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Identification of c-Kit receptor as a regulator of adult neural stem cells in the mammalian eye: interactions with Notch signaling

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Abstract

Neural stem cells are present in specific regions of the adult central nervous system (CNS). Recent evidence suggests that the ciliary epithelium (CE), a CNS derivative, in the adult mammalian eye, harbors a quiescent population of neural stem cells. Here, we report the identification of c-Kit signaling as one of the regulators of adult CE neural stem cells in vitro. c-Kit receptors are expressed in proliferating adult CE neural stem cells and colocalized with neural progenitor markers. Perturbation of c-Kit signaling influences the self-renewal and differentiation of CE neural stem cells, thus demonstrating the role of c-Kit signaling in the maintenance of these cells. In addition, we observed an influence of c-Kit-mediated signaling on the expression of Notch1, another critical regulator of neural stem cells. Our observations suggest that, given the importance of preservation of a stem cell pool for generating different cell types at different times, multiple signaling pathways act in concert for the maintenance of neural stem cells.

Keywords: Neural stem cells; Ciliary epithelium; c-Kit receptor; Notch1

Introduction

The development of vertebrate eyes is intrinsically related to the formation of central nervous system (CNS). It begins with the emergence of a single eye field in the midline of the anterior neural plate (Chow and Lang, 2001). Later, the single eye field splits into two optic primordia, which subsequently evaginate as bilateral optic vesicles. The neurosensory retina, derived from the inner neuroepithelial layer of the optic vesicle, is regarded as the primary site of neurogenesis in the developing eye. The outer neuroepithelial layer of the optic cup differentiates into a single cell layer, called the retinal pigmented epithelium (RPE), which supports the structure and function of the retina. The neuroepithelial region between the developing retina and RPE contributes to a bilayer structure called the ciliary epithelium (CE) that regulates accommodation and aqueous humor production. Recent evidence suggests that the CE in the adult eye of warm-blooded animals, including mammals, harbors neural stem cells. This notion is supported by three reports that the CE in chick (Fischer and Reh, 2000), rat (Ahmad et al., 2000), and mouse (Tropepe et al., 2000) contains cells with proliferating potential. These cells represent a mitotic quiescent population of cells that proliferate in response to exogenous growth factors in vivo (Fischer and Reh, 2000; Zhao et al., 2003) or in vitro after their removal from the niche (Ahmad et al., 2000; Tropepe et al., 2000). Analyses of their proliferative and differentiation potential have shown that the CE stem cells display the features of neural stem cells; that is, they can self-renew and are multipotent in terms of their ability to generate both neurons and glia (Ahmad et al., 2000; Tropepe et al., 2000). In addition, they possess the ability to differentiate into cells that display some of the features of retinal cells (Das et al., 2002; Tropepe et al., 2000). Therefore, these cells appear to be evolutionarily analogous to retinal stem cells found in the peripheral margin of the adult retina, called the ciliary margin zone (CMZ), in lower vertebrates like fish and frogs, which generate retinal neurons throughout life (Ahmad, 2001; Perron and Harris, 2000; Raymond and Hitchcock,

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2000; Reh and Fischer, 2001). While these studies identified the existence of neural progenitors in adult mammalian eye and demonstrated their stem cell properties, they did not shed light on the mechanisms that regulate their maintenance and differentiation.

Here, we report the identification of c-Kit signaling as one of the regulators of CE neural stem cells in vitro. The c-Kit receptor, encoded by the Dominant White Spotting (W) locus, belongs to platelet-derived growth factor (PDGF) receptor family (Besmer et al., 1986; Qiu et al., 1988; Ullrich and Schlessinger, 1990). Evidence, based on a variety of approaches, has shown that the normal functioning of c-Kit and its ligand, stem cell factor (SCF), is essential for gametogenesis, hematopoiesis, and melanogenesis and therefore, for the maintenance of stem cells belonging to three different lineages (Dolci et al., 1991; Heinrich et al., 1993, 1995; Lowry et al., 1991; Migliaccio et al., 1993). c-Kit and SCF are expressed in both peripheral nervous system (PNS) and CNS neurons, and it is thought that signaling through c-Kit receptor might be involved in survival, development, and maturation of neurons (Carnahan et al., 1994; Hirata et al., 1993; Jin et al., 2002; Keshet et al., 1991; Matsui et al., 1991; McLaughlin, 2000; Zhang and Fedoroff, 1998). We demonstrate, for the first time, that c-Kit signaling plays an important role in self-renewal and differentiation of retrospectively identified and enriched CE neural stem cells. In addition, we observed interactions of c-Kit with signaling mediated by Notch1, another regulator of neural stem cells. Our observations suggest that, given the importance of preservation of a stem cell pool for generating different cell types at different times, multiple signaling mechanisms are recruited, to act in concert, for the maintenance of neural stem cells.

Experimental procedures

In vivo activation of CE neural stem cells

Postnatal day 14 (PN14) rats were anaesthetized. Growth factors (insulin: 1 μg/eye; FGF2: 20 ng/eye) and BrdU (1 μg/eye) were delivered into the right eye by intraocular injection using a glass micropipette attached to a Hamilton syringe. The left eye, injected with PBS and BrdU, was used as control. Animals were sacrificed 4 days after treatment, and eyes were enucleated for immunohistochemical analysis, as previously described (Zhao et al., 2002).

Isolation and culture of CE neural stem cells

Isolation and culture of stem cells from CE were carried out as previously described (Ahmad et al., 2000). Briefly, eyes were enucleated, and cornea, lens, and iris were removed. A strip of ocular tissue containing CE was obtained by cutting at the anterior edge of the pars plana. The pigmented portion of CE was separated from the nonpigmented portion and was incubated in HBSS (pH 7.0) containing collagenase (Sigma; 78 U/ml) and hyaluronidase (Sigma; 38 U/ml) for 35 min at 37°C. The pigmented portion of CE was dissociated by trypsinization for another 30–35 min. The dissociated cells were cultured in DMEM/F12, 1 × N2 supplement (GIBCO), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin) supplemented with FGF2 (10 ng/ml; Collaborative Research), and EGF (20 ng/ml; Collaborative Research) at a density of 5×10^4 (high density) to 4×10^3 (low density) cells/cm^2 for 5–7 days to generate neurospheres. To ascertain the clonal generation of neurospheres, cell dissociates from CE of the green [constitutively expressing the green fluorescent protein (GFP)] and wild-type mice were cocultured at a high density as described above. For LDA analysis, cell dissociates were diluted to give an initial concentration of 7500 cells/ml from which serial dilutions in 200 μl aliquots were plated in individual wells of 96-well plate. Culture was carried out for 7 days, after which the fraction of wells not containing neurospheres for each cell-plating density was calculated. The negative logarithm of fraction of negative wells was plotted against the number of cells plated per well to provide a straight line in a semi-logarithm plot. The zero term of Poisson equation ($F_0 = e^{-x}$, where F_0 is the fraction of well without neurospheres and x is the mean number of cell per well) predicts that when 37% of test wells are negative, there is an average of one stem cell per well. To examine the differentiation potential of CE neural stem cells, neurospheres were exposed to 10 μM BrdU (Sigma) for the final 48 h to tag the dividing cells and plated on poly-D-lysine (500 μg/ml) and laminin (5 μg/ml)-coated 12-mm glass coverslips. To promote differentiation, mitogens were substituted with brain-derived neurotrophic factor (BDNF; 1 ng/ml), retinoic acid (RA; 1 μM), and 0.5% FBS, and culture was continued for 5–7 days. Cells were fixed using cold 4% paraformaldehyde for immunocytochemical analysis.

Microarray analysis

Neurospheres were generated as described above, and their stem cell properties were determined before collecting for the microarray analysis. The majority of cells in neurospheres were dividing and expressed neural stem cell marker, nestin (see Figs. 2A and B). Neurospheres in proliferating (presence of mitogens) and differentiating (absence of mitogens and presence of serum) conditions were frozen in liquid nitrogen. RNA from the neurospheres was isolated using RNeasy mini Kit (Qiagen). First strand synthesis was done using Superscript II reverse transcriptase (Gibco) and T7(dT)24 primer at 42°C for 1 h in a total volume of 20 μl. T4gp32 was added to enhance the first strand synthesis (Nycz et al., 1998; Rapley, 1994). This was followed by second strand synthesis using DNA polymerase I at 16°C for 2 h, followed by incubation with T4 DNA polymerase (10 U) at 16°C for 5 min. The reaction was

stopped by adding 0.5 M EDTA and cleaned up with phenol–chloroform–isoamyl alcohol method. The final pellet was resuspended in RNA/DNA free water. In vitro transcription was done with biotin-labeled nucleotides and T7 RNA polymerase at 37°C for 5 h, followed by cleaning up using RNeasy column. Twenty micrograms of each cRNA was fragmented by adding the appropriate amount of fragmentation buffer and placing at 95°C heat block for 35 min. To ensure complete fragmentation, 1 μg of cRNA was run on a 2% agarose gel. Fifteen micrograms of each sample was mixed with other ingredients (listed in the Affymetrix protocol) to make a hybridization solution of 300 μl. Hybridization to microarrays was carried out by Research Genetics, Inc., using rat genome U34A (RGU34A) microarray chips containing sequences corresponding to 8323 known rat neurobiology genes and 417 ESTs. The hybridized arrays were scanned using Agilent GeneArray scanner, and data were analyzed using Affymetrix MicroArray Suite Software. The raw data were initially normalized against housekeeping genes represented in the array. The analysis of hybridization data, using the Affymetrix MicroArray Suite Software, provided the average difference (AD) values for the expression 8740 genes, for two different conditions (i.e., CE neurospheres in proliferating and differentiating conditions). Thus, for every gene, two AD values were obtained for each condition. The AD values were subjected box plot analysis to normalize any difference between samples. The box plots of all possible values, plotted on log2 scale, were similar, therefore no further normalization was applied. The relative expression of transcripts in four groups was measured using ABS call and DIFF call. The ABS call, based on *P* values, obtained using Wilcoxon signed ranked test, determined whether a particular transcript corresponding to a particular gene is present or absent. Genes thus sorted were ordered by fold change values, that is, the expression ratio, corresponding to the two groups. The microarray results were corroborated by RT–PCR and immunocytochemical analyses.

