Segregation of Human Neural Stem Cells in the Developing Primate Forebrain


Many central nervous system regions at all stages of life contain neural stem cells (NSCs). We explored how these disparate NSC pools might emerge. A traceable clone of human NSCs was implanted intraventricularly to allow its integration into cerebral germinal zones of Old World monkey fetuses. The NSCs distributed into two subpopulations: One contributed to corticogenesis by migrating along radial glia to temporally appropriate layers of the cortical plate and differentiating into lamina-appropriate neurons or glia; the other remained undifferentiated and contributed to a secondary germinal zone (the subventricular zone) with occasional members interspersed throughout brain parenchyma. An early neurogenesis program allocates the progeny of NSCs either immediately for organogenesis or to undifferentiated pools for later use in the "postdevelopmental" brain.

As cells with stemlike qualities have come to be identified within a widening range of organs [e.g., (1, 2)], new questions have arisen about their relevance to normal development. The central nervous system (CNS) may serve as a bellwether for insights in this field. NSCs have been identified in the mammalian CNS, including humans (3–9), at stages from fetus to adult in a surprisingly wide range of regions (10–13). NSCs, defined as self-renewing, propagating primordial cells each with the capacity to give rise to differentiated progeny within all neural lineages in all regions of the neuraxis, are posited to exist in the embryonic and fetal ventricular germinal zone (VZ) where they participate in CNS organogenesis (5, 14, 15). Cells equally "stemlike" in their potential have been identified at later stages (including old age) from a variety of regions: subventricular (SVZ) (13–17) and ependymal (18) zones of the forebrain, subgranular zone of the hippocampus (6–10, 19), retina (20) and optic nerve (10, 11), cerebellum (12), spinal cord (21), and even cortical parenchyma (10, 13, 22). How might these observations be reconciled? Are such stemlike pools, particularly those isolated from various parenchymal regions at "postdevelopmental" periods, of physiological relevance or artifacts of experimental manipulation (10, 11)? Do these populations represent the same lineage or unique pools (17)? Of what relevance are these cells to normal human CNS development and repair? We hypothesized that multiple stem cell pools, descendants of a common NSC, emerge during early cerebrogenesis as cells are used in organogenesis and concurrently

References and Notes

2. R. Gibrat, Les Inégalités Économiques; Applications: aux inégalités des richesses, à la concentration des entreprises, aux populations des villes, aux statistiques des familles, etc., d'une loi nouvelle, la loi de l'effet proportionnel (Librairie du Recueil Sirey, Paris, 1931).
8. Although any finite sample will have moments, by definition, the nonexistence of moments in the context of real data implies that the moments give no indication of convergence as the number of data points increase.
14. Census data is based on Small Business Administration (SBA) tabulation; available at www.sba.gov/advo/stats/data.html.
also set aside to establish a reservoir for subsequent use in homeostasis and repair. This could represent a developmental strategy in which plasticity is programmed into the CNS at the single-cell level from early stages of embryogenesis.

We sought to determine how progeny of a single traceable clone of NSCs get segregated during development by using a system that might also lend insight into human development. We grafted a clone of NSCs of human derivation (3, 23) into the developing brains of fetal bonnet monkeys (Macaca radiata), an Old World species (Web note 1) (24). We asked what the fate would be of human cells transplanted at a time when neocortical cell genesis, migration, and differentiation are intensive (25–27). The primate neocortex, at the appropriate developmental stage, allows a distinction between layers of active neuron birth and layers where neurogenesis has been completed and glial cells are instead acquired (27) (Web note 2) (24) (Fig. 1, schematics I and II). One can discern experimentally the responses to local developmental cues simply by assaying the spatial segregation and patterns of differentiation of NSCs of a single clone in a given animal’s brain after a single transplantation procedure. (A summary of simian cortical development is provided in an expanded legend to Fig. 1 in Web note 2 (24)). Under transabdominal ultrasonic guidance, bonnet monkey fetuses at 12 to 13 weeks gestation received a single in utero injection of $2 \times 10^{5}$-cloned related undifferentiated NSCs [prelabeled with the nuclear marker 5-bromo-2$'$-deoxyuridine (BrdU)] into the left lateral cerebral ventricle, allowing the cells access to the VZ from which the cerebral cortex is derived (23). [At 12 to 13 weeks, VZ cells normally cease giving rise to the neurons in layers IV to VI and begin contributing to neurogenesis in layers II and III (27) (Fig. 1, schematic I).] Pregnancy was allowed to continue to the completion of most
cortical neurogenesis at ~16 to 17 weeks gestation (Fig. 1, schematic II), when the fetuses were delivered by Cesarean section and their brains were processed for histological analysis (28) (Fig. 2). Distribution of donor human NSCs (hNSCs) in the monkey brains was monitored by immunocytochemical staining for the BrdU marker (Figs. 1 and 3) (28). To provide further independent confirmation of the cells’ origin, we used, in parallel, antibodies against additional donor-specific markers, including the human-specific nuclear mitotic antigen (NuMA) as well as other species-specific tags (28). The phenotypes of these cells were characterized by immunocytochemistry (28) (Fig. 3).

