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Hirschsprung Disease Is Linked to Defects in Neural Crest Stem Cell Function

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Abstract

Genes associated with Hirschsprung disease, a failure to form enteric ganglia in the hindgut, were highly up-regulated in gut neural crest stem cells relative to whole-fetus RNA. One of these genes, the glial cell line– derived neurotrophic factor (GDNF) receptor *Ret*, was necessary for neural crest stem cell migration in the gut. GDNF promoted the migration of neural crest stem cells in culture but did not affect their survival or proliferation. Gene expression profiling, combined with reverse genetics and analyses of stem cell function, suggests that Hirschsprung disease is caused by defects in neural crest stem cell function.

Although stem cell properties have been characterized in many tissues (1), we are only beginning to understand how stem cell function is regulated at the molecular level. Gene expression profiles have been described for uncultured hematopoietic stem cells and cultured central nervous system neurospheres (2–8), but not for prospectively identified, uncultured neural stem cells. Because stem cell properties change in culture (9–11), the gene expression profile of uncultured neural stem cells might better reflect their properties in vivo.

Molecular links between stem cell function and disease are of particular interest. Many diseases involve defects in neural development and may be caused by mutations that impair neural stem cell function. One potential example is Hirschsprung disease, a relatively common (1 in 5000 births) gut motility defect caused by a failure to form enteric nervous system ganglia in the hindgut. This can lead to fatal distention of the gut (megacolon). Although a number of the mutations that cause Hirschsprung disease have been identified (12), the ways in which these mutations affect neural development have been controversial, and it is unknown whether they affect gut neural crest stem cell (NCSC) function.

Gut NCSCs are self-renewing and multipotent, give rise to diverse types of neurons and glia in vivo, and persist in the gut throughout adult life (13–15). Uncultured gut NCSCs can be isolated by flow cytometry by selecting freshly dissociated fetal gut cells that express the highest levels of p75 (the neurotrophin receptor) and α_4 integrin (14). These $p75^+\alpha_4^+$ cells represent only 1 to 2% of cells in the E14.5 (embryonic day 14.5) rat gut (14). Of the single $p75^+\alpha_4^+$ cells that were added to culture, 60 ± 9% survived to form colonies, and 80 ± 7% of these colonies contained neurons (peripherin), glia [glial fibrillary acidic protein (GFAP)], and myofibroblasts [smooth muscle actin (SMA)]. These colonies typically contained 1×10^5 to 2×10^5 cells after 14 days of culture. These colonies are characteristic of NCSCs (13,14,16, 17).

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We compared the gene expression profiles of gut NCSCs and whole-fetus RNA using oligonucleotide arrays (26,379 probe sets). Three independent 10,000-cell aliquots of freshly isolated, uncultured gut NCSCs were sorted by flow cytometry. Target RNA was independently extracted from the NCSCs and from three E14.5 fetuses, amplified through two rounds of in vitro transcription, and hybridized to each set of arrays.

The reproducibility of sample isolation and amplification was high. The variability among gut NCSC samples (mean ± SD: $R^2 = 0.975 \pm 0.004$) and among whole-fetus samples ($R^2 = 0.981 \pm 0.003$) was comparable to what would be expected from chipto-chip variation ($R^2 = 0.973$ for the same sample on different chips). In contrast, the correlation coefficient between whole-fetus and gut NCSC samples was $R^2 = 0.855 \pm 0.006$. Arrays probed with whole-fetus or gut NCSC RNA contained 13,189 (50.0%) or 12,424 (47.1%) probe sets, respectively, at which transcript expression was detected. Genes corresponding to 475 probe sets were expressed at higher levels (by a factor of > 3; $P < 0.05$) in gut NCSCs, and 970 probe sets were expressed at higher levels in whole-fetus RNA (Table 1 and tables S1 and S2).

To assess the accuracy of the microarray results, we compared the expression of a subset of genes by quantitative (real-time) reverse transcription polymerase chain reaction (qRT-PCR). The same trends in expression levels were observed by microarray analysis and qRT-PCR in 20 of 21 cases (Table 2). Also, genes that encoded cell surface proteins and that appeared to be expressed by NCSCs by microarray analysis were also expressed at the protein level by flow cytometry (Table 2). The only exception was α_1 integrin (CD49A), for which low-intensity signals were apparent by microarray analysis but which was undetectable by flow cytometry (18). Overall, the results from microarray analysis, qRT-PCR, and flow cytometry were consistent.

