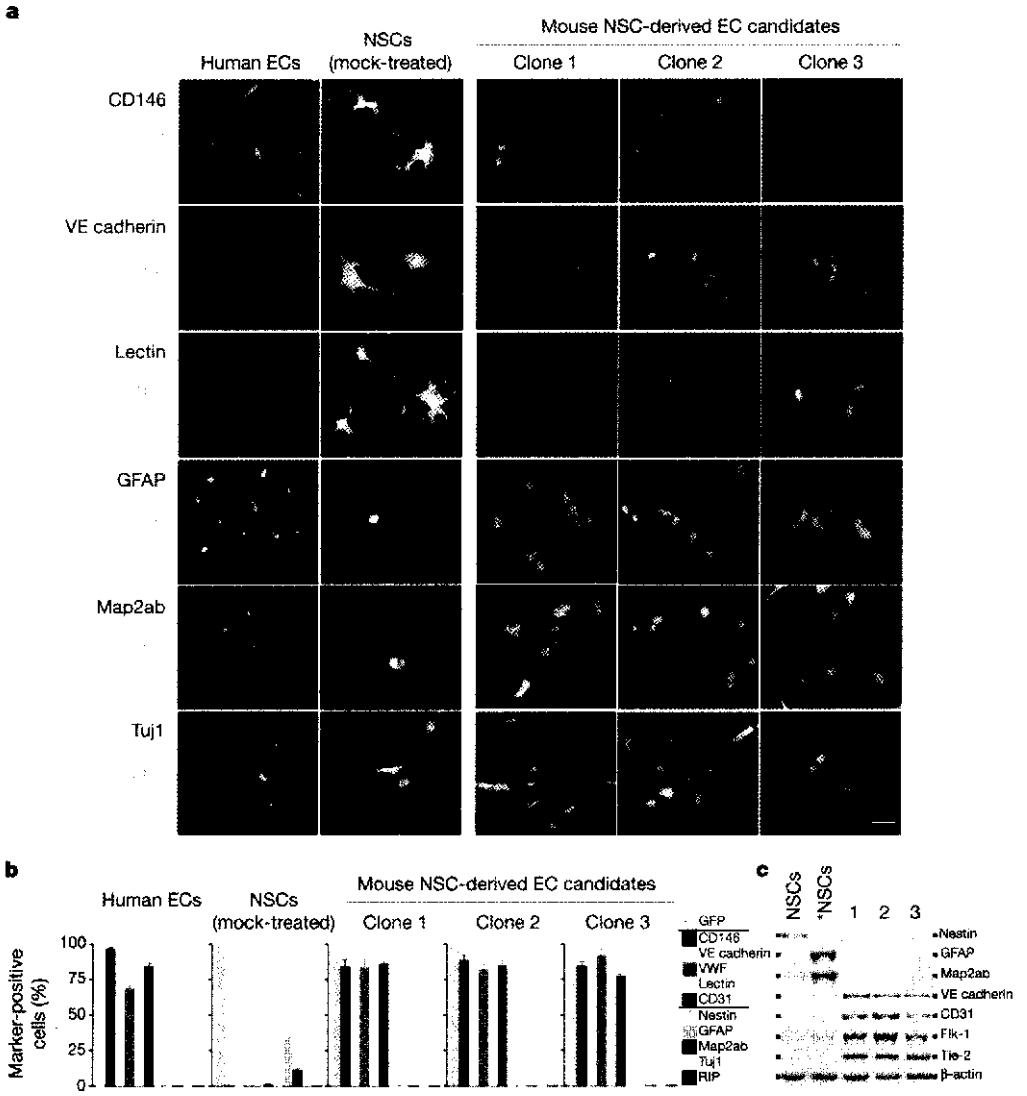


Figure 2 Endothelial markers are expressed by NSC-derived endothelial cell candidates clonally purified from human endothelial cell co-culture. **a–c**, Expression of endothelial, neuronal and glial markers in human endothelial cells, mock-treated NSCs and three clonal lines of NSC-derived endothelial cell candidates (clones 1–3). **a**, Fluorescent images of each cell type. Scale bar, 20 μm. **b**, Quantification of the percentage of marker-positive cells by fluorescent imaging. Mean values ± s.d. are shown. **c**, Reverse transcriptase-polymerase chain reaction analysis of nestin, Map2ab, GFAP, VE cadherin, CD31, Flk-1, Tie-2 and β-actin messenger transcript expression in NSCs maintained under growth conditions, mock-treated NSCs (column marked with an asterisk) and NSC-derived endothelial cell candidates (columns labelled 1–3).

cells from the neural lineages, instead inducing endothelial characteristics.