Unilaterally injected hNSCs distributed themselves throughout both cerebral hemispheres symmetrically and at most levels of the neuraxis, settling in diverse widespread regions of the telencephalon, principally at the frontal and frontoparietal levels (Fig. 3). Although the individual hNSCs were clonally related, they appeared to segregate into two subpopulations (Fig. 3), as follows.

Cells in subpopulation I (red stars in Fig. 1, schematic II) were found to be clustered and segregated (Web note 2) (24) (Fig. 1, schematics I and II). One can discern experimentally the responses to local developmental cues simply by assaying the spatial segregation and patterns of differentiation of NSCs of a single clone in a given animal’s brain after a single transplantation procedure. (A summary of simian cortical development is provided in an expanded legend to Fig. 1 in Web note 2 (24)). Under transabdominal ultrasonic guidance, bonnet monkey fetuses at 12 to 13 weeks gestation received a single in utero injection of $2 \times 10^{5}$-cloned related undifferentiated NSCs [prelabeled with the nuclear marker 5-bromo-2$'$-deoxyuridine (BrdU)] into the left lateral cerebral ventricle, allowing the cells access to the VZ from which the cerebral cortex is derived (23). [At 12 to 13 weeks, VZ cells normally cease giving rise to the neurons in layers IV to VI and begin contributing to neurogenesis in layers II and III (27) (Fig. 1, schematic I).] Pregnancy was allowed to continue to the completion of most
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Cells in subpopulation I (red stars in Fig.
3) appeared to traverse great distances (~1.6 cm or ~1600 times a migrating cell body diameter) from the periventricular germinal zones along host radial glial processes (Fig. 1A) to terminate at developmentally and temporally appropriate cortical laminae and differentiated into several neuronal (Fig. 3, A to D) and glial (Fig. 3, E to G) cell types. Those hNSCs that migrated to the superficial neurogenic cortical layers II and III (Fig. 1A, schematic II) appropriately became neurons (Fig. 3, A, arrow), identified by dual immunoreactivity to antibodies to NeuN, calbindin, and neurofilament (Fig. 3, B to D, arrows), intermixed with the monkey’s own neurons (arrowheads). The majority of the hNSC-derived neurons were found in cortical layers II and III [which, at the time of transplant, profited from an intensive supply of newly formed neurons (27, 29)]. Those hNSC-derived cells that stopped and integrated within the deeper cortical layers IV to VI differentiated appropriately into glial cells (Fig. 1, B and C, schematic II), identified by immunoreactivity to glial fibrillary acidic protein (GFAP) (for astrocytes) or to 2′,3′-cyclic nucleotide 3′-phosphohydrolase (CNPase) (for oligodendrocytes) (Fig. 3, E to G). Glial cells of donor origin were also appropriately observed in the marginal zone (MZ, layer I) (Fig. 1, schematic II) and in subcortical regions. Some donor cells contributed also to the radial glial cell population.

Cells in subpopulation 2 (blue dots in Fig. 3) were small, undifferentiated BrdU-positive cells lacking neuronal processes and were dispersed throughout the SVZ as single cells or small clusters intermingled with the germinal cells of the host (Fig. 3, H and I). When double-stained for cell type–specific antigens, these cells expressed vimentin (an immature progenitor/stem cell marker) (Fig. 3I and inset) but were negative for all other markers of differentiation. The majority of such undifferentiated hNSC-derived cells remained within the SVZ [none in the ependyma (18)]. The SVZ has been implicated in postnatal and adult homeostatic mechanisms.

