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Transcriptome analysis of neural progenitor cells by a genetic dual reporter strategy

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Abstract

Global analysis of stem/progenitor cells promises new insight into mechanisms that govern self-renewal and cellular potential, an unresolved question of stem/progenitor cell biology. Despite rapid advance of genome-wide profiling methods, the difficulty in cell purification remains a major challenge for global analysis of somatic stem/progenitor cells. Genetic tagging with a reporter provides a powerful tool for identification and isolation of a specific mature cell type, however, for stem/progenitor cells, reporter retention by progeny may be a concern for impurity. Here we describe a genetic system combining a progenitor cell specific label with a second tag for marking differentiation. We present evidence that differential labeling of neural progenitor cells and their progeny enables prospective purification of these two cell types, whereas isolation based on a single marker compromises the purity of the intended progenitor population. Comparative expression profiling between the purified progenitors and progeny documents a neural progenitor cell transcriptome and uncovers an important role of TAM receptor tyrosine kinases in the maintenance of neural progenitor cells. This study establishes a general strategy for isolation of somatic stem/progenitor cells and provides a transcriptome database of neural progenitor cells useful for identification of causal factors of neural progenitor cell state, global dissection of epigenetic control of cellular potential, as well as for developing biomarkers or targets of brain cancer stem/initiating cells for therapeutic interventions.

Keywords

Neural progenitor cell; purification; transcriptome; neurogenesis; cerebral cortex

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Introduction

The development of genome-wide survey methods has enabled study of molecular regulations of stem/progenitor cells at the system level. Results obtained from embryonic stem cells (ESC) through global profiling of gene expression, DNA methylation, or histone modification, have started to provide new insights on the extent and dynamics of molecular integrations critical for many aspects of self-renewability and pluripotency¹⁻⁴. With more advanced and sensitive techniques being rapidly developed, the purity of stem/progenitor cell samples becomes a critical factor to affect the quality of a global analysis. This aspect is particularly challenging in global study of primary stem/progenitor cells derived from a developing embryo or an adult tissue, as there is in general a lack of defined surface markers which can be reliably used for stem/progenitor cell purification. In the case of neural stem/progenitor cells, for example, CD133⁵ was widely used as a surface marker; however, recent reports have suggested that expression of CD133 is associated with some neural stem/progenitor cells but not others⁶⁻⁸.

Genetic reporter expression in a mature cell is an effective method for cell tagging. In the case of labeling a primitive cell, however, conventional genetic tagging based solely on stem/progenitor cell specific expression⁹⁻¹³ may not be sufficient, as a technical concern would be that the exogenous reporter protein intended for stem/progenitor cell specific expression may be retained by a progeny, thereby compromising the specificity of cell tagging.

In this study, we have devised a genetic strategy for achieving specific labeling and isolation of somatic stem/progenitor cells. We have tested this approach for the purification of neural progenitor cells, for which a reliable isolation method is lacking. The new approach utilizes a progenitor cell specific promoter and a promoter of an early progeny to drive the expression of two different reporters, respectively. We present evidence that this dual labeling strategy can allow effective separation of neural progenitor cells from progeny thereby achieving prospective isolation of the two types of cells. Using purified neural progenitor cells and their neuronal progeny derived from the developing mouse cerebral cortex, we have examined gene expression patterns of endogenous neural progenitor cells and obtained a cortical neural progenitor cell transcriptome. Using this transcriptome, we further present data suggesting an important role of TAM receptor tyrosine kinases in neural progenitor cell maintenance.

Materials and Methods

Preparation of dissociated cortical cells

The DCX-RFP and Nestin-GFP transgenic mice were generated using expression constructs as previously described¹⁴. E15.5 cortices were dissociated by trituration in HBSS (Mediatech) with 5mM of EDTA (Invitrogen) and 25µg/ml of DNase I (Roche). Cells were washed once with HBSS and re-suspended in DMEM/F12.

Cell purification by FACS

Fluorescence-activated cell sorting (FACS) was performed with a 4-laser MoFlo Immunocytometry System (Dako, Glostrup, Denmark). Compensation was properly set based on single GFP and single RFP controls. Negative cells, cell debris or cell doublets were excluded with gating of side scatter, forward scatter and pulse width (one example of sorting scheme was shown in Supplementary Figure S1). Summit software was used for acquisition and data analysis.