Perturbation of c-Kit and Notch signaling

Cell dissociates from CE were cultured for the generation of neuropspheres as described above. CE cells were exposed to varying concentrations of SCF (Peprotech; 10–100 ng/ml) for 5–6 days (Dahlen et al., 2001; Nakahara et al., 2002) and to BrdU (10 μM) in the final 48 h, followed by counting the number of neurospheres generated and number of BrdU-positive cells in the neurospheres. Controls included CE neural cultured in the presence of EGF and FGF2 as described above. Attenuation of SCF-induced c-Kit signaling was achieved by adding varying concentrations of human c-Kit antibody that cross-reacts with rat and mouse c-Kit (Santa Cruz; 0.1–1 μg/ml) in SCF containing culture to block c-Kit receptor (Dahlen et al., 2001; Yan et al., 2000). Similar experiments were carried out on cell dissociates obtained from primary neurospheres to evaluate the effect of perturbation of c-Kit signaling on

Table 1
List of primers and their respective sequences used for RT–PCR analysis

Gene	Primer sequence	Annealing temperature (°C)	Product size (bp)	Gene bank accession number
β-actin	Forward: 5′ GTGGGGCGCCCCAGGCACCA3′ Reverse: 5′ CTCCTTAATGTCACGCACGATTTC3′	50	543	XM037235
Nestin	Forward: 5′ TGGAGCAGGAGAAGCAAGGTCTAC3′ Reverse: 5′ TCAAGGGTATTAGGCAAGGGGG3′	56	295	NM012987
β-tubulin III	Forward: 5′ TGCGTGTGTACAGGTGAATGC3′ Reverse: 5′ AGGCTGCATAGTCATTTCCAAG3′	52	250	NM139254
GFAP	Forward: 5′ ATCTGGAGAGGAAGGTTGAGTCG3′ Reverse: 5′ TGGCGGCGATAGTCATTAGA3′	58	310	NM017009
Notch1	Forward: 5′ TCTGGACAAGATTGATGGCTACG3′ Reverse: 5′ CGTTGACACAAGGGTTGGACTC3′	56	329	NM008714
Hes1	Forward: 5′ GCTTTCCTCATCCCCAATG3′ Reverse: 5′ CGTATTTAGTGTCCGTCAGAAGAG3′	56	224	NM024360
Hes5	Forward: 5′ TGGAGATGCTCAGTCCCAAG3′ Reverse: 5′ GCTTTGCTGTGCTTCAGGTAG3′	56	199	NM024383
MRP322	Forward: 5′ GGAAGCACTCCCTTAGCAGATTC3′ Reverse: 5′ CTTGACCCACCTCAGTCTTTGC3′	58	280	U23146
BDNF	Forward: 5′ GCTCAGCAGTCAAGTGCCTTTG3′ Reverse: 5′ TGCCTTTTGTCTATGCCCCTG3′	58	324	S71196
Semaphorin III	Forward: 5′ GGAAGAGTCCTTATGACCCCAAAC3′ Reverse: 5′ TGGCACAGAGCAAATCAGGC3′	56	389	X95286
c-Kit RTK	Forward: 5′ AAGGCACAGAAGGAGGCACTTAC3′ Reverse: 5′ TCCAAATGGTGACACAGACGC3′	58	263	D12524
Musashi-1	Forward: 5′ TGAAAGAGTGTCTGGTGATGCG3′ Reverse: 5′ GCCTGTTGGTGGTTTTGTCG3′	52	309	NM148890
Fut9	Forward: 5′ GTTTTACCTGGCGTTTGAGAACTC3′ Reverse: 5′ TGGAAGTAGCGGCGATAGACAG3′	56	237	NM053465

the generation of secondary neurospheres. To determine the intracellular components of c-Kit signaling involved in the regulation of CE neural stem cells, cells preexposed to SCF were cultured in varying concentrations of rapamycin (Sigma; 5–25 nM) (Otto et al., 2001). The number of neurospheres generated and proliferating cells (BrdU-positive) were counted. An aliquot of neurospheres in each of the experiment was analyzed for BrdU and nestin immunoreactivities to ascertain their progenitor nature. To rule out the toxicity of rapamycin, cell viability was assayed at different concentration of drug using Viability/Cytotoxicity Kit as per vender's instructions (Molecular Probes). Cell viability was also assayed for other experimental conditions described above. To investigate the effect of c-Kit signaling on the differentiation of CE neural stem cells, neurospheres were cultured under differentiating conditions as described above in the presence of SCF and SCF+c-Kit antibody for 5 days and subjected to immunocytochemical analysis. To attenuate Notch signaling in CE, neurospheres were cultured in the presence of 5 μM of *N*-[*N*-(3,5-difluorophenacetyl-L-alanyl)]-*S*-phenylglycine t-butyl ester (DAPT), a γ-secretase inhibitor (Calbiochem, CA) as previously described (Dovey et al., 2001; James et al., 2004). The specificity of DAPT influence on Notch signaling was confirmed by analyzing the relative levels of transcripts corresponding to the downstream effectors of Notch, Hes1.

Immunocytochemistry and cell quantification

Immunocytochemical analysis was carried out for the detection of BrdU and cell specific markers as previously described (Zhao et al., 2002). Briefly, paraformaldehyde fixed cells were incubated in PBS containing 5% NGS and 0%, 0.2%, or 0.4% Triton X100 followed by an overnight incubation in antibodies against Notch1, Nestin, β-tubulin III, GFAP, c-Kit, Musashi-1, SSEA-1, and BrdU at 4°C. Cells were examined for epifluorescence following incubation in IgG conjugated to Cy3/FITC. Images were captured using cooled CCD-camera (Princeton Instruments) and Openlab software (Improvision). To determine the percentage of specific cell types in a particular condition, total number of cells and the number of cells with specific immunoreactivity were counted in 10–12 randomly selected fields in two to three different coverslips. Each experiment was repeated at least three times. Values are expressed as

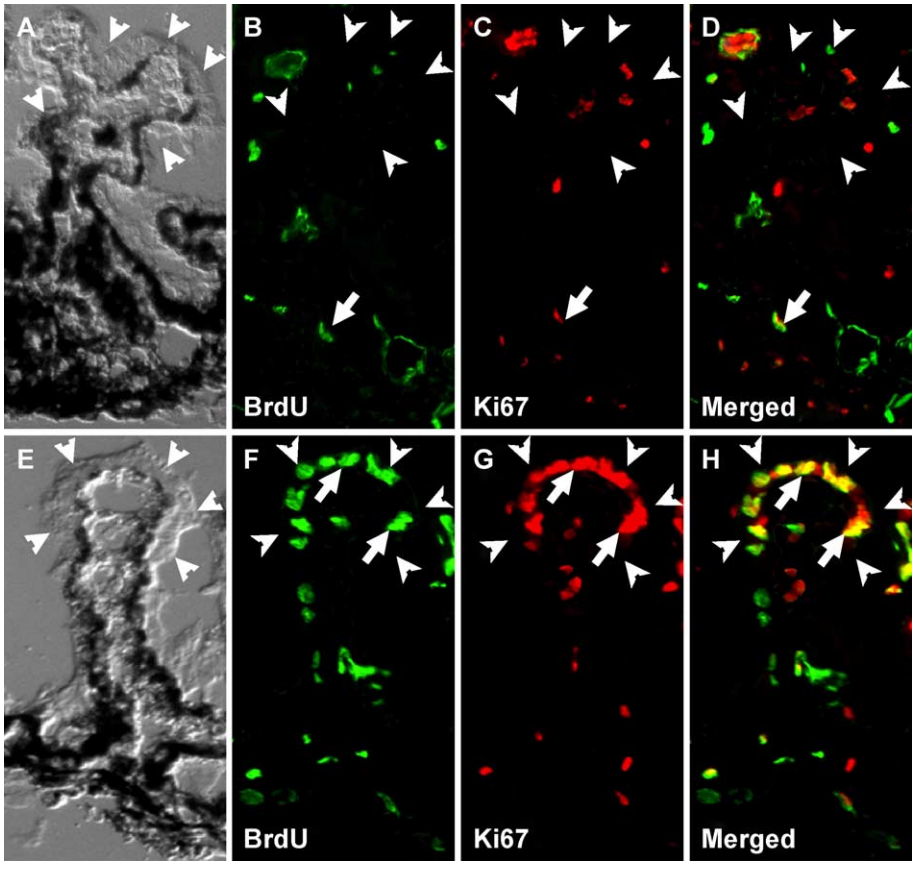

Fig. 1. The CE harbors quiescent population of cells, which proliferate in response to mitogens in vivo. PN14 rats were given intraocular injections of a mixture of FGF2, insulin, and BrdU in the right eyes and were sacrificed after 4 days. Left eyes received injection of PBS and BrdU and served as controls. Immunohistochemical analysis carried out on 10-μm cryostat sections of eyes revealed more BrdU- and Ki67-positive cells (arrows) in the CE (arrowheads) of growth factor-treated group (E–H) than in the controls (A–D).

mean ± SEM. Statistical analysis was done using Student *t* test to determine the significance of the differences between different conditions.

RT–PCR analysis

Isolation of total RNA from undifferentiated and differentiated CE neural stem cells progenitors and cDNA synthesis was carried out as previously described (Bhattacharya et al., 2003). Approximately 4 μg of RNA was transcribed into cDNA in total volume of 50 μl. Specific transcripts were amplified with gene specific forward and reverse primers using a step cycle program for 25 cycles on a Robocycler (Stratagene). Products were visualized by ethidium bromide staining after electrophoresis on 2% agarose gel. Gene specific primers used for RT–PCR analyses are given in Table 1.

Results

Retrospective identification and enrichment of CE neural stem cells

To identify CE neural stem cells in vivo, the CE of rats were exposed to exogenous FGF2 and insulin by intraocular injection. More BrdU-positive cells, coexpressing cell proliferation marker Ki67, were detected as compared to those in control eyes suggesting that CE harbors quiescent population of cells that proliferate in response to exogenous stimuli (Figs. 1A–H). To identify genes involved in the maintenance of CE neural stem cells, we carried out transcription profiling of these cells under proliferating and differentiating conditions. Since a reliable method for prospective identification of these cells is not currently available, CE neural stem cells were enriched retrospectively and their stem cell properties confirmed in vitro before subjecting them for transcription profiling. Cell dissociates from CE were cultured in serum-free medium containing EGF and FGF2. In this condition, the differentiated cells die, and only a minor subset of cells with proliferative potential survive. These cells generate neurospheres that

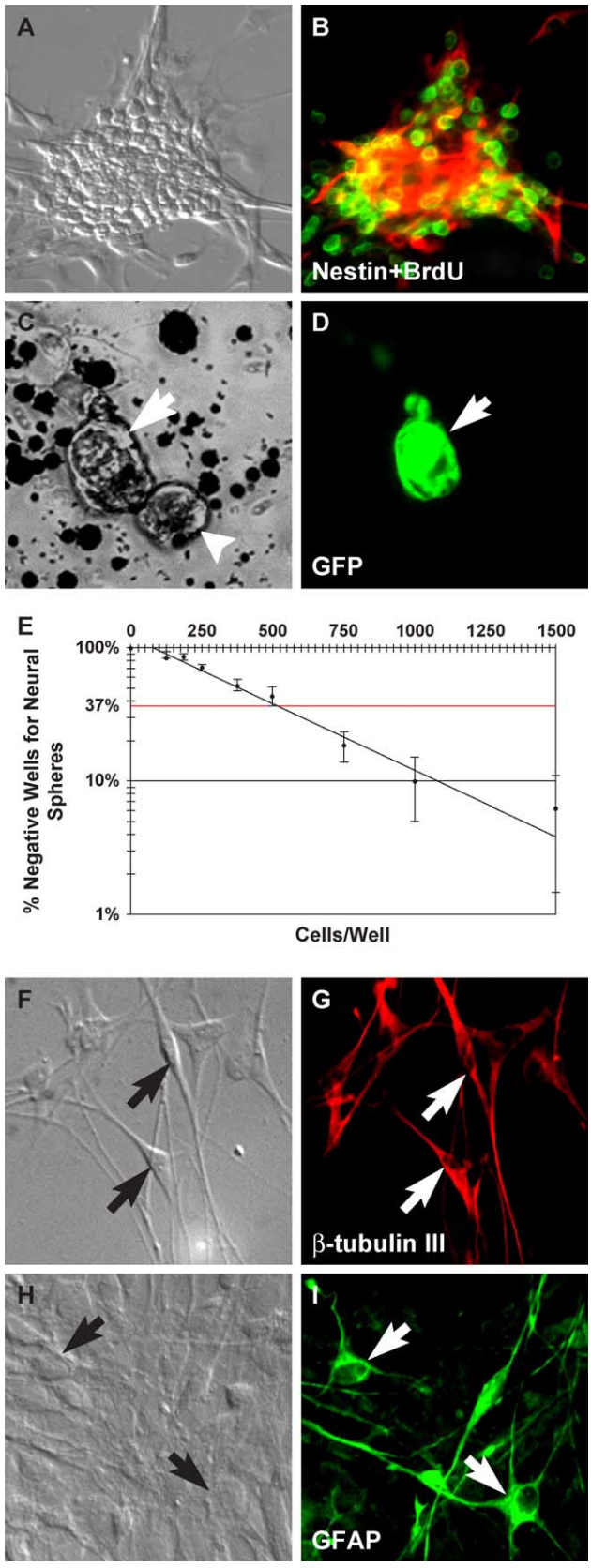

Fig. 2. CE neural stem cells are proliferative and multipotential in vitro. CE cells were isolated and cultured in the presence of EGF and FGF2 to enrich neural stem cells. Neurospheres, thus generated, spread out when cultured on glass poly-D-lysine+laminin-coated coverslips. Immunocytochemical analysis revealed that cells in neurospheres incorporated BrdU (arrows) and expressed neuroectodermal stem cell marker, nestin (A, B). To ascertain the clonal generation of the neurospheres, cell dissociates from CE of green mice and nongreen mice were cultured together in the presence of mitogens. The neurospheres generated consisted of either green or nongreen cells suggesting that their generation is clonal (C, D). LDA analysis was carried out to reconfirm clonal generation of neurospheres and to find the frequency of neural stem cells in the CE (E). These cells when cultured under differentiating conditions expressed a neuronal marker, β-tubulin III (F, G; arrows) and a glial marker, GFAP (H, I; arrows), suggesting that they are multipotent. Magnification ×200.