Genes that have been linked to Hirsch-sprung disease were frequently expressed at higher levels in gut NCSCs. Of the 10 known genes that were most highly expressed in gut NCSCs relative to whole-fetus RNA, mutations in four of these genes have been linked to Hirschsprung disease: *Ret*, *Sox10*, *Gfra-1*, and *endothelin receptor type B* (*EDNRB*) (12) (Table 1 and Table 2).

To ensure that these genes were expressed in NCSCs rather than contaminating restricted neural progenitors or differentiated cells, we used qRT-PCR to compare their expression in E14.5 gut $p75^+\alpha_4^+$ NCSCs with E14.5, E19.5, or postnatal day 4 (P4) gut cells that expressed moderate levels of $p75^{med}$ that are enriched for restricted progenitors and more differentiated cells (fig. S1). *Ret*, *Sox10*, *Gfra-1*, and *EDNRB* were all expressed at significantly higher levels in NCSCs ($P < 0.01$). Most of the other 17 genes tested were also expressed at significantly different levels in NCSCs as compared with $p75^{med}$ gut cells. Thus, there are significant differences in gene expression between gut NCSCs and restricted neural progenitors/differentiated cells.

The genes that were up-regulated in gut NCSCs relative to whole fetal RNA were not necessarily NCSC-specific. Whereas some of these genes (*Ret*, *Sox10*, *Gfra-1*, and *EDNRB*) were expressed at lower levels by $p75^{med}$ gut cells, other genes (*DβH*) were expressed at comparable or higher levels by $p75^{med}$ cells (fig. S1). Nonetheless, *Ret*, *Sox10*, *Gfra-1*, and *EDNRB* were all expressed at high levels by gut NCSCs, which raised the possibility that

Supporting Online Material
www.sciencemag.org/cgi/content/full/301/5635/972/DC1
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mutations in these genes cause severe defects in enteric nervous system development by impairing the function of gut NCSCs.

Mutations in *GDNF*, its receptor *Ret*, or its coreceptor *Gfra-1* all lead to Hirschsprung disease in humans and aganglionic megacolon in mice (19–26). GDNF promotes the survival, proliferation, and migration of mixed populations of neural crest cells in culture (27–30). However, Ret protein was reported to be expressed by restricted gut neural crest progenitors but not by migrating trunk NCSCs (31). These data raise the question of whether GDNF and Ret regulate gut NCSC function.

To analyze Ret receptor expression, we stained live gut NCSCs from the stomach and intestines with an antibody to Ret (Fig. 1). Virtually all gut NCSCs expressed Ret protein on the cell surface. In contrast, other populations of migrating and postmigratory trunk NCSCs failed to express Ret (18,31). To study the function of Ret, we cultured E13.5 to E14.5 rat guts in collagen gels supplemented with GDNF (10 ng/ml). In the presence of GDNF, large numbers of cells migrated into the collagen gel (Fig. 2, A to D). Cells also migrated in the general direction of beads soaked in GDNF (Fig. 2E). This is consistent with reports that GDNF is expressed in the gut in advance of migrating neural crest cells and is chemoattractive for neural crest cells in culture (29,30).

To test whether GDNF promoted the migration of NCSCs (a small minority of gut neural crest cells), we extracted the migrated cells from the gel and cultured them at clonal density (13). In five independent experiments, 2.5 ± 1.2% of migrating cells formed multilineage NCSC colonies. More than 13 times as many NCSCs could be extracted from collagen gels supplemented with GDNF as from control cultures (Fig. 2H). This increase appears to be entirely explained by a promotion of migration, as GDNF did not affect the survival (Fig. 2I), proliferation (Fig. 2J), or differentiation of NCSCs into neurons and glia (Fig. 2K) under these culture conditions. Consistent with previous reports (27,28,32), GDNF did appear to promote the proliferation and/or survival of restricted neural crest progenitors under the same conditions (fig. S2).

To test whether NCSCs fail to migrate in vivo in the absence of GDNF signaling, we examined NCSC migration in the guts of *Ret*-deficient mice. Few neural crest cells migrate beyond the esophagus in $Ret^{-/-}$ mice (20,33), but the NCSCs in these mice have not been studied. In the esophagus of E13.5 mice, we found a factor of 4 reduction in the frequency of $Ret^{-/-}$ NCSCs (Fig. 2L), although this difference was not statistically significant because one of the $Ret^{-/-}$ mice had normal numbers of NCSCs in the esophagus. The proliferation and differentiation of these $Ret^{-/-}$ NCSCs in culture were indistinguishable from $Ret^{+/+}$ or $Ret^{+/-}$ NCSCs (fig. S3), suggesting that there was no intrinsic defect in their stem cell potential. In contrast, in the stomach and intestine we found a factor of 20 reduction in the frequency of NCSCs in $Ret^{-/-}$ mice (Fig. 2L). A failure of $Ret^{-/-}$ NCSCs to migrate beyond the esophagus is sufficient to explain the absence of enteric ganglia in the distal stomach and intestines of $Ret^{-/-}$ mice.