Having been observed to form liver, epithelial, blood and muscle cells, NSCs were initially proposed to have a highly plastic differentiation programme that could readily bypass the neural lineages[3,23,24]. A growing number of studies now suggest that stem cells, including NSCs, adopt the phenotypes of other cells by fusion, an event that may previously have been mistaken for stem-cell plasticity[1,2,5,7,8]. It is currently unclear whether cell fusion can account exclusively for all reports of stem-cell plasticity[4,8,25]. Nonetheless, the conversion of NSCs to endothelial-like cells might entail the cell-fusion-mediated formation of mouse NSC–human endothelial cell hybrids that epistatically express human endothelial cell phenotypes and function.

Analysing the chromosomal content of cultured stem-cell derivatives has proven to be effective in the detection of stem-cell fusion[1,2,8]. On the basis of metaphase chromosomal spreads of previously reported mouse–human hybrids, low-passage mouse NSC–human endothelial cell hybrids would be predicted to have approximately 40 mouse and 10–40 human chromosomes[26,27]. As shown in Fig. 4a, human endothelial cells manifested 46 chromosomes that were metacentric or acrocentric, whereas mouse NSCs contained 40 telocentric chromosomes. Consistent with a non-fusion event, the screening of 141 chromosomal spreads revealed approximately 40 telocentric mouse chromosomes, but never any human chromosomes, within low-passage endothelial-like cells derived from NSCs (Fig. 4a). Additionally, a species-specific antibody that recognizes human ribonuclear proteins reacted strongly with the nuclei of human endothelial cells but did not notably label the nuclei in any of the three clonal, NSC-derived endothelial-like lines (Fig. 4b). These experiments strongly suggest that each of the three mouse NSC-derived endothelial-like cell lines maintained endothelial phenotypes independently of genomic lineage determinants acquired from human endothelial cells by cell fusion.

To address a potential role for cell fusion at the initiation of the NSC-to-endothelial cell transition, human endothelial cells were killed with paraformaldehyde before co-culture with NSCs. Paraformaldehyde pre-fixation of human endothelial cells is predicted to stabilize cellular components, rendering human endothelial cells refractory to cell fusion (Fig. 4c). This method has been shown to prevent fusion of live sperm cells with fixed sea urchin eggs, while not disrupting receptor-mediated recognition and association of these cell types[28]. As expected, paraformaldehyde treatment disrupted the exclusion of propidium iodide by human endothelial cells, indicating that these cells were non-viable (Fig. 4d). After extensive washing to remove excess paraformaldehyde, GFP-labelled NSCs were cultured with fixed human endothelial cells for 5 days. During the course of this study, we found that lectin preferentially bound to mouse NSC-derived endothelial-like cells with several-fold higher affinity than human endothelial cells. Lectin was therefore used as an endothelial probe to facilitate identification of NSC-derived endothelial-like cells in the context of fixed human endothelial cell co-cultures. This procedure led to the conversion of 2–4% of NSCs to GFP^+/$lectin^+$ double-labelled cells relative to fixed COS7 cell co-cultured NSCs (Fig. 4e, f). Collectively, these experiments indicate that cell fusion did not play a part in the initial expression or maintenance of endothelial traits by NSC-derived cells. The data instead support the existence of relatively paraformaldehyde-resistant cell surface molecules (such as transmembrane proteins) that convey endothelial differentiation or survival signals from human endothelial cells to NSC-derived cells.

Stem-cell plasticity was thought to form the foundation for one of the multiple prospective uses of adult stem cells in regenerative medicine[29]. Data demonstrating that stem cells could fuse with and subsequently adopt the phenotypes of other cell types indicated that the very co-culture assays originally interpreted to support plasticity instead were artefacts of cell fusion[1–6]. By showing that NSCs unexpectedly differentiated to the endothelial lineage in the absence of cell fusion, we reintroduce the idea that NSCs are inherently plastic.