predominantly consists of dividing cells that express neuroectodermal stem cell markers, nestin (Figs. 2A and B). The clonal generation of neurospheres was ascertained by two different approaches. First, cell dissociates from CE of green mice (constitutively expressing GFP) and wild-type mice were cocultured in the presence of mitogens. Neurospheres were generated that consisted of either green cells or nongreen cells confirming that their generation is clonal and not due to cell aggregation (Figs. 2C and D). Second, we carried out the LDA analysis that demonstrated a single limiting cell type for the generation of neurospheres whose frequency approximates to 0.2% (i.e., 1 in 500 CE neural stem cells) (Fig. 2E). To investigate the differentiation potential of these cells, neurospheres were generated as described above. To induce neural differentiation, mitogens were removed from the culture medium and substituted with 0.5% FBS. In this condition, cells in the neurospheres quit mitosis and differentiated along neuronal and glial lineage demonstrating that like neural stem cells elsewhere in the CNS (Temple, 2002), they are multipotential, capable of generating both neurons and glia (Figs. 2F–I). Cell dissociates of primary neurospheres, when cultured in the presence of mitogens, generated secondary neurospheres consisting of multipotential cells, thus confirming that a subset of CE neural stem cells possesses self-renewal property, the cardinal feature of stem cells (see Figs. 8A–F).

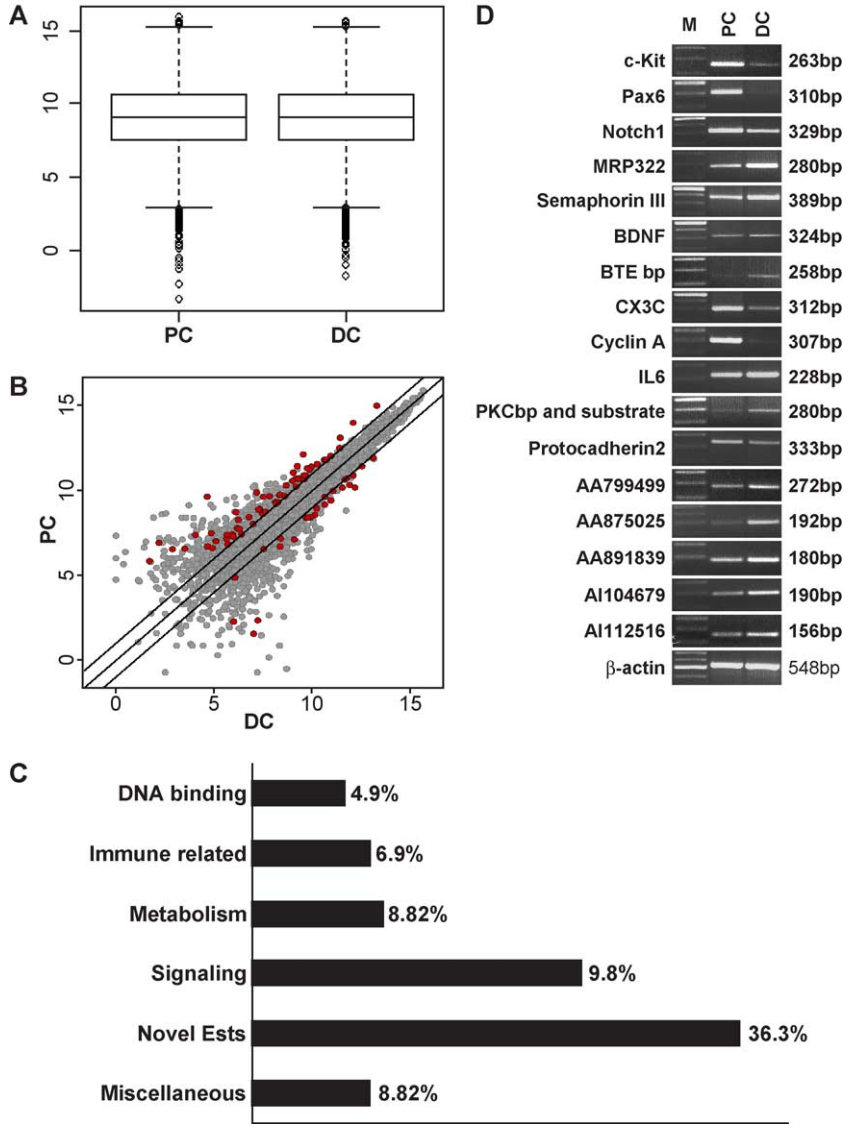

Fig. 3. Identification of *c-Kit* in CE neural stem cells. CE-derived neurospheres in proliferating and differentiating conditions were subjected to microarray analysis to identify genes involved in the regulation of CE neural stem cells. Box plot analysis for the expression of 8740 genes in proliferating and differentiating condition displays the extent of intersample variations (A). Genes identified with significant fold changes between the two conditions were subjected to scatter plot analysis (B) to identify outlying genes (red spots) whose expression differ between neurospheres in proliferating and differentiating conditions. These genes are classified into functional categories (C), and the differential patterns of expression of some of these genes, as determined by microarray analysis, are confirmed by RT–PCR analysis (D). PC indicates proliferating condition; DC, differentiating condition; M, marker lane.

Table 2
Differentially expressed genes and their functional classifications

Accession No.	Fold Change	Descriptions	
AF035632	-2	Syntaxin 12	Cytoskeleton
U09401	-2.1	Rattus norvegicus clone p12.3 tenascin mRNA	
U15550	-2.4	Tenascin-C	
Y09945	-3	Putative integral membrane transport protein (UST1r)	
U09022	2.4	15 kDa perforatorial protein PERF 15	
U23146	-3	Mitogenic regulation SSeCKS (322)	DNA binding/ Transcription
AB011369	-2.5	RBCK2	
D12769	-3.1	BTE binding protein	
D37934	-2.3	5E5 antigen	
D84418	2.6	Chromosomal protein HMG2	
S74393	4.5	Pax-6	
U17565	2	Intestinal DNA replication protein	
M15562	-2.7	MHC class II RT1.u-D-alpha chain	Immune related
M18853	-3.1	T-cell receptor active alpha-chain C-region	
S79711	-4.8	CD3 gamma-chain	
U45965	-8.8	Macrophage inflammatory protein-2 precursor	
U65217	-5.0	MHC class II antigen RT1.B beta chain mRNA	
X13044	-4.2	MHC-associated invariant chain gamma	
X14319	-15.9	T-cell receptor beta chain	
X63434	-3.1	urokinase-type plasminogen activator	
Y08358	-5.4	Eotaxin	
AF072411	2.5	Fatty acid translocase/CD36	Metabolism
AJ011116	4.9	Endothelial nitric oxide synthase	
D29646	-3	ADP-ribosyl cyclase_/ cyclic ADP-ribose hydrolase (CD38)	
D45862	-3.2	ob mRNA	
D63886	2	MT3-MMP-del	
J04791	-2	Ornithine decarboxylase (ODC)	
L03294	-2	Lipoprotein lipase	
L22339	-2.9	N-hydroxy-2-acetylaminofluorene (ST1C1)	
U24282	3	Type III iodothyronine deiodinase (diolII)	
U39943	-3	Cytochrome P450 monooxygenase (CYP2J3)	
AF015304	2.4	Equilbrative nitrobenzylthioinosine-sensitive nucleoside transporter	Miscellaneous
U66322	25.6	Dithiolethione-inducible gene-1 (DIG-1)	
U83119	-2	L1 retrotransposon ORF2	
X07266	3.4	Gene 33 polypeptide	
X59864	3.7	ASM15 gene	
X60767	2.7	cdc2 promoter region	
Y00497	-2.1	Manganese-containing superoxide dismutase (MnSoD)	
AFFX-CreX-5	-3	P1 cre recombinase protein	
AFFX-BioDn-3	-2.5	bioD gene dethiobiotin synthetase	
AB004278	2.8	Protocadherin 2	Signaling
AF012891	-3	Frizzled related protein frpAP	
AF019624	-6	Myostatin	
AF030088	-2.6	Activity and neurotransmitter-induced early gene 3 (ania-3)	
AF030358	2.2	Chemokine CX3C	
AF034899	-2.2	Olfactory receptor-like protein (SCR D-9)	
AF039583	-10.7	Decay accelerating factor GPI-form precursor (DAF)	
AF053988	-3.4	Tissue-type vomeronasal neurons putative pheromone receptor V2R1-1	
D12524	3.2	c-kit receptor tyrosine kinase	
D16840	2.3	Angiotensin II type 2 receptor	
D28560	2.1	D28560 RATNPHIII Rat mRNA for phosphodiesterase I	
D43778	2.2	Angiotensin II type 2 receptor	
L25633	-2.6	Neuroendocrine-specific protein (RESP18)	
M26744	-2	Interleukin 6 (IL6)	
M55049	-7.5	Interleukin-2 receptor alpha chain (CD25)	
M64092	-3.3	cAMP-dependent protein kinase inhibitor protein	
M81855	-3.5	Mdr mRNA	
M85301	-2.6	Sodium-hydrogen exchange protein-isoform 4 (NHE-4)	
S71196	-4.1	S1 brain-derived neurotrophic factor	
U01908	3	Type-2 angiotensin II receptor	
U22296	-3.8	Casein kinase 1 gamma 1 isoform mRNA	
U41453	-2.4	PKC binding protein and substrate	
U67138	-4.7	PSD-95/SAP90-associated protein-2	
U78517	-2.2	cAMP-regulated guanine nucleotide exchange factor II (cAMP-GEFII)	
X95286	-2.6	Semaphorin III/collapsin-1	

(*continued on next page*)

Table 2 (*continued*)