Because GDNF did not affect NCSC survival or proliferation in culture, the precipitous reduction in NCSC frequency in the stomach and intestine is likely caused primarily by a defect in migration. However, Ret signaling may also be required for the survival or proliferation of NCSCs before E12.5 in the esophagus or before their entry into the esophagus (32). Most neural crest cells that colonize the gut are Ret-dependent and derive from the vagal neural crest, whereas a minority of neural crest cells that colonize the esophagus are Ret-independent and derive from the trunk neural crest (33). One possibility is that NCSCs are depleted from the esophagus and virtually absent from the stomach and intestine because only trunk-derived NCSCs are able to migrate into the foregut of $Ret^{-/-}$ mice. This would suggest that Ret signaling is required not only for the migration of NCSCs within the gut but also for the migration of

most vagal-derived NCSCs into the esophagus. Irrespective of the precise fate of *Ret*$^{-/-}$ vagal-derived NCSCs, these data demonstrate that *Ret* is required for the colonization of the gut by NCSCs.

It is likely that loss-of-function mutations in *Gfra-1*, *EDNRB*, and *Sox10* also lead to Hirschsprung disease by impairing gut NCSC function. Sox10 has recently been shown to regulate the multipotency of NCSCs (34).

The mutations responsible for about one-half of Hirschsprung cases have not yet been identified (35). Given that mutations in 4 of the 10 most up-regulated genes in gut NCSCs have already been shown to cause Hirschsprung disease, the remaining genes that are highly up-regulated in gut NCSCs represent a resource of candidates that could also cause or modify the risk of Hirschsprung disease when mutated.

Two studies recently identified subsets of genes that were up-regulated in three stem cell populations, relative to other cells, and concluded that the genes they identified were indicative of "stemness" or the "molecular signature of stem cells" (6,7). Only one gene, α_6 *integrin*, was up-regulated in gut NCSCs (NCSC/fetus = 4.6, $P < 0.0001$) and was present on both of these previously published lists (table S3). α_6 *integrin*$^{-/-}$ mice develop to birth but then die neonatally as a result of severe blistering in the skin and other epithelia (36). Keratinocyte stem cells and spermatogonial stem cells also express α_6 integrin (37,38). It will be interesting to determine whether α_6 integrin is necessary for stem cell function in multiple tissues.

Our results demonstrate the value of combining the analysis of stem cell phenotype and function with microarray analysis and reverse genetics. The results we obtained by microarray analysis were consistently confirmed by qRT-PCR (Table 2), flow cytometry (Fig. 1), and functional analysis (Fig. 2). We believe this combination of approaches will provide critical insights into the cellular and molecular mechanisms underlying diseases.

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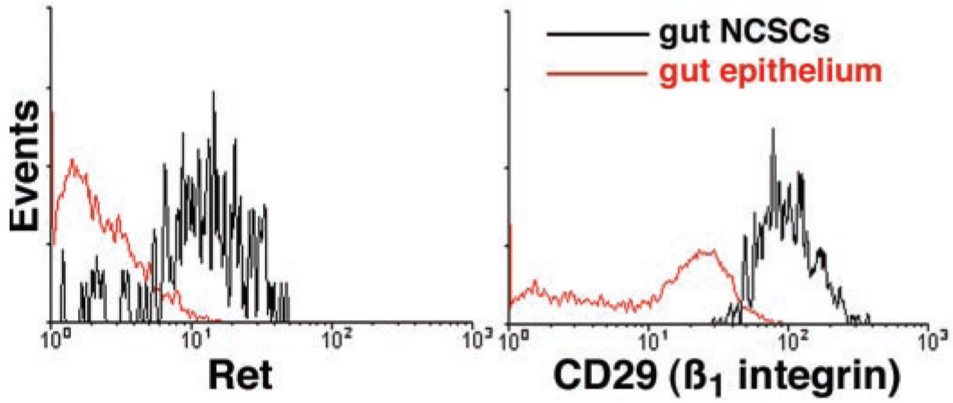

Fig. 1.