This demonstration of NSC plasticity *in vitro* may reflect the potential for NSCs to differentiate to endothelial cells *in vivo*. Consistent with this possibility, when clonal GFP-labelled NSCs were transplanted into the telencephalon of embryonic day-14 mice, 1.6% of NSCs converted to cells that co-expressed endothelial phenotypes (that is, bound to lectin and expressed VE cadherin) by embryonic day 16 (Fig. 4g, h). Most transplanted NSCs (>80%) differentiated to glial cells (data not shown). This *in vivo* assay system did not recapitulate the high ratio of endothelial cells to NSCs in our *in vitro* experiments, possibly explaining the relative frequency of NSC-to-endothelial cell conversion *in vivo* (1.6%) versus *in vitro* (6%). Nonetheless, NSC-derived endothelial-like cells showed the characteristic cup-shaped morphology of endothelial cells and also labelled for VE cadherin at intercellular junctions with endogenous endothelial cells (Fig. 4g, h). Optical sectioning of NSC-derived endothelial cells by confocal microscopy showed that these cells had a single nucleus, suggesting that NSCs had differentiated to endothelial-like cells *in vivo* (Fig. 4g, h).

This work encourages a new look at angiogenesis, the process whereby endothelial cells dissociate from pre-existing vessels, proliferate and aggregate to form new vessels directed at oxygen- and nutrient-deprived tissue[11]. The traditional view of angiogenesis suggests that newly formed endothelial cells can arise through the proliferation of pre-existing endothelial cells[11]. An alternative possibility raised by this work is that NSCs give rise not only to neurons and glial cells but also to endothelial cells that promote vascularization of the surrounding tissue. Further experimentation

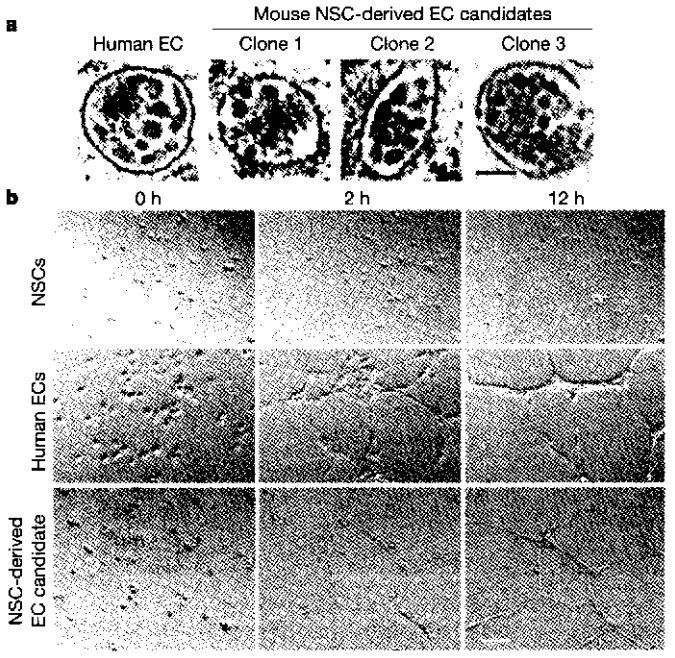


Figure 3 NSC-derived endothelial cell candidates show endothelial Weibel–Palade bodies and form capillary networks *in vitro*. **a**, Electron microscopy of subcellular Weibel–Palade bodies in human endothelial cells and NSC-derived endothelial cell candidates. Scale bar, 100 nm. **b**, NSCs, human endothelial cells and NSC-derived endothelial cell candidates (clone 1 is shown) were introduced onto Matrigel-coated plates at the 0 h time point and monitored for their capacity to form vascular cords. Scale bar, 100 μm.