Accession No.	Fold Change	Descriptions	
AA799449	-2.8	99% homologous to R.norvegicus similar to nucleosome assembly protein	Novel Ests
AA799499	-2.2	79.65% homologous to R.norvegicus similar to NADH dehydrogenase (ubiquinone) 1 beta	
AA799567	-8.5	49.05% homologous to M.musculus cDNA sequence BC011487	
AA819187	-3.2	95.86% homologous to R.norvegicus CD38 mRNA for ADP-ribosyl cyclase	
AA850781	-2.1	98.55% homologous to R.norvegicus similar to peptidylprolyl isomerase D	
AA859612	4.5	37.58% homologous to R.norvegicus strain BN/SsNHsdMCW mitochondrion	
AA859837	-2.3	96.62% homologous to R.norvegicus guanine deaminase (Gda)	
AA866489	-2.1	22.59% homologous to M.musculus G protein-coupled receptor 155	
AA875025	-15.0	87.04% homologous to M.musculus cellular retinoic acid binding protein I	
AA875033	2.4	93.17% homologous to R.norvegicus embryonic vascular EGF repeat-containing protein	
AA875509	-2.2	100% homologous to R.norvegicus similar to mdm2 gene product	
AA875609	-3.1	84.62% homologous to R.norvegicus similar to SNF2 alpha	
AA891839	-2.9	25% homologous to M.musculus mitochondrial ribosomal protein L50	
AA892986	-2.8	20.34% homologous to M.musculus RIKEN cDNA 9230117N10 gene	
AA894148	-2.2	56.15% homologous to Rat apolipoprotein A-IV gene	
AA899106	-2	68.15% homologous to M.musculus cyclin D2	
AA925248	2.5	66.8% homologous to R.norvegicus mRNA for sodium channel	
AA945054	-4.3	87.19% homologous to R.norvegicus cytochrome b5 (Cyb5)	
AA946368	4	50.46% homologous to R.norvegicus cd36 antigen (Cd36)	
AA946503	2.2	99.39% homologous to Rat mRNA for alpha-2u globulin-related protein	
AI070295	-3.4	52.84% homologous to M.musculus growth arrest and DNA-damage-inducible 45 alpha	
AI102868	-2.7	43.97% homologous to R.norvegicus phosphoserine aminotransferase mRNA	
AI103396	-3.3	69.98% homologous to R.norvegicus strain BN/SsNHsdMCW mitochondrion	
AI104077	-7.3	91.38% homologous to M.musculus clone:D830015C09	
AI104679	-2.2	44.89% homologous to M.musculus clone:2310016K22	
AI112516	-2.2	37.84% homologous to R.norvegicus zinc finger protein 36, C3H type-like 1	
AI171355	-3.8	95.72% homologous to R.norvegicus mitochondrial gene for cytochrome b	
AI180108	-2.2	93.25% homologous to R.norvegicus similar to Wbscr1	
AI180410	-2	95.65% homologous to R.norvegicus prolactin-like protein C 1	
AI233219	-2.2	99.58% homologous to R.norvegicus endothelial cell-specific molecule 1 (Esm1)	
H31479	2	93.87% homologous to M.musculus tumor suprressor in lung cancer 1 mRNA	
H33093	-2	34.57% homologous to Mouse DNA sequence from clone RP23-10K12 on chromosome 15	
AI639162	-2	38.99% homologous to M.musculus clone:1110046O21	
AI639376	-2	52.58% homologous to M.musculus golgi autoantigen, golgin subfamily a	
AI639338	-3	27.01% homologous to M.musculus src-like adaptor	
AI639119	-2.5	85.05% homologous to M.musculus clone:5430439G14	
AI639173	-4.9	7.66% homologous to H.sapiens chromosome 8, clone RP11-380B11	

c-Kit is predominantly expressed in CE neural stem cells

To identify genes involved in the maintenance of CE neural stem cells, we carried out microarray analysis of transcripts expressed in CE neurospheres under proliferating and differentiating conditions. Probes corresponding to transcripts from the two groups were hybridized with oligonucleotides on rat genome U34A Affymetrix arrays. The analysis of hybridization data, using the Affymetrix MicroArray Suite Software, provided the average difference (AD) values for the expression 8740 genes. The AD values were subjected to box plot analysis to normalize any difference between samples. The box plots of all possible values, plotted on log2 scale, were similar, therefore no further normalization was applied (Fig. 3A). The relative expression of transcripts in two groups was measured using ABS call and DIFF call. Transcripts thus sorted were ordered by fold change values, that is, the expression ratios of the two groups. Fig. 3B shows a scatter plot analysis identifying candidate transcripts that passed the selection

process described above and have a fold change above 2 or below 1/2. Using the above criterion, 102 genes were selected and assigned to different functional groups (Fig. 3C and Table 2). Results of transcription profiling of some of these genes were corroborated by RT–PCR analysis (Fig. 3D and Table 2). In the present study, *c-Kit*, was selected for a more detailed evaluation of function because of its established role in the regulation of stem cells belonging to different lineages. Transcripts corresponding to *c-Kit* were detected in the pigmented portion of CE where stem cells are predominantly localized and not in nonpigmented portion of CE, suggesting that c-Kit is associated with CE neural stem cells in vivo (Fig. 4E, inset). Immunocytochemical detection of c-Kit showed that the proportion of cells expressing c-Kit immunoreactivity decreased significantly in neurospheres under differentiating conditions as compared to those in proliferating conditions (4.29% ± 0.57% vs. 73.04% ± 2.33%, $P < 0.001$), further corroborating the results of the microarray analysis (Figs. 4A–E). Analysis of progenitor properties of c-Kit-positive cells showed that the majority of them were BrdU-positive (85.83% ± 2.46%) and expressed progenitor markers nestin (44.27% ± 1.57%) and Notch1 (60.41% ± 2.41%) suggesting their neural progenitor nature (Figs. 5A–M).

Perturbation of c-Kit signaling influences proliferation and neurosphere formation

The expression of c-Kit in the majority of proliferating CE neural stem cells and the fact that signaling through this receptor maintains a variety of stem cells suggested that it might play a similar role in these cells. This notion was tested by perturbing c-Kit signaling and evaluating the consequences on cell proliferation and generation of neurospheres (Fig. 6). c-Kit was activated by its ligand, SCF. The addition of SCF to cultures of CE neural stem cells led to a dose-dependent increase in the generation of neurospheres (number of neurospheres per well) and cell proliferation (percentage of cells that were BrdU-positive) as compared to control cells growing in the presence of FGF2 and EGF only (Figs. 6A and B). To determine if effects of SCF were mediated through c-Kit, SCF treatment was carried out in the presence of c-Kit antibody that blocks the ligand–receptor interactions (De Miguel et al., 2002; Feng et al., 2000). Effects of SCF on cell proliferation and neurosphere generation were abrogated in the presence of the neutralizing antibody (Figs. 6D and E). During these experiments, cell viability remained unchanged (Figs. 6C and F). Next, we analyzed the intracellular aspects of c-Kit signaling in the regulation of CE neural stem cells. Several lines of evidence have shown that the intracellular aspects of c-Kit signaling, pertaining to the maintenance of stem cells, are mediated by rapamycin-sensitive FRAP/mTOR pathway (Blume-Jensen et al., 1998; Feng et al., 2000). We observed a dose-dependent decrease in SCF-induced cell proliferation

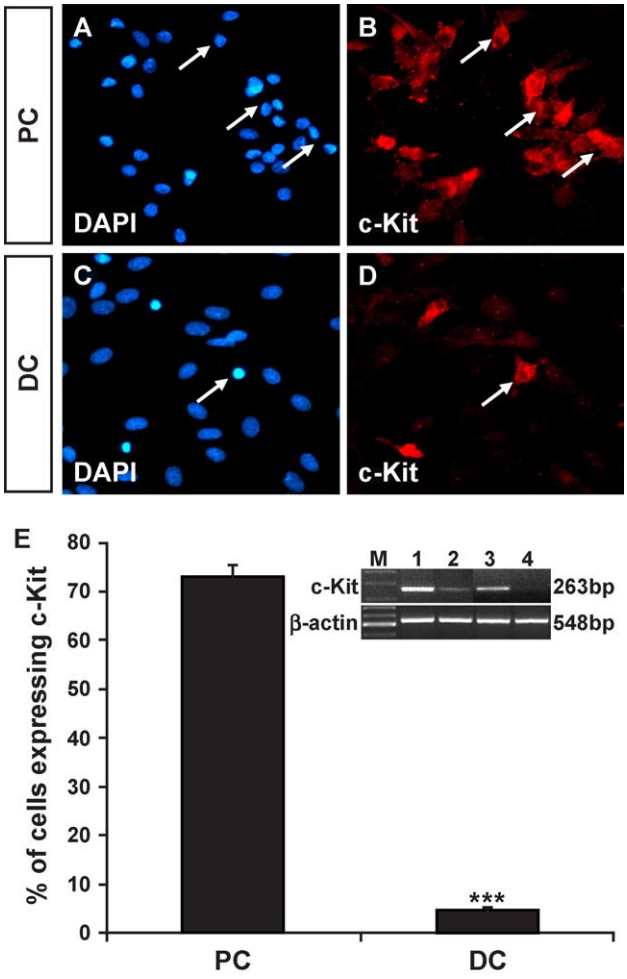

Fig. 4. c-Kit is expressed predominantly in proliferating CE neural stem cells. Immunocytochemical analysis of c-Kit was carried out on cells in neurospheres in proliferating and differentiating conditions to further corroborate microarray results. The proportion of CE neural stem cells expressing c-Kit (arrows) decreased significantly (*$P < 0.001$) in differentiating conditions (C, D, and E) as compared to those in proliferating conditions (A, B, and E). RT–PCR analysis of transcripts corresponding to *c-Kit* shows their expression in CE neural stem cells in proliferating and differentiating conditions and in pigmented portion of ciliary epithelium, in vivo (E, inset). PC indicates proliferating condition; DC, differentiating condition; PE, pigmented epithelium; NPE, nonpigmented epithelium; M, marker lane. Data are expressed as mean ± SEM from triplicate culture of three different experiments. ***$P < 0.001$. Magnification ×200.

and generation of neurospheres in the presence of rapamycin, suggesting that c-Kit-induced activation of FRAP/mTOR plays a significant role in regulating CE neural stem cell proliferation (Figs. 6G and H). Cell viability remained unchanged during these experiments (Fig. 6I). Next, to know if endogenous SCF played a role in the regulation of CE neural stem cells, the generation of neurospheres was examined in the presence of c-Kit antibody only. There was a dose-dependent decrease in the proportion of neurospheres generated in the presence of c-Kit antibody as compared to control [908.48 ± 48.19 vs. 735.69 ± 34.42 (0.1 μg/ml)/605.04 ± 37.1 (0.5 μg/