![Fig. 3. Segregation of the fates of hNSCs and their progeny into two subpopulations in the brains of developing Old World monkeys. Schematics (left) and photomicrographs (right) illustrating the distribution and properties of clonal hNSC-derived cells. (A) An hNSC-derived BrdU-positive cell (black nucleus, arrow) —likely a neuron according to its size, morphology, large nucleus, and location—is visualized (under Nomarski optics) intermingled with the monkey’s own similar neurons (arrowheads) in neocortical layers II and III. The neuronal identity of such donor-derived cells is confirmed by immunocytochemical analysis in (B) to (D). (B, C, and E to G) High-power photomicrographs of human donor-derived cells integrated into the monkey cortex double-stained with antibodies against BrdU and cell type–specific markers: (B) NeuN and (Bd) and (C) calbindin for neurons (arrows, donor-derived cells; arrowheads, host-derived cells); (E) CNPase for oligodendrogia (arrow, BrdU-positive black nucleus in CNPase-positive brown cell; arrowhead indicates long process emanating from the soma). (F and G) GFAP for astroglia [anti-body to Brd U revealed via fluorescein in (F); antibody to GFAP revealed via Texas Red in (G)]. The human origin of the cortical neurons is further independently confirmed in (D) where the human-specific nuclear marker NuMA (black nucleus) is colocalized in the same cell with neurofilament (NF) immunoreactivity (brown). Progeny from this same hNSC clone were also allocated to a second cellular pool—subpopulation 2 [blue dots in the schematic and pictured in (H) and (I) (arrows)]—that remained mainly confined to the SVZ and stained only for an immature neural marker [vimentin (brown) colocalized with BrdU (black nucleus)] better visualized in inset (arrows); arrowhead indicates host vimentin-positive cell.] Some members of subpopulation 2 were identified within the developing neocortex (blue dots) intermixed with differentiated cells. (F) and (G) use immunofluorescence; the other immunostains use a DAB-based color reaction. The photomicrographs were taken from different animals as representative of all animals. ve, lateral cerebral ventricle; arrow, BrdU-positive donor-derived cell; arrowhead, BrdU-negative, host-derived cell except in (F). Scale bars, 30 μm [(A) to (C)], 20 μm [(D) to (I)].]
(16, 17, 30, 31) and as an ongoing source of cortical neurons after overt cortical development has ceased (32–34). A small number of subpopulation 2 cells, however, were present within the striatum and cortex, intermixed with the differentiated cells (Fig. 3). These cells may provide a local resident pool for self-repair and plasticity and may represent the stem-like cells extracted by several investigators (10, 13, 15, 22, 35). This observation favors the interpretation that such reported cells are not simply the result of dedifferentiation of committed progenitors, an artifact of experimental manipulation, as has occasionally been speculated (10, 11).

Our data provide a plausible dynamic for how multiple, disparate stem cell populations are generated as part of a single strategy of NSC allocation. The clonal progeny of a given NSC segregate to yield some differentiated cells for organogenesis (e.g., subpopulation 1) and other cells (e.g., subpopulation 2) for deposition in secondary germinal zones (e.g., the SVZ) as a reservoir. The NSCs that have been isolated from adults are likely descendants of the same NSCs that contributed to embryonic and fetal CNS development and thus do not represent a unique pool. In this view, ongoing lifelong self-repair and plasticity are a fundamental developmental program set in place during early stages of brain organogenesis. Grafted hNSCs appear to become integrated into the morphogenetic program of the developing primate host brain (Figs. 1 and 3) (36). Although it was not technically possible in these monkeys to quantitatively rigorously the percentage of grafted cells that survived, the histological images show that a large number of donor-derived cells were present bilaterally in all recipients (37, 38). That hNSCs can migrate through the large expanse of the primate cerebral cortex, not merely through the much smaller rodent brain (5–7), suggests that migration may be a fundamental stem cell property limited only by available terrain (large or small). In rodents, NSCs have been shown to be well-suited for transplant-based approaches to gene therapy and/or cell replacement in diseases characterized by extensive or global abnormalities (39). Our results suggest that this approach may similarly be feasible in large primates and possibly humans.