Neurosphere formation and differentiation

GFP⁺RFP⁻, RFP⁺, and GFP⁺ cells were isolated by FACS, counted and plated into 24 well plate in a density of 10cells/μl or less¹⁵ with neurosphere formation media (DMEM/F12, 2mM L-Glutamine, 25mM HEPES, 10U/ml Heparin, Penicillin/Streptomycin, B27, 20ng/ml bFGF, 20ng/ml EGF) followed by culturing (37°C) for about a week. Control cortical cells were mock FACS sorted. Primary spheres were documented by quantification followed by imaging, or were dissociated with Accutase for secondary culture. To induce differentiation, neurospheres were cultured in the absence of bFGF/EGF for 3 days and then fixed with 4% paraformaldehyde for immunostaining.

Microarray

Three independent pools of GFP⁺RFP⁻ and RFP⁺ cells, respectively, were isolated from E15.5 cortices of reporter mice. Total RNAs were isolated from individual pools of freshly purified cells using a Qiagen RNeasy mini RNA extraction kit. Biotinylated single strand cDNA was generated using 100ng total RNA. Hybridization cocktails containing 2.5 μg of fragmented, end-labeled cDNA were prepared and applied to GeneChip mouse Gene 1.0 ST arrays, which interrogates more than 28,000 genes. Following hybridization (16 hours), the arrays were washed and stained with the GeneChip Fluidics Station 450 and scanned at 5-μm resolution using the Affymetrix GCS 3000 7G and GeneChip Operating Software v. 1.4 to produce intensity files. The three independent RNA samples produced consistent hybridization results with little deviation. Raw intensity measurements of all probe sets were background corrected, normalized and converted into transcript expression levels using the Affymetrix's Expression Console v1.1.1. The Bioconductor "ArrayTools" package was used to identify genes differentially expressed between GFP⁺RFP⁻ and RFP⁺ cells. Significant genes were selected with a cut-off of FDR (false discovery rate) <0.01 and fold change > 2. Profiling data are deposited with GEO, Gene Expression Omnibus (accession number GSE22946).

Expression analysis by real-time RT-PCR

Synthesis of cDNA from total RNA was done using Qiagen Omniscript RT kit. mRNA was quantified by real-time quantitative PCR using Applied Biosystems 7300 Fast Real-Time PCR System (Applied Biosystems) with SYBR Green PCR reagents (Applied Biosystems). Relative gene expression was normalized by housekeeping gene β-actin.

RNA *in situ* and Immunohistochemistry

RNA *in situ* and immunohistochemistry were done as previously described¹⁶. Primary antibodies included anti-DCX (Santa Cruz, 1:50), anti-Axl (Santa Cruz, 1:100), anti-Ki67 (Novacastra Laboratories Ltd, 1:100), anti-Pax6 (Developmental Studies Hybridoma Bank, 1:200), anti-BrdU (Abcam, 1:100), and anti-βIII-tubulin (Covance, 1:500). All secondary antibodies were from Jackson ImmunoResearch (Rhod Red-X-AffiniPure and Cy2 AffiniPure conjugated) and were used with a dilution of 1:200.

Fluorescent images were taken with a confocal microscope Zeiss LSM 510 Upright 2 photon or Olympus Inverted IX81. RNA *In situ* images were captured on Leica S6D Dissection Scope connected with Spot Insight GE Camera.

In utero electroporation

In utero electroporation was done as previously described^{16, 17}. Expression plasmids carry an Ubiquitin-EGFP expression cassette for convenient tracing of transfected cells. In brief, expression plasmids were electroporated into embryonic cortices at E13.5. Transfected brains were dissected out at E15.5, fixed with 4% paraformaldehyde, cryoprotected with