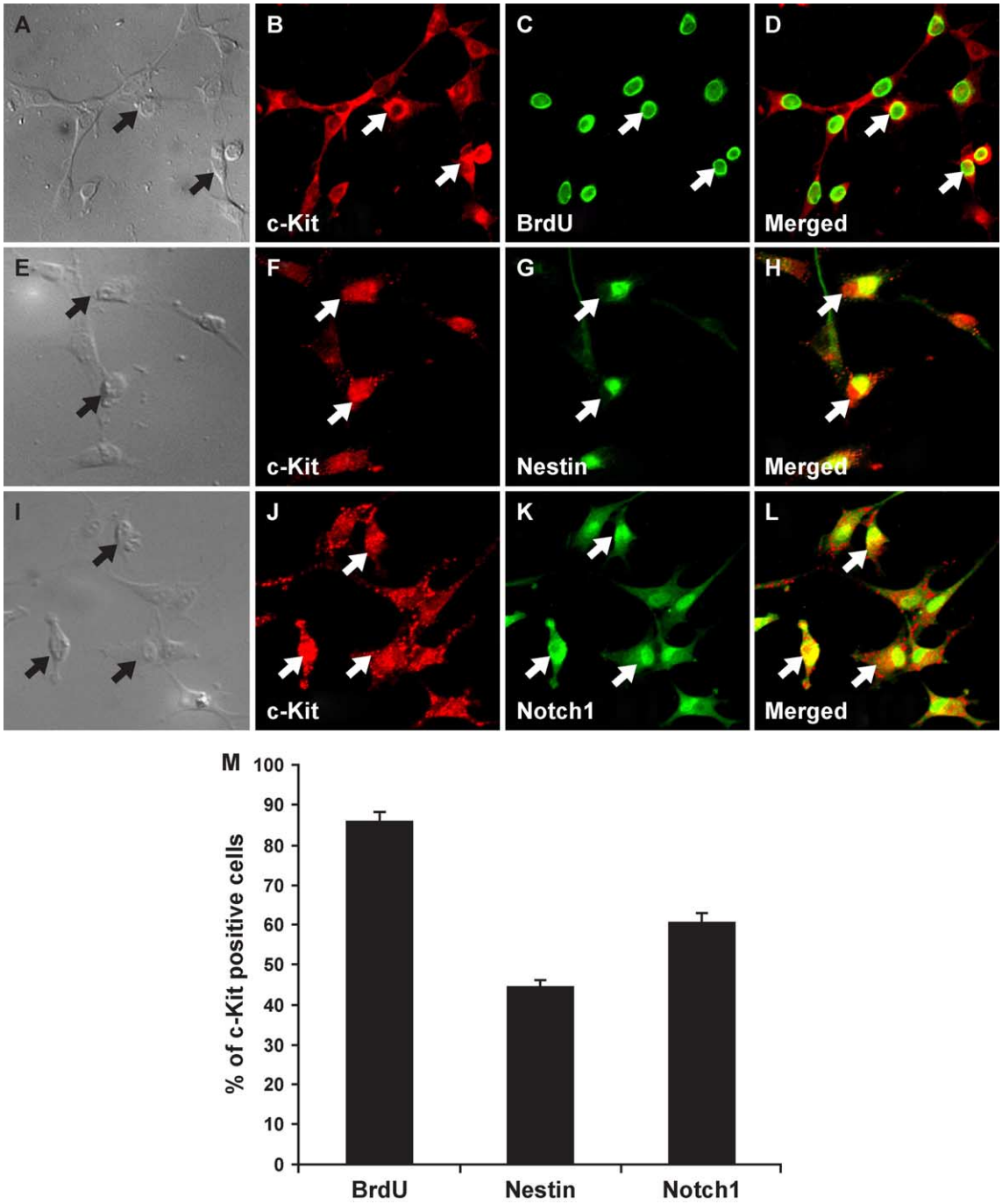

Fig. 5. c-Kit is coexpressed in CE neural stem cells with nestin and Notch1. To determine the progenitor nature of c-Kit-positive cells, a double immunocytochemical analysis was carried out to colocalize neural stem cells markers. The majority of c-Kit-positive cells (arrows) expressed BrdU (A–D; M). A relatively smaller proportion of c-Kit-positive cells (arrows) expressed nestin (E–H; M), and the majority of c-Kit-positive cells coexpressed Notch1 (I–L; M). Data are expressed as mean ± SEM from triplicate cultures of three different experiments. Magnification ×200.

ml), $P < 0.01$, /556.4 ± 24.28 (1 μg/ml), $P < 0.001$], suggesting that endogenous SCF influences the proliferation of CE neural stem cells (Fig. 7). To confirm the influence of c-Kit signaling on self-renewal of CE neural stem cells, single primary neurospheres were dissociated and cultured in individual wells of 96-well plate to generate clonal secondary neurospheres. Cells in the secondary neurospheres were proliferating and expressed neural stem cell marker nestin (Figs. 8A and B). Under differentiation condition, they expressed pan neural markers, β-tubulin III (Figs. 8C and D) and GFAP (Figs. 8E and F). The influence of perturbation of c-Kit signaling on the generation of secondary neurospheres was evaluated (Fig. 8G). As observed for the primary neurospheres, the proportion of secondary neurospheres was significantly higher in the presence of SCF than in

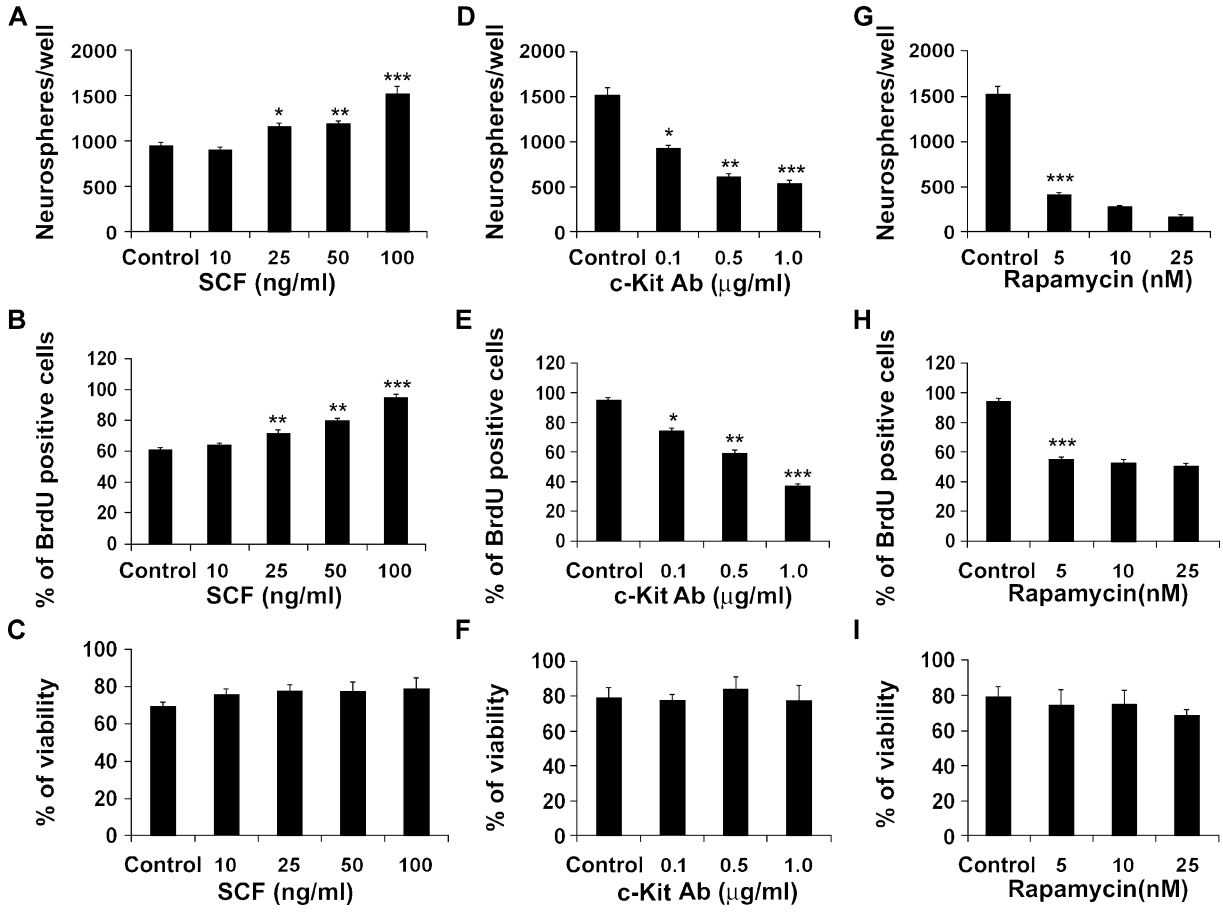

Fig. 6. c-Kit signaling influences cell proliferation and primary neurosphere formation. To determine the role of c-Kit signaling in neural stem cell maintenance, CE neural stem cells neurospheres were cultured in the presence of SCF (A–C), SCF+c-Kit antibody (D–F), or rapamycin (G–I), and the effects on generation of neurospheres, BrdU incorporation, and viability were evaluated. While the presence of SCF (A, B) in the culture medium increased the number of neurospheres and proportion of BrdU-positive cells (A, B), the presence of SCF+c-Kit antibody (D, E) or rapamycin (G, H) decreased both the number of neurospheres and proportion of BrdU-positive cells significantly as compared to controls. In each experimental group, cell viability remained unchanged (C, F, and I). Controls in A, B, and C represent CE neural stem cells cultured in EGF and FGF2 only, while in D–I represent those growing in the presence of 100 ng/ml of SCF+mitogens. Data are expressed as mean ± SEM from triplicate cultures of three different experiments. *$P < 0.05$; **$P < 0.01$, and ***$P < 0.001$.

controls. The proportion of SCF-induced secondary neurospheres decreased significantly when c-Kit antibody or rapamycin was included in the culture, suggesting the influence of c-Kit signaling on the self-renewal of the CE neural stem cells. To address the issue that observations described above were specifically related to neural stem cell populations, the number of proliferating cells expressing neural stem cell markers, nestin (Lendahl et al., 1990), Musashi-1 (Sakakibara et al., 1996), and SSEA-1 (Capela and Temple, 2002) was determined in the presence of SCF and SCF+c-Kit antibody (Fig. 9). The proportion of BrdU-positive cells, expressing neural stem cell markers, increased in the presence of SCF as compared to controls (nestin: 67.65% ± 3.33% vs. 40.81% ± 2.6%, $P < 0.05$; Musashi-1: 75.58% ± 2.08% vs. 41.93% ± 2.39%, $P < 0.05$; SSEA-1: 65.98% ± 3.75% vs. 36.48% ± 3.84%, $P < 0.05$). In contrast, there was a significant decrease in the proportion of cells expressing neural stem cells markers in SCF+c-Kit antibody as compared to those in SCF alone (nestin: 67.65% ± 3.33% vs. 25.47% ± 2.99%, $P < 0.01$; Musashi-1: 75.58% ± 2.08% vs. 24.51% ± 0.8%, $P < 0.01$; SSEA-1: 65.98% ± 3.75% vs. 15.51% ± 1.26%, $P < 0.01$), confirming the role of c-Kit in the regulation of CE neural stem cells. Results obtained by immunocytochemical analysis were corroborated by RT–PCR analysis; the levels of transcripts corresponding to *nestin*, *Musash1*, and *Fut9*, an alpha-fucosyltransferase which synthesizes SSEA-1/LexA (Cailleau-Thomas et al., 2000), increased, and decreased in comparison to control in the presence of SCF and SCF+c-Kit antibody, respectively (Fig. 9T).