References and Notes
23. See also Web note 1 (24). Cells from a stable, self-maintaining clone of hNSCs (clone H6), originally isolated from the VZ of a 15-week human fetal calvarium and generated, grown, and characterized as previously described (5), were preconditioned in culture (at a density of 5 × 10^5 cells/ml) with BrdU (10 μM, Sigma) for 48 hours before implantation in order to be subsequently identifiable in vivo by their darkly stained BrdU-immunopositive nuclei. (The cells were derived from a postnatal stage more immature than that of the monkey recipients.) These hNSCs were then resuspended in phosphate-buffered saline (PBS) at 1.7 × 10^7 cells/ml (as previously described [5]). This allowed 60 to 70% of the cells to become BrdU-labeling toxic; the BrdU remains detectable in vivo for at least 5 to 10 cell divisions, beyond the number known to occur for stem cells in vivo. Three pregnant bonnet monkeys were anesthetized with ketamine [0.5 mg/kg intramuscularly (IM)], ketamine [10 mg/kg IM], and atropine sulfate [0.4 mg/kg IM]. Ketamine [0.02 mg/kg IM] was administered every 15 min during the grafting procedure. The skin of the mother’s abdomen was shaved, the abdomen was sterilized with betadine/alcohol, and locally anesthetized with lidocaine. The fetal head was palpated through the abdominal wall and visualized by ultrasound. Under ultrasound guidance, an 18G spinal needle attached to a 10-ml syringe was inserted through the abdominal and uterine walls into the left lateral ventricle of the fetal brain. 1.53 to 2.21 × 10^6 hNSCs were injected in a volume of 0.9 to 1.3 ml of PBS through a 25-gauge needle inserted into the cerebral ventricle allows reliable and uniform access of the hNSCs to the VZ into which they integrate avidly (5, 14); K. Campbell et al., Neuro, 15, 1259 (1999); H. D. Lacorazza et al., Development 125, 424 (1996); C. A. Walsh, C. L. Cepko, Nature 362, 633 (1993); C. P. Austin, C. L. Cepko, Development 110, 713 (1990). The procedure took ~30 min/animal. Antibiotics were administered subcutaneously daily for 3 days. Two of the pregnant monkeys also received cyclosporin (Sandoz) [15 mg/kg intravenously (IV)]; cyclosporin treatment continued at the same daily dose per orum (PO) throughout the survival period. The third mother received no immunosuppression. After the procedure, animals were housed individually in cages and maintained on a regular feeding schedule. No pain, bruising, inflammation, or behavioral changes were observed and pregnancies continued normally. One month after engraftment, each of the pregnant monkeys was anesthetized and placed on an isoflurane inhalation anesthetic. The women were antisepsically prepared, and the fetus was removed by Cesarean section and killed by a pentobarbital overdose. All surgeries were successful and the mothers returned to their breeding groups after 3 weeks of recovery in individual housing. The removed fetuses were perfused with chilled heparinized saline (240 ml) followed by 4% paraformaldehyde (240 ml). Each brain was bulked and was stored in a freezer at −20ºC. Over 6 hours, the tissue was then cryoprotected in 30% sucrose in PBS and kept at 4ºC until further processing. For histological analysis, the brains were processed in the following order: cryosection, 40 µm; staining protocol (Vector) with Ni/Co enhancement. Sections were immunoglobulin (Vector). The subsequent color reaction was developed according to the ABC Vectastain protocol (Vector) with Ni/Co enhancement. Sections were dehydrated, mounted in Entellan (Merck), and examined with a Nikon microscope equipped with Nomarski optics. Parallel staining with multiple human-specific monoclonal antibodies to NeuN (Chemicon, 1:40 and Calbiochem, 1:400), human ribonuclease protein (Chemicon, 1:20), and the human EGF receptor (Upstate Biotech) was performed according to standard protocols also with the Vectastain kit. Double-immunostaining was carried out with antibodies to BrdU or NeuN and the following cell type-specific antibodies to NeuN (gift from R. Mullen), antibody to calbindin (Chemicon), antibody to NF (Roche), antibody to CNPase (Chemicon), and antibody to vimentin (Chemicon). The procedure was a differential

R E P O R T S
Allele-Specific Receptor-Ligand Interactions in *Brassica* Self-Incompatibility