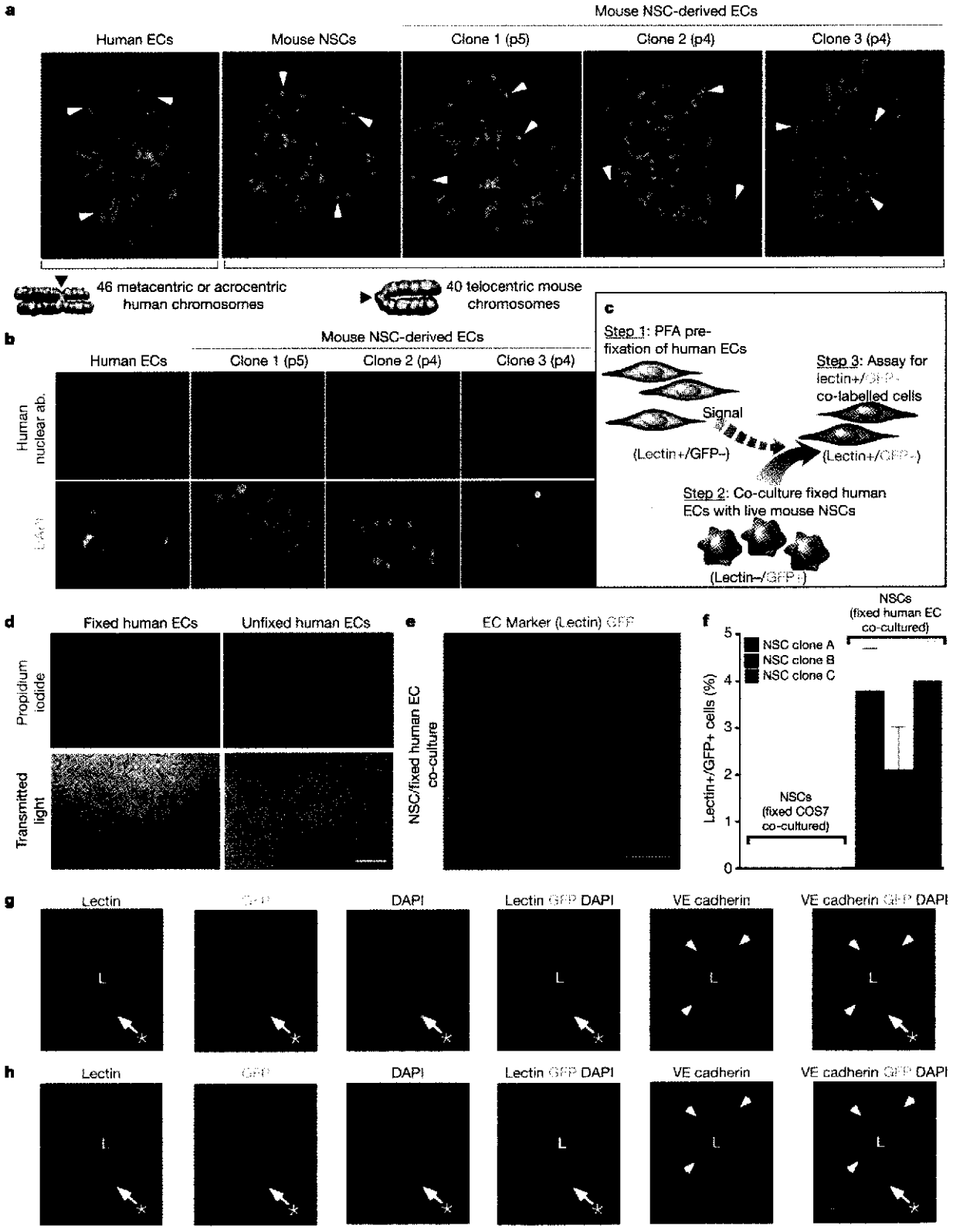


Figure 4 Cell fusion is unlikely to contribute to the generation of NSC-derived endothelial-like cells. **a**, Metaphase chromosome spreads of human endothelial cells, mouse NSCs and clones 1–3 of mouse NSC-derived endothelial-like cells are shown. Clones 1–3 were karyotyped at passages 5 (p5), 4 (p4) and 4, respectively. Several centromeres are highlighted (arrowheads). **b**, Human endothelial cells and clones 1–3 of NSC-derived endothelial-like cells at passages 5, 4 and 4, respectively, stained with human-specific ribonuclear protein antibody and DAPI. **c**, Procedure for the culture of live GFP-labelled NSCs with fixed human endothelial cells. **d**, Fixed human endothelial cells stained with propidium iodide. Scale bar, 100 μm. **e**, **f**, Fixed human endothelial cells induced clones A–C of GFP-labelled NSCs to bind the endothelial probe lectin. Scale bar, 20 μm (**e**). Mean values ± s.d. are shown (**f**). **g**, **h**, GFP-labelled NSCs converted to lectin and VE cadherin co-expressing endothelial cells within the neocortex of the subventricular zone in embryonic day-16 mice (indicated by arrow and an asterisk). Two sections of one sample capillary are shown. The lumen of the capillary (L) and the adherent VE cadherin junctions between endothelial cells (arrowheads) are highlighted. Scale bar, 10 μm.

will be necessary to distinguish whether NSCs contribute to vascular outgrowths at a frequency inherent to the normal physiology of the embryonic and adult central nervous systems. □

Methods

Cell culture

Whole brain NSCs of adult mice that ubiquitously express GFP (C57BL/6-TGN (ACTbEGFP)10sb; The Jackson Laboratory) were purified in a two-step process first entailing the fractionation of mitotic NSCs from non-mitotic cells using a Percoll gradient[30]. Gradient-enriched NSCs were then clonally isolated by a single-cell-per-well FACS-based selection of GFP-positive cells. On the day of plating, visual verification by phase contrast and GFP-fluorescence microscopy confirmed that clonal lines A–C originated from a single cell. NSCs were proliferated in DMEM/F-12 medium (Omega Scientific) supplemented