Interactions between c-Kit and Notch signaling

Notch is another key regulator of neural stem cells. Mice deficient in various components of Notch signaling are observed to have depleted populations of neural stem cells (Hitoshi et al., 2002; Nakamura et al., 2000). We were interested in learning if c-Kit signaling could influence Notch signaling, since Notch is coexpressed with c-Kit in CE neural stem cells and Notch signaling has been observed

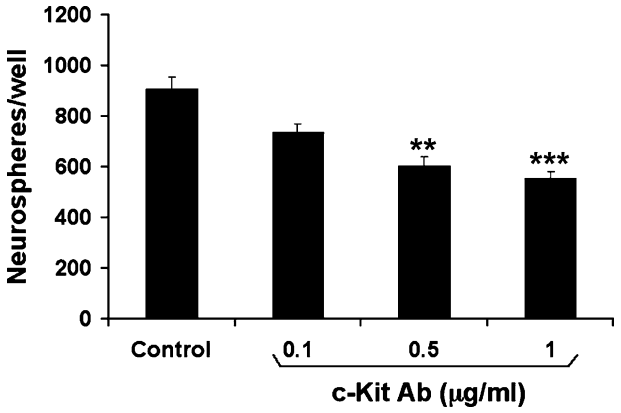

Fig. 7. Endogenous SCF is involved in c-Kit-mediated cell proliferation. To determine the influence of endogenous SCF, CE neurospheres were cultured in the increasing concentration of c-Kit antibody. There was a significant decrease in the number of neurospheres formed in the presence of 0.5 μg/ml (**$P < 0.01$) and 1 μg/ml (***$P < 0.001$) of c-Kit antibody. Data are expressed as mean ± SEM from triplicate cultures of three different experiments.

to be positively regulated by factors that influence proliferation of stem cells in general (Ahmad et al., 1998; Faux et al., 2001). Such cross-regulation would indicate a cooperative mechanism for the maintenance of neural stem cells. Activation of c-Kit signaling by SCF led to a significant increase in the proportion of cells expressing Notch1 as compared to control cells (57.72 ± 9.4 vs. 33.43 ± 2.4, $P < 0.05$) in medium containing only EGF and FGF2 (Figs. 10A–G). In contrast, there was a significant decrease in Notch1-positive cells in the presence of SCF+c-Kit antibody as compared to EGF and FGF2 controls (11.67 ± 2.81 vs. 33.43 ± 2.4, $P < 0.05$). The results obtained from immunocytochemical analyses were corroborated by RT–PCR analysis of transcripts corresponding to Notch1 and Hes1, the transcriptional repressor through which Notch signaling inhibits the target genes; the levels of *Notch1* and *Hes1* transcripts increased and decreased in comparison to controls in the presence of SCF and SCF+c-Kit antibody, respectively (Fig. 10H). To ensure that the change in Notch activity is due to perturbation in c-Kit signaling and not due to changes in cell population in neurospheres after 7 days in culture, we examined the levels of transcripts corresponding to *Notch1* and *Hes1* at different time intervals following the addition of c-Kit antibody to SCF-containing medium. Transcripts corresponding to neural stem cell markers were analyzed to determine the temporal effect of the attenuation of c-Kit signaling on progenitor properties of CE neural stem cells (Fig. 10I). A temporal decrease in levels of *Notch1* and *Hes1* transcripts along with those corresponding to neural stem cell markers was observed as compared to controls, suggesting a direct influence of c-Kit signaling on Notch activities and progenitor properties of neural stem cells. These observations suggested that signaling through c-Kit, besides having a direct effect, might also regulate CE neural stem cells indirectly (see below), by positively influencing key components of Notch signaling.

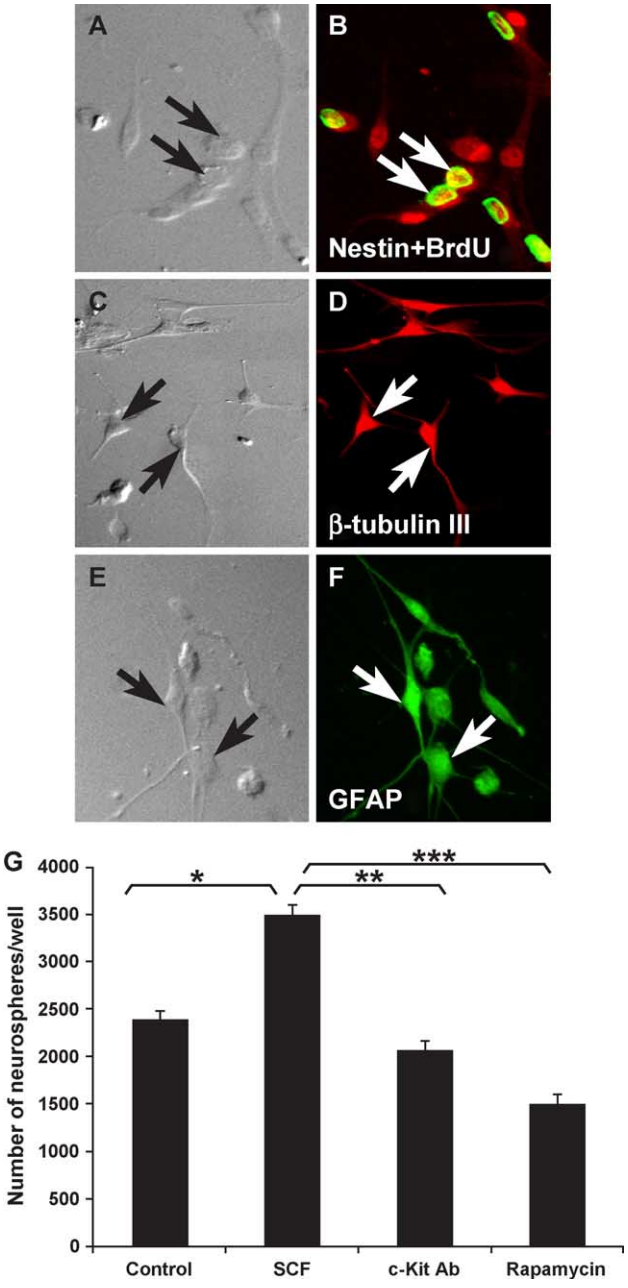

Fig. 8. c-Kit signaling influences the generation of secondary CE neurospheres. To confirm the role of c-Kit signaling in the self-renewal, single primary neurospheres were dissociated and cultured in individual wells in 96-well plate. Majority of the proliferating cells expressed nestin (A, B), and when they differentiated, they expressed neuronal marker, β-tubulin III (C, D) and glial cell marker, GFAP (E, F). Dissociates from single primary neurospheres were cultured in the presence of SCF (100 ng/ml), SCF+c-Kit antibody (1 μg/ml) and rapamycin (25 nM), respectively, and the effect of generation of secondary neurospheres was evaluated. While the presence of SCF in the culture medium increased the number of secondary neurospheres significantly, the presence of c-Kit antibody or rapamycin caused a significant decrease (G). Control represents CE neural stem cells cultured in EGF and FGF2 only. Data are expressed as mean ± SEM from triplicate cultures of three different experiments. *$P < 0.05$ when compared to control, **$P < 0.01$ and **$P < 0.001$ when compared to SCF.

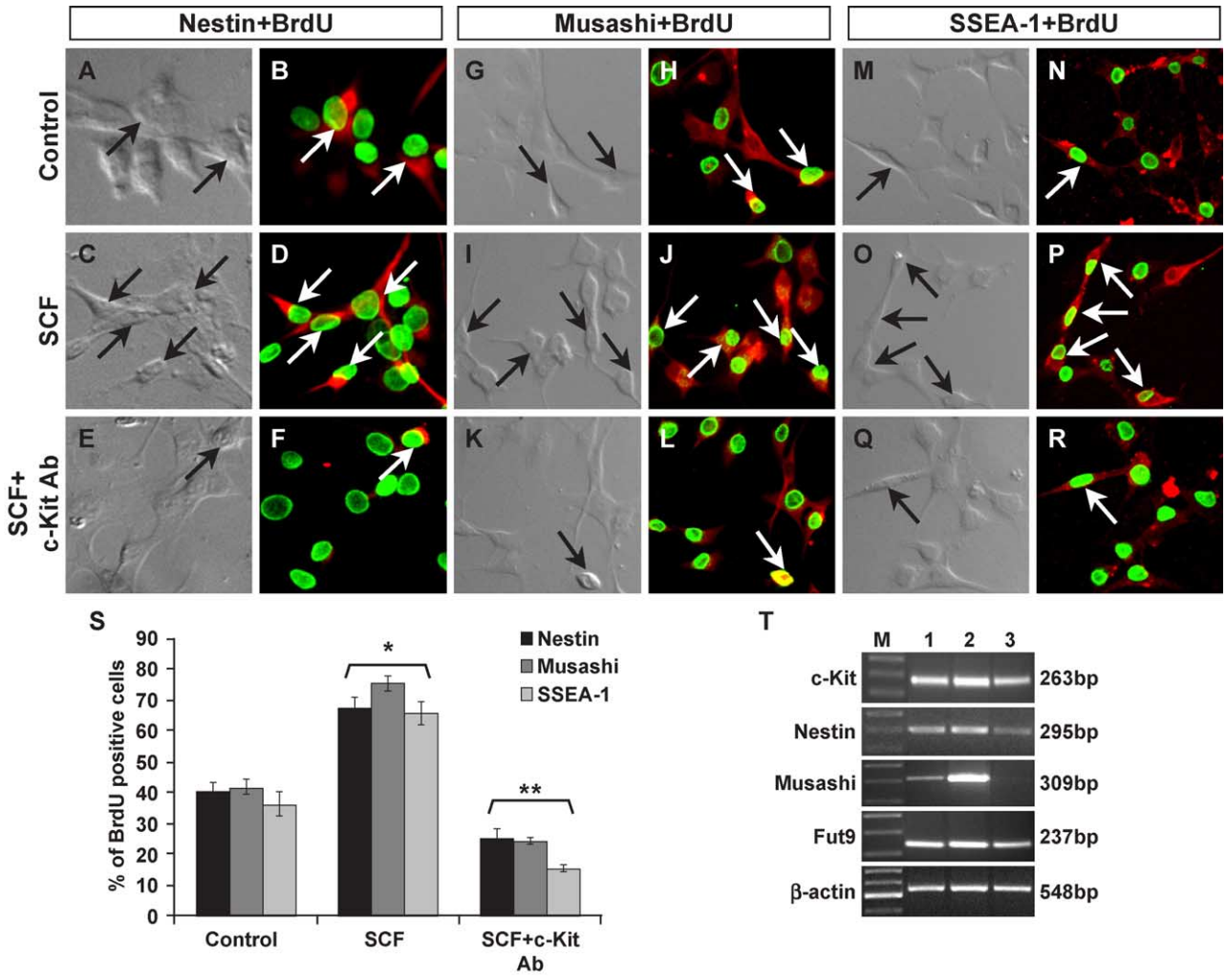

Fig. 9. c-Kit signaling influences proliferation of neural stem cells. To determine that c-Kit signaling specifically influences neural stem cell population, the effect of perturbation of c-Kit signaling was evaluated on the proportion of proliferating cells expressing neuroectodermal stem cell markers nestin, Musashi1, and SSEA-1. More BrdU-positive cells (arrows) were observed expressing nestin (A–F, S), Musashi1 (G–L, S), and SSEA-1 (M–R, S) in the presence of SCF alone than in the presence of SCF and c-Kit antibody. The immunocytochemical results were confirmed by RT–PCR analysis (T). The relative levels of transcripts corresponding to *nestin*, *Musashi-1*, and *Flut9* (an alpha-3-fucosyltransferase which synthesizes SSEA-1) decreased in the presence of SCF+c-Kit antibody (lane 3) as compared to those in SCF alone (lane 2) or without SCF (lane 1). Control represents CE neural stem cells cultured in EGF and FGF2 only. Data are expressed as mean ± SEM from triplicate cultures of three different experiments. *$P < 0.05$; **$P < 0.001$; M indicates marker lane. Magnification ×400.