Flow-cytometric analysis of Ret, and CD29 (β_1 integrin) expression by E14.5 gut $p75^+\alpha_4^+$ NCSCs and E14.5 gut $p75^-\alpha_4^-$ epithelial progenitors from the same dissociated guts. As summarized in Table 2, the gut NCSCs consistently expressed Ret and CD29. In contrast, gut epithelial progenitors did not detectably express Ret but heterogeneously expressed CD29.

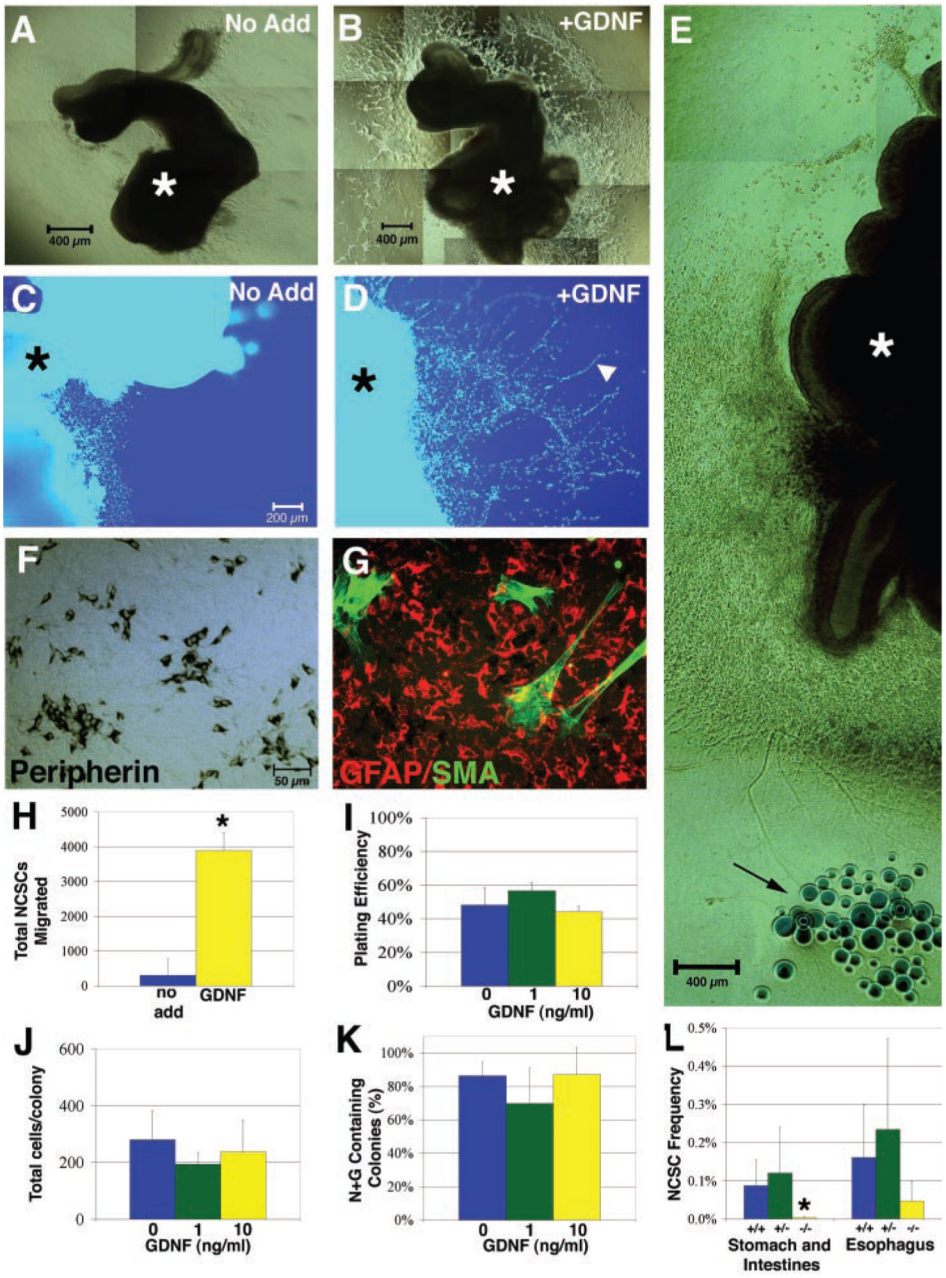

Fig. 2.