with N2 (Gibco), 2 mM L-glutamine, 20 ng ml^{-1} fibroblast growth factor-2 and 20 ng ml^{-1} epidermal growth factor. Purified human umbilical artery endothelial cells (human endothelial cells) were grown in EGM2–MV endothelial medium that contains vascular endothelial growth factor, fibroblast growth factor-2, epidermal growth factor, insulin growth factor 1 and 5% serum (BioWhittaker/Clonetics).

Clonal lines of NSCs were grown as a monolayer and shifted from growth medium into serum-rich EGM2–MV medium for 5 days to quantify NSC differentiation to neurons, glial cells and CD146-expressing cells.

For co-cultures, NSCs and human endothelial cells (or COS7/NIH3T3) were trypsinized and resuspended in NSC growth or EGM2–MV medium. Seventy-five NSCs were then mixed with 750–7,500 human endothelial cells in 400 μl EGM2–MV per well of a 24-well plate (Costar). Co-culture was continued until 80% confluence was attained (2–5 days). Cells were fed 200 μl of EGM2–MV on day 3. Cells that stained positive for CD146 in the characteristic staining pattern shown in Fig. 1b were scored as positive. Four thousand human endothelial cells were plated per well of a 24-well plate and grown to 80% confluence before being fixed with 0.5% paraformaldehyde for 15 min at 25 °C. Fixed cells were washed extensively, first with PBS and then overnight with EGM2–MV. The next day, 300 GFP-labelled NSCs (clones A–C) were cultured with fixed human endothelial cells for 5 days and then stained with lectin, as described below. The frequency of conversion of NSCs to GFP$^+$/lectin$^-$ cells was calculated by normalizing the number of GFP$^+$/lectin$^+$ cells resulting from human endothelial cell co-culture with the number of GFP$^+$/lectin$^+$ cells arising from paraformaldehyde-fixed COS7 co-culture.

Capillary formation

Endothelial cells were induced to form vascular cords by pre-coating 24-well plates with collagen/laminin (Matrigel; BD Biosciences). Each cell type was plated in EGM2–MV lacking serum, epidermal growth factor and insulin growth factor I and then was incubated at 37 °C for 12 h. Cells were monitored by Hoffman modulation contrast optics using a × 10 lens.