Perturbation of c-Kit signaling influences neural differentiation

Evaluation of c-Kit signaling during myogenesis has suggested that activation of c-Kit, besides inducing cell proliferation, may influence the process of differentiation (Jayaraman and Marks, 1993). To determine if c-Kit signaling might influence the differentiation of CE neural stem cells, neurospheres maintained under undifferentiated conditions were shifted to a medium containing 0.5% FBS (control) and SCF/SCF+c-Kit, and the expression of markers corresponding to neuronal (β-tubulin III) and glial (GFAP) differentiation was analyzed (Figs. 11A–M). The proportion of β-tubulin III and GFAP-positive cells decreased significantly in the presence of SCF as compared to control cells (β-tubulin III: 3.64% ± 0.3% vs. 22.74% ± 2.27%, $P < 0.001$; GFAP: 5.0% ± 0.41% vs. 31.0% ± 4.18%, $P < 0.001$). In contrast, blocking of c-Kit receptor by c-Kit antibody restored the proportion of these cells in cultures (β-tubulin III: 18.72% ± 1.17%; GFAP: 33.31% ± 2.64%), suggesting that inhibition of c-Kit signaling may precede or accompany the differentiation of CE neural stem cells. Results obtained by immunocytochemical analysis were corroborated by RT–PCR analysis; transcripts corresponding to *β-tubulin III/GFAP* decreased or increased in comparison to control in the presence of SCF and SCF+c-Kit antibody, respectively (Fig. 11M, inset). To test the possibility that SCF might be increasing the number of neural stem cells/progenitors, we cultured CE neurospheres in the presence of mitogens and SCF for 5 days and then shifted these cells to a differentiation

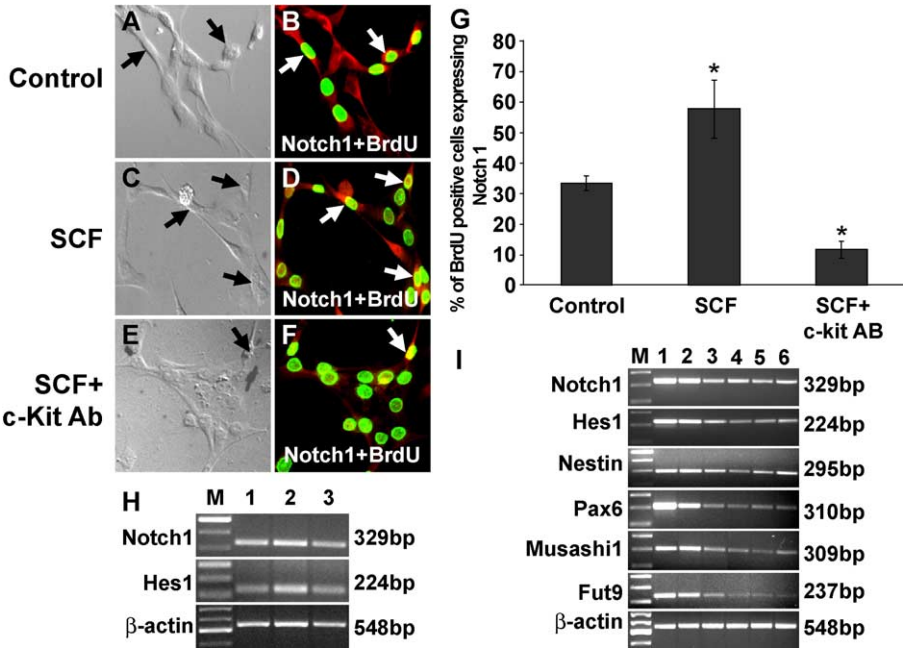

Fig. 10. c-Kit positively regulates of Notch signaling in CE neural stem cells. To investigate the influence of c-Kit on Notch signaling in CE neural stem cells, CE neurospheres were cultured in the presence of SCF or SCF+c-Kit antibody. Immunocytochemical analysis revealed a significantly higher proportion of BrdU-positive cells expressing Notch1 (arrows) in the presence of SCF (C, D, and G) than in controls (A, B, and G). The positive influence of SCF on Notch1 expression was abrogated in the presence of c-Kit antibody (E, F, and G). The immunocytochemical results were confirmed by RT–PCR analysis (H); the relative levels of transcripts corresponding to *Notch1* and *Hes1* increased in the presence of SCF (lane 2) as compared to those in the presence of SCF+c-Kit antibody (lane 3) or without SCF (lane 1). Analysis of transcripts corresponding to *Notch1*/*Hes1*/progenitor markers at different time intervals following exposure of neurospheres to c-Kit antibody in the presence of SCF reveals a decrease in their levels at 6 (lane 2), 12 (lane 3), 24 (lane 4), 48 (lane 5), and 96 (lane 6) hours as compared to those at 0 (lane 1) hour. Controls represent CE neural stem cells cultured in EGF and FGF2 only. Data are expressed as mean ± SEM from triplicate cultures of three different experiments. *$P < 0.001$. M indicates marker lane. Magnification ×200.

medium containing 0.5% FBS. We observed that there was a significant increase in the proportion of β-tubulin III-positive cells in cultures that were preexposed to SCF as compared to those that were not (32.81% ± 2.89% vs. 22.74% ± 2.27%, $P < 0.05$) and that this effect was abrogated when cells were preexposed to SCF+c-Kit antibody (SCF: 32.81% ± 2.89% vs. SCF+c-Kit antibody: 17.18% ± 2.93%, $P < 0.01$), suggesting that SCF might promote neurogenesis by increasing the population of neural stem cells/progenitors. Next, we wanted to know if the effect of c-Kit signaling on neural differentiation is direct or mediated through Notch signaling or both. The effect of c-Kit signaling on neural differentiation was examined in the presence of DAPT, an inhibitor of Notch signaling (Dovey et al., 2001; James et al., 2004). Neurospheres were cultured in the presence of DAPT (5 μM), DAPT+SCF, and SCF alone, and cells expressing β-tubulin III and GFAP immunoreactivities were examined (Fig. 12A) and immunocytochemical results were corroborated by RT–PCR analysis of transcripts corresponding to these markers (Fig. 12B). Levels of transcripts corresponding to Hes1, a target gene of Notch signaling, were analyzed as a measure of decrease in the signaling in response to DAPT. There was a significant increase in the proportion of cells expressing β-tubulin III (46.31% ± 5.88% vs. 22.74% ± 2.27%, $P < 0.01$) and GFAP (43.35% ± 4.9% vs. 31.0% ± 4.9%, $P < 0.05$) in the presence of DAPT as compared to controls, suggesting that inhibition of Notch signaling promotes differentiation; that is, Notch signaling inhibits differentiation. However, the proportion of these cells decreased in the presence of SCF+DAPT as compared to DAPT alone (β-tubulin III: 30.29% ± 4.31% vs. 46.31% ± 5.88%; $P < 0.05$, GFAP: 34.55% ± 1.35% vs. 43.35% ± 4.9%), suggesting that SCF can have negative influence on differentiation even in the absence of Notch signaling; that is, c-Kit has an additional inhibitory influence on differentiation besides that mediated by Notch signaling. Therefore, when both c-Kit and Notch signaling are active as in the case of neurospheres exposed to SCF only, the proportion of β-tubulin III and GFAP-positive cells decreased as compared to those in the presence of SCF+DAPT (β-tubulin III: 3.64% ± 0.3% vs. 46.31% ± 5.88%, $P < 0.001$, GFAP: 5.0% ± 0.41% vs. 43.35% ± 4.9%, $P < 0.001$). These results suggest that the influence of c-Kit on neural differentiation is mediated directly and indirectly via Notch signaling.

Discussion

c-Kit signaling is essential for the maintenance of stem cells during hematopoiesis, gametogenesis, and melanogenesis (Huang et al., 1990; Williams et al., 1990; Zsebo et al., 1990). Consistent with these observations, the lack of c-Kit

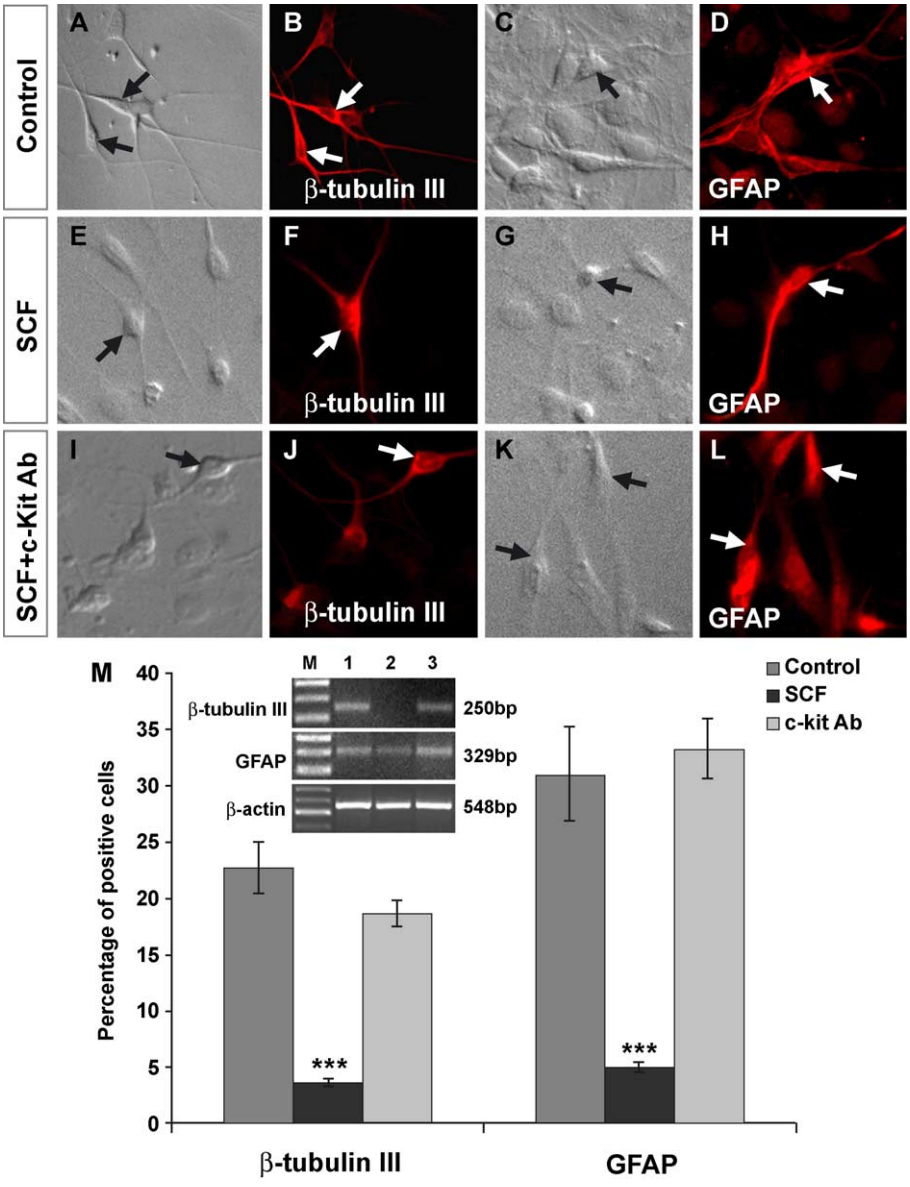

Fig. 11. c-Kit inhibits differentiation of CE neural stem cells. To determine the influence of c-Kit signaling on stem cell differentiation, CE cells were cultured in the presence of EGF+FGF2 for 5–6 days to generate neurospheres and then shifted to differentiation medium (0.5% FBS) containing SCF or SCF+c-Kit antibody for another 5–6 days. Immunocytochemical analysis revealed significantly fewer β-tubulin III- (arrows) and GFAP-positive cells (arrows) in the presence of SCF (E–H; M) than in controls (A–D, M). The negative influence of SCF on stem cell differentiation was abrogated in the presence of c-Kit antibody (I–L, M). The immunocytochemical results were confirmed by RT–PCR analysis (N, O); the relative levels of transcripts corresponding to *β-tubulin* and *GFAP* decreased in the presence of SCF (N, lane 2) as compared to those in the presence of SCF+c-Kit antibody (N, lane 3) or without SCF (N, lane 1). Control represents CE neural stem cells cultured in differentiation medium containing 0.5% FBS only. Data are expressed as mean ± SEM from triplicate cultures of three different experiments. ***$P < 0.001$, *$P < 0.05$. M indicates marker lane. Magnification ×200.