GDNF signaling promotes gut NCSC migration and is required for the migration of NCSCs into the intestines. [(A) to(E)] In nine independent experiments, E13.5 toE14.5 rat guts (*) were dissected and cultured in collagen gels. In the absence of GDNF (**A** and **C**), few cells migrated out of the gut, whereas in the presence of GDNF (10 ng/ml) (**B** and **D**), a large number of cells migrated into the collagen gel [(A) and (B): tiled phase-contrast images; scale bars, 400 μm; (C) and (D): Hoechst 33342–stained nuclei; scale bar, 200 μm]. In GDNF-supplemented cultures, many cells migrated along neurites that extended into the collagen [(D), arrowhead]. (**E**) Neural crest cells migrated in the direction of beads (arrow) soaked in GDNF. Scale bar, 400 μm. (**F** and **G**) Migrating cells that were extracted from the gel and cultured at clonal density formed large multilineage NCSC colonies containing neurons [peripherin^{+},

shown in (F)], glia [GFAP$^+$, shown in (G)], and myofibroblasts [SMA$^+$, shown in (G)]. Scale bar in (F) and (G), 50 μm. (**H**) In three independent experiments, 13 times as many (*$P < 0.001$) NCSCs were extracted and cultured from GDNF-supplemented gels. In five to seven independent experiments, GDNF did not affect the ability of single E14.5 gut NCSCs to survive (**I**) or proliferate over the first 6 days in culture (**J**). (**K**) In four independent experiments, GDNF also did not affect the percentage of $p75^+\alpha_4^+$ NCSCs that differentiated to form colonies containing neurons and glia in culture. (**L**) The frequency of NCSCs that could be cultured from *Ret*$^{-/-}$ esophagus was reduced by a factor of 4 ($P = 0.07$), but in three independent experiments, *Ret*$^{-/-}$ NCSCs were nearly absent from the stomach and intestines (factor of >20 reduction; *$P < 0.05$). Similar results were obtained in two experiments using E15.5 guts. GDNF also did not affect E12.5 or E14.5 NCSC survival, or proliferation in chemically defined standard medium lacking chick embryo extract (fig. S5).

Table 1

Known genes that were more highly expressed in gut NCSCs relative to whole-fetus RNA by a factor of >5 [only expressed sequence tags that were highly similar (HS) to known genes were listed]. *Ret*, *Sox10*, *Gfra1*, and *EDNRB* have been linked toHirschsprung disease by previous studies (12).