Microscopy

Culture medium was washed away for 10–15 min with 0.1 M TBS buffer before fixation with 4% paraformaldehyde. Fixed cells were incubated with primary antibodies overnight in 0.1 M TBS buffer supplemented with 3% normal donkey serum and 0.25% Triton X-100, or with lectin for 1 h in 10 mM HEPES buffer pH 7.6, 150 mM NaCl and 1 mM $CaCl_2$. The primary antibodies used were: CD146 (1 μg ml^{-1}, mouse; Chemicon), Map2ab (1:250, mouse; Sigma), Tuj1 (1:1,000, mouse; Covance Inc.), GFAP (1:2,500, rabbit; Dako), S–100β (1:2,500, rabbit; Sigma), VE cadherin (3 μl ml^{-1}, rabbit; Alexis Biochemicals), anti-mouse VE cadherin (1:100, rat; Research Diagnostics), VWF (1:1,000, sheep; laboratory reagent), CD31 (2.5 μl ml^{-1}, rat; Caltag Laboratories), nestin (1:1,000, mouse; Pharmingen), RIP (1:50, mouse; Hybridoma Bank), human nuclei (1:300, mouse; Chemicon), endothelial nitric oxide synthetase (1.5 μl ml^{-1}, mouse; Research Diagnostics), connexin 40 (12 μl ml^{-1}, rabbit; Zymed), claudin 5 (6 μl ml^{-1}, rabbit; Lab Vision), thrombomodulin (6 μl ml^{-1}, mouse; Lab Vision) and endoglin (12.5 μl ml^{-1}, mouse; Lab Vision). Lectin was used at 2 μg ml^{-1} (Alexa 594- or 647-conjugated; Molecular Probes). Where applicable, CY3- or Texas Red-conjugated secondary antibodies specific to the appropriate species were used (1:250; Jackson Laboratory).

Images were obtained with either a multi-photon equipped confocal system (Bio-Rad) or an Eclipse E800 microscope (Nikon)/Spot Camera system (Diagnostic Instruments).

Quantification of marker expression was carried out using an Eclipse E800 microscope (Nikon) /Spot Camera system (Diagnostic Instruments). Each marker was quantified as a percentage of 4,6-diamidino-2-phenylindole (DAPI)-positive cells that were also marker positive. Results of three to four experiments are shown.

For electron microscopy, EGM2–MV was removed and cultured cells were fixed by the addition of 3% glutaraldehyde/0.1 M phosphate buffer followed by 0.1% osmium tetroxide. Cells were dehydrated in 80% ethanol, scraped off the plate with a Teflon blade and pelleted. Cells were then embedded in EPON resin mixture. Ultrathin sections of a grey interference colour (~40 nm) were observed with a Jeol 100CX electron microscope.

Karyotyping

Cells were conditioned in EGM2–MV supplemented with 60 μg ml^{-1} bromodeoxyuridine (Sigma), 0.3 μg ml^{-1} FuDr (5-fluoro-2′-deoxyuridine; Sigma) and 2 μl ml^{-1} colcemid (Gibco BRL) for 5 h at 37 °C. After trypsinization, cells were washed with PBS and then incubated in 0.06 M KCl for 15 min at 37 °C. Chromosomes were then fixed in methanol/acetic acid (3:1) overnight, attached to slides by flaming and stained with DAPI.

Reverse transcriptase-polymerase chain reaction

A complementary DNA template was synthesized using SuperScript reverse transcriptase-polymerase chain reaction kit (Invitrogen) and subjected to 20–33 rounds of polymerase chain reaction in the presence of one unit of Taq polymerase (Promega), 4 mM $MgCl_2$, 400 μM deoxyribonucleotide triphosphates and 1 μg of each oligonucleotide primer per 50 μl reaction. Nested primers internal to the initial primers were used to confirm the identity of first-round polymerase chain reaction fragments. All primers were designed to hybridize with the murine version of each transcript tested. β-actin was used as an internal standard.

In utero transplantation of NSCs

Approximately 1 μl containing 10^5 GFP-expressing NSCs (line 2, clonally-derived NSCs) were injected into the lateral ventricle of embryonic day-14 ICR mice *in utero*. The NSC-transplanted embryonic brains were collected on embryonic day 16 and fixed with 4% paraformaldehyde overnight. Fixed brains were sectioned at 14 μm thickness on a Microm HM 560 cryostat and probed with lectin (Alexa 647-conjugated; Molecular Probes) and VE cadherin (1:30; Research Diagnostics), as described above. Endothelial marker-expressing cells originating from GFP-labelled NSCs were quantified as a percentage of GFP-labelled cells that integrated into neural tissue.

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