causes multiple defects that include anemia, sterility, and depigmentation, and *c-Kit* null mutant mice die during the first postnatal week due to hematopoietic defect (Bernstein et al., 1990; Broudy, 1997; Fleischman et al., 1991; Waskow et al., 2002). Evidence is emerging that c-Kit signaling may play a role in both the developing and adult nervous system. For example, a variety of approaches suggest that in the adult nervous system, the signaling may subserve diverse functions such as establishing neuronal connection (Hirata et al., 1993; McLaughlin, 2000), promoting neuronal survival (Hirata et al., 1995), and potentiating synaptic activities (Katafuchi et al., 2000; Motro et al., 1996). In the developing nervous system, particularly in the PNS, c-Kit signaling has been implicated in survival, migration, proliferation, and differentiation of neural crest precursors (Carnahan et al., 1994; Langtimm-Sedlak et al., 1996). More recently, c-Kit and SCF have been suggested to participate in neurogenesis in the adult CNS (Jin et al., 2002). c-Kit expression (Eriksson et al., 2000; Morii et al., 1994) and the activity of its promoter (De Sepulveda et al., 1995; Eriksson

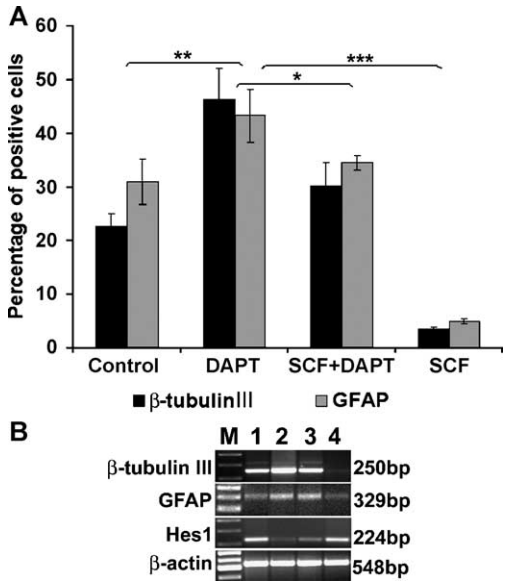

Fig. 12. c-Kit influences cell differentiation directly and indirectly through Notch signaling. To know if c-Kit influence on neural differentiation is direct or indirect through Notch signaling or both, neurospheres were cultured in the presence of DAPT to attenuate Notch signaling, SCF to activate c-Kit signaling, and SCF+DAPT to examine the effect of c-Kit signaling in the absence of Notch signaling. Proportion of cells expressing β-tubulin and GFAP immunoreactivities and transcripts corresponding to β-tubulin III and GFAP were analyzed. Transcripts corresponding to *Hes1* were analyzed as a measure of attenuation of Notch signaling. There was significant increase in the proportion of *β-tubulin III*- and *GFAP*-positive cells in the presence of DAPT as compared to controls, which was compromised when neurospheres were treated with both SCF and DAPT, therefore suggesting an effect of SCF on differentiation, independent of Notch signaling. Exposure of neurospheres to SCF only led to a decrease in proportion of these cells which were significantly greater than those in the presence of SCF+DAPT, suggesting an indirect effect of c-Kit signaling on differentiation, presumably mediated through Notch signaling. The immunocytochemical results were corroborated by RT–PCR analysis of *β-tubulin III*, *GFAP*, and *Hes1* transcripts in conditions described above. 1 = controls; 2 = DAPT treatment; 3 = SCF+DAPT treatment; 4+ SCF treatment; M = markers. Data are expressed as mean ± SEM from triplicate cultures of three different experiments. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

et al., 2000) have been reported in adult and developing retina. Also, c-Kit expression has been reported in the RPE and iris pigmented epithelium (Kociok et al., 1998). However, the developmental and functional implications of c-Kit activity in retina and other ocular tissues remain unknown. Given the role of c-Kit signaling in the maintenance of hematopoietic stem cells, primordial germ cells, and melanoblasts, the expression of c-Kit in proliferating adult CE neural stem cells suggested that it might be involved in regulating their maintenance. We confirmed this possibility by two different approaches. First, we exposed cells to SCF and observed that the proportion of proliferating cells and the number of both the primary and secondary neurospheres increased significantly. The increase in the proportion of proliferating cells and the number of neurospheres (primary as well as the secondary) was abrogated when cells were exposed to c-Kit antibody, either in the presence or absence of SCF, suggesting that signaling through c-Kit receptor influences their proliferation and self-renewal.

The binding of SCF causes c-Kit dimerization and receptor autophosphorylation. The activated receptor, in turn, phosphorylates different substrates to activate distinct pathways. These include phosphatidylinositol-3-kinase (PI3K), Ras, the Janus kinase, and Src (Blume-Jensen et al., 1998; Rameh and Cantley, 1999; Ueda et al., 2002). Both PI3K and Src can activate FKBP-12–rapamycin-associated protein (FRAP; also known as mTOR or RAFT1) pathway via AKT. FRAP/mTOR belongs to the kinase family that influences the progression of cells from G1 to S phases of the cell cycle by regulating the rate of translation of mRNAs (Gingras et al., 2001; Hentges et al., 2001; Kuruvilla and Schreiber, 1999). This is achieved by regulating the activity of general translational machinery and/or translation of specific mRNAs, such as those that encode cyclin D1 (Grewe et al., 1999) by activating S6 ribosomal subunit (Gingras et al., 2001; Hentges et al., 2001; von Manteuffel et al., 1997) and initiation factor eIF4e (Gingras et al., 2001). In addition, FRAP/mTOR may influence G1 to S phase transition by regulating the expression of cyclin D1 mRNA (Grewe et al., 1999; Hashemolhosseini et al., 1998). It is likely that the effects of c-Kit on adult CE neural stem cells, like those observed for other stem cells, are mediated by FRAP/mTOR pathway as SCF-induced proliferation and generation of neurospheres were abrogated in the presence of rapamycin, a potent inhibitor of FRAP/mTOR. However, FRAP/mTOR may not be the only regulators of proliferation of the adult CE neural stem cells as BrdU uptake is reduced only by approximately 50% in the presence of rapamycin as compared to controls. It is likely that residual BrdU uptake is due to the presence of mitogens, that is, FGF2 and EGF, in the culture medium. The decrease in the proportion (approximately 86%) of neurosphere formation was more than the decrease in the proportion (approximately 65%) of BrdU-positive cells, which suggests that FRAP/mTOR influences self-renewal not only by promoting cell proliferation, but also by keeping cells from differentiating. Similar mechanism has been proposed for Wnt signaling-mediated self-renewal in hematopoietic stem cells (Reya et al., 2003).

The elaboration of bipolar processes by adult CE neural stem cells following the decrease in c-Kit signaling suggested that besides influencing proliferation, c-Kit might affect differentiation. In support of this hypothesis, we observed that, when c-Kit signaling is enhanced in conditions that promote differentiation, the proportion of cells expressing neuronal and glial markers decreased significantly. This inhibitory effect on neural cell differentiation was reversed by attenuating c-Kit signaling, attesting to its negative influence on the differentiation process. Similar observations have been reported in a myogenic cell line where inhibition of c-Kit signaling by rapamycin was accompanied with myogenic differentiation (Jayaraman and Marks, 1993). These observations, including ours, that

c-Kit signaling negatively affects differentiation are in contrast to the observation that SCF promotes neurogenesis (Jin et al., 2002). The fact that the CE neural stem cells that were preexposed to SCF, when shifted to differentiating conditions, generated more neural cells as compared to controls suggests that there was an increase in the number of neural progenitors in response to accentuation in c-Kit signaling. This was reflected in an increase in the number cells expressing neural marker under differentiating conditions. Therefore, the increase in neurogenesis previously reported (Jin et al., 2002) might reflect an increase in the number of neural stem cells/progenitors and not their differentiation in response to SCF.

It is possible that the influence of c-Kit on the maintenance of CE neural stem cells is mediated via its influence on Notch signaling. Notch1 receptor is a key regulator of stem cells. Notch signaling regulates differentiation in variety of tissues including the CNS by keeping a population of progenitors uncommitted, until proper cues of differentiation appear in the environment (Artavanis-Tsakonas et al., 1999). Analyses of ES cells with deficiency in downstream regulators of Notch signaling such as Hes1 and RBP-Jk and the study of stem cells isolated from embryonic telencephalon of mice lacking Hes1 or Hes5 have demonstrated that Notch signaling is a critical regulator of the maintenance of neural stem cells (Hitoshi et al., 2002; Nakamura et al., 2000; Ohtsuka et al., 2001). It has been suggested that mitogens such as FGF2 and EGF may act in concert with Notch signaling for the maintenance of a pool of neural stem cells (Ahmad et al., 1998; Faux et al., 2001). Since the levels of transcripts corresponding to both Notch1 and c-Kit were higher in CE neural stem cells in proliferating conditions as compared to those in differentiating conditions, we sought to investigate the interactions between of c-Kit on Notch signaling to promote the maintenance of CE neural stem cells. We observed a positive influence of c-Kit signaling on Notch1 expression, suggesting cooperativity between them for the maintenance of neural stem cells. An increase in Notch signaling in response to c-Kit activity is likely to keep cells uncommitted. There are two likely mechanisms by which c-Kit signaling might activate Notch1 expression. These mechanisms are not mutually exclusive. c-Kit signaling may influence Notch1 expression through the JAK-STAT pathway; one of the downstream substrates activated by c-Kit signaling is the JAK tyrosine kinase (Brizzi et al., 1999; Deberry et al., 1997; Gotoh et al., 1996; Weiler et al., 1996), and activation of this substrate in response to CNTF has been demonstrated to up-regulate Notch1 expression in neural stem cells (Chojnacki et al., 2003). In addition to influencing the expression of Notch1, c-Kit signaling may influence the translation of Notch1 transcripts via the FRAP/mTOR pathway as it has been observed for other specific mRNAs (Gingras et al., 2001; Grewe et al., 1999). While the effect of c-Kit signaling on cell proliferation is well established, its influence on cell differentiation is not well known. It is likely that it influences cell differentiation directly and indirectly via Notch signaling, whose main role is to keep progenitor population in an uncommitted state. This notion is supported by the observation that neural differentiation is profoundly inhibited in the presence of SCF and that this inhibition is maintained, albeit at a lower levels, when Notch signaling is attenuated. Our results demonstrate that Notch signaling negatively influences both neural and glial differentiation of CE neural stem cells. However, in certain instances, particularly during late neurogenesis, Notch signaling is gliogenic (Wang and Barres, 2000). Since CE neural stem cells appear to be antecedent of neural stem cells that characterize early retinal histogenesis, Notch signaling may function in its classical inhibitory role, with a generalized negative influence on both neuronal and glial differentiation (Ahmad et al., 2004; James et al., 2004). Taken together, our observations suggest that c-Kit-mediated signaling plays a critical role in the maintenance of CE neural stem cells by regulating both the cell proliferation and differentiation in cooperation with other signaling pathways such as those mediated by Notch1 receptor. Since our analysis involved retrospectively enriched CE neural stem cells, the next step will be to determine if c-Kit signaling and its interactions with Notch signaling are operational in vivo.

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