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**Genetic self-incompatibility in *Brassica* is determined by alleles of the transmembrane serine-threonine kinase SRK, which functions in the stigma epidermis, and of the cysteine-rich peptide SCR, which functions in pollen. Using tagged versions of SRK and SCR as well as endogenous stigma and pollen proteins, we show that SCR binds the SRK ectodomain and that this binding is allele specific. Thus, SRK and SCR function as a receptor-ligand pair in the recognition of self pollen. Specificity in the self-incompatibility response derives from allele-specific formation of SRK-SCR complexes at the pollen-stigma interface.**

In self-incompatible *Brassica* plants, self-pollinations and crosses between genetically related individuals are nonproductive because self-related pollen grains are inhibited upon contact with the epidermal cells of the stigma, a structure that caps the female reproductive organ. Specificity in this self-incompatibility (SI) response is determined by haplotypes of the polymorphic S locus. The self-recognition molecules encoded by this locus include the single-pass transmembrane receptor–like serine-threonine kinase SRK, which functions in the stigma epidermis (1–3) and becomes phosphorylated upon self-pollination (4), and the cysteine-rich peptide SCR, which functions in pollen (5, 6). These two molecules are highly polymorphic, with allelic forms of SRK and SCR exhibiting 10 to 30% and >60% divergence, respectively (1, 3–8). Views of SRK as a ligand-activated receptor kinase and SCR as its ligand are consistent with the predicted molecular properties of these molecules and the rapidity of the SI response (1, 9). The SCR peptide is localized on the surface of pollen grains (10). During self-pollination, SCR is predicted to bind the receptor domain of its cognate SRK, thereby triggering an intracellular phosphorylation cascade that leads to inhibition of pollen hydration and germination. Specificity in the SI response is thought to result from haplotype-specific activation of SRK by SCR. Here, we describe experiments that demonstrate a physical and haplotype-specific interaction between SCR and the ectodomain of SRK.

To investigate the SRK-SCR interaction, we generated tagged versions of the two proteins. Recombinant eSRK6 consisting of the ectodomain of SRK6 (from the S6 haplotype) and carrying a COOH-terminal FLAG epitope tag, was expressed as a soluble secreted glycoprotein in *Nicotiana benthamiana* leaves using the potato virus X expression system (11). eSRK6 protein migrated as two molecular mass forms of ~63 and 70 kD on SDS–polyacrylamide gel electrophoresis (SDS-PAGE), which presumably reflect differential glycosylation of eSRK6–FLAG in *Nicotiana* leaves. SCR6 and SCR13 (the SCRs of the S6 and S13 haplotypes, respectively) were expressed in bacteria as secreted periplasmic proteins carrying a COOH-terminal myc-His6 tag (12). They exhibited expected masses of ~8 and 9 kD, respectively, but they migrated as doublets, possibly due to inefficient cleavage of the periplasmic signal peptide in bacteria.

Recombinant SCR-myc-His6 was shown to be biologically active in pollination bioassays (12). Pretreatment of stigmas with purified “self” SCR protein (i.e., S6S6 stigmas with SCR6-myc-His6 or S13S13 stigmas with SCR13-myc-His6) mixed with pollen-coat protein carrier (12) caused these stigmas to inhibit the germination of normally compatible S6S6 pollen on S6S6 but not on S13S13 stigmas.

![Fig. 1. Effect of purified recombinant SCR protein on cross-pollen tube development.](image)

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28. An evaluation of surviving grafted cells in all three animals revealed ~150 to 200 cells per 35-μm coronal section in the most densely engrafted areas (usually at level 1; see Fig. 2) and ~10 cells per section in the more sparsely engrafted regions. Whether this distribution reflects a genuine anterior-posterior developmental gradient or simply a stochastic distribution of cells or the product of transplantation technique cannot yet be determined. An estimate of ~107 hNSC-derived cells were detectable per monkey brain. hNSCs segregated in about a 3:7 ratio proportion between subpopulation 1 and subpopulation 2, respectively. Of subpopulation 1 cells in the cortex, 7 to 8% were neurons, 80% were astrocytes, and 12% were oligodendrocytes; the neurons were almost invariably in the appropriate laminae II and III.
29. Two of the three pregnant monkeys received cyclosporin, as described in (23). No histological evidence of inflammatory reaction or of cell rejection was seen in any of the three specimens. Experiments of longer duration and with grafting at more mature ages will be necessary to test whether rejection might ultimately have occurred; however, there is the suggestion that, at least at certain stages, an immunotoler-ance for NSCs might exist.
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