Probe set	Unigene title	Unigene ID	NCSC	Fetus	NCSC/fetus
Ret	*Ret proto-oncogene*	Rn.44178	9596	167	57.3
DβH	*Dopamine β-hydroxylase*	Rn.87166	1757	81	17.6
ESTs	HS to *RAT CD9 ANTIGEN*	Rn.2091	1612	92	16.1
ESTs	HS to *T42204 chromatin structural prot. homolog Supt5hp*	Rn.97299	1282	15	12.8
Sox10	*SRY-box containing gene 10*	Rn.10883	1272	23	12.7
Gfra1	*Glial cell line-derived neurotrophic factor receptor alpha*	Rn.88489	3846	304	12.6
ESTs	HS to *ubiquitin-like 3*	Rn.12128	1195	74	12.0
GPRK5	*G protein-coupled receptor kinase 5*	Rn.6500	1175	109	10.8
Gas7	*Growth arrest specific 7*	Rn.17160	3319	309	10.7
EDNRB	*Endothelin receptor type B*	Rn.11412	1159	117	9.9
Cart	*Cocaine and amphetamine regulated transcript*	Rn.89164	1246	128	9.8
ESTs	HS to *40S RIBOSOMAL PROTEIN S16*	Rn.29791	14572	1648	8.8
Cnp	*2',3'- Cyclic nucleotide 3'-phosphodiesterase*	Rn.31762	3163	373	8.5
Cdh2	*Cadherin 2, type 1, N-cadherin (neuronal)*	Rn.17239	2342	289	8.1
Dapkl	*Death-associated like kinase*	Rn.2311	1124	141	8.0
Hdlbp	*Lipoprotein-binding protein*	Rn.8515	790	69	7.9
Chn2	*Chimerin (chimaerin) 2*	Rn.10521	784	92	7.8
	Rat copper transporter 1	Rn.2789	770	95	7.7
Rasa3	*RAS p21 protein activator 3*	Rn.23055	1437	194	7.4
RT1Aw2	*RT1 class Ib gene*	Rn.39743	826	117	7.0
Rbp1	*Retinol-binding protein 1*	Rn.902	12135	1739	7.0
ESTs	HS to *CYSTEINE-RICH INTESTINAL PROTEIN*	Rn.8405	1802	259	6.9
Ckb	*Creatine kinase, brain*	Rn.1472	5964	860	6.9
Npy	*Neuropeptide Y*	Rn.9714	4185	608	6.9
Homer3	*Homer, neuronal immediate early gene, 3*	Rn.55092	673	75	6.7
Chrna5	*Acetylcholine receptor alpha 5*	Rn.40125	665	48	6.6
ESTs	HS to *NCR1 nuclear receptor corepressor 1 (N-COR1)*	Rn.22385	637	60	6.4
ESTs	HS to *TRA2 mouse TNF receptor associated factor 2*	Rn.14615	893	141	6.3
Cdc37	*CDC37 (cell division cycle 37, S. cerevisiae, homolog)*	Rn.17982	614	83	6.1
	Jun V-jun sarcoma virus 17 oncogene homolog (avian)	Rn.44320	3479	580	6.0
1G5	*Vesicle-associated calmodulin-binding protein*	Rn.9958	809	137	5.9
ESTs	HS to *MSTP043 protein*	Rn.16962	4203	712	5.9
Cyba	*Cytochrome b558 alpha-subunit*	Rn.5856	1003	170	5.9
Erp29	*Endoplasmic reticulum protein 29*	Rn.32904	588	−3	5.9
ESTs	HS to *BLMH RAT BLEOMYCIN HYDROLASE*	Rn.4278	583	88	5.8
Bckdha	*Branched alpha-ketoacid dehydrogenase subunit E1 alpha*	Rn.3489	599	109	5.5
Rpl30	*Ribosomal protein L30*	Rn.36878	3186	585	5.4
ESTs	HS to *S30034 translocating chain-associating memb. prot.*	Rn.3476	1150	214	5.4
ESTs	HS to *poliovirus receptor homolog precursor*	Rn.2144	699	131	5.3
Spin2b	*Serine protease inhibitor*	Rn.91257	779	148	5.3
ESTs	HS to *plasma retinol-binding protein*	Rn.3477	527	86	5.3
	Karyopherin, beta 1	Rn.11061	3358	639	5.3
Arpc1b	*Actin-related protein complex 1b*	Rn.2090	2757	527	5.2
	Oxysterol binding protein-like 1A	Rn.19665	520	50	5.2
ESTs	HS to *JN0124 glycine dehydrogenase*	Rn.17101	511	63	5.1
	Cyclin D1	Rn.22279	3426	678	5.1

Table 2

Comparison of the expression of selected genes in E14.5 gut NCSCs and whole fetuses by microarray analysis, qRT-PCR, and flow cytometry. Microarray intensities <100 were similar to background and were set to 100 for purposes of calculating ratios. All values represent the means of three independent samples (*$P < 0.01$). Genes that encode cell surface proteins against which antibodies were available were also analyzed by flow cytometry (ND, not determined). See fig. S4 for qRT-PCR details.

		Microarray			qPCR NCSC/fetus	Flow cytometry NCSCs
		Fetus	NCSC	NCSC/fetus		
NCSC > fetus	*Ret*	167	9596	57*	110	Expressed
	DβH	81	1757	18*	8.2	ND
	CD9	92	1612	16*	17	Expressed
	Sox10	23	1272	13*	17	ND
	Gfra1	304	3846	13*	14	ND
	EDNRB	117	1159	10*	14	ND
	CD29	9842	17189	1.7*	1.6	Expressed
	α_4*integrin*	142	246	1.7	12	Expressed
NCSC ~ fetus	*CD81*	9668	11369	1.2*	0.41	Expressed
	PCNA	15504	14189	0.93	0.77	ND
	Topo2a	14343	13373	0.93	0.77	ND
	CD24	18946	15295	0.81	0.63	Expressed
	f-spondin	879	714	0.81	0.65	ND
	Cdc25B	1427	1144	0.80	0.65	ND
	Dlx5	992	784	0.79	0.56	ND
	Hmgb2	10604	6438	0.61	0.70	ND
Fetus > NCSC	α_1 *integrin*	1336	323	0.24*	0.26	Undetectable
	CD59	4357	855	0.20*	0.45	ND
	Map2	4881	969	0.20*	0.10	ND
	Igfbp3	3171	541	0.17*	0.29	ND
	Nr2f1	7148	1200	0.17*	0.14	ND