30% sucrose, OTC embedded, and sectioned at 12 μ m for imaging acquisition or immunohistochemistry. For functional analysis of TAM receptors, the center coronal sections along the anterior-posterior axis of the injected region of individual brains were used for quantification (more than six injected brains were analyzed for each DNA plasmid). Distribution of transfected cells in the radial domain of the cortex were quantified based on the VZ/SVZ, IZ, and CP demarcation, delineated with Hoechst nuclear staining. Quantification was done using Image-Pro Plus 5.1 program. For acutely dissociated cell culture, green fluorescent areas of the injected cortices (E15.5) were collected and dissociated in HBSS and washed twice with HBSS. Cell pellets were resuspended in D-MEM/F12 medium supplemented with B27 (1:50 v/v), penicillin (100 units/ml) and streptomycin (100 μ g/ml), counted, plated (5×10^5 cells/well) onto poly-D-lysine (PDL) coated coverslips placed in a 24-well plate, and cultured at 37 °C. Two hours after incubation, cells were fixed with 4% paraformaldehyde and processed for immunocytochemistry.

Knockout mice analyses

Axl single and Axl/Mer double knockout mice were reported previously¹⁸. Pregnant female mice were labeled with BrdU (100 mg/kg) for 24 hours and brains were then collected at E15.5, fixed with 4% paraformaldehyde, cryoprotected with 30% sucrose, OTC embedded, and sectioned at 12 μ m for immunohistochemistry with anti-BrdU, anti-Ki67 or anti- β III-tubulin antibody. Fluorescent images were taken with a confocal microscope Zeiss LSM 510 Upright 2 photon or Olympus Inverted IX81. Cell cycle exit index was calculated as the percentage of BrdU⁺Ki67⁻ differentiating cells in the total population of BrdU⁺ cells (proliferating and differentiating progenitor cells).

Results

A genetic dual reporter system for neural progenitor cell isolation

To overcome the problem of potential progenitor reporter retention in a progeny, we have devised a genetic differential cell tagging approach, employing a progenitor cell specific promoter in conjunction with a promoter of an early progeny to drive the expression of green fluorescent protein (GFP) and red fluorescent protein (RFP), respectively. Our rationale is that the use of a promoter of an early progeny can help mark the fate change in progenitor cells thereby distinguishing the self-renewing progenitors from the differentiating ones, including cells undergoing normal differentiation during development and cells undergoing spontaneous differentiation often seen during *in vitro* tissue sample handling. Purification of progenitor cells could thus be achieved, in principle, by isolation of the population of cells that only express GFP but not RFP. We tested this strategy in the developing cerebral cortex, as the cortex provides a model system for study of neural progenitor cells with unique technical advantages. In particular, it can provide sufficient amount of cells for global analysis and allow convenient access for experimental validation of profiling data.

In the developing mouse cerebral cortex, neural progenitor cells and young neurons can be marked by the expression of nestin and doublecortin (DCX), respectively. We therefore chose to use nestin- and DCX-specific expression of reporters to test the dual labeling strategy for the purpose of neural progenitor cell purification. The nestin promoter and DCX promoter have been characterized and documented in previous studies^{14,19}. For differential labeling of cortical cells, we generated transgenic mice using doublecortin promoter-driven monomeric RFP (DCX-RFP) and nestin promoter/enhancer-driven EGFP (Nestin-GFP) transgenes. Expression patterns of RFP and GFP in the brains of transgenic mice showed that the two reporters were expressed overall in a pattern consistent with the endogenous DCX and nestin proteins. RFP was found in the regions harboring young neurons, for

example outside the ventricular zone (VZ) of the cortex (Figure 1A), while GFP was mostly concentrated in the areas surrounding various brain ventricles, for example in the VZ of the cortex (Figure 1B). The brain patterns of RFP and GFP expression were consistently observed in independent transgenic lines, suggesting that the DCX and nestin promoters could reliably direct reporter expression in a manner consistent with the endogenous genes. We selected one RFP line (Figure 1A) and one GFP line (Figure 1B) to test for double reporter mice. Figure 1C shows RFP and GFP expression patterns in the cortex of the DCX-RFP/ Nestin-GFP double transgenic mice. In the cortical VZ, the majority of cells express GFP but not RFP. When extending into the intermediate zone (IZ), however, there is an apparent overlap of GFP and RFP expression, an indication of possible retention of progenitor reporter in the progeny.

To directly test for reporter expression in cortical cells, we collected embryonic day 15.5 (E15.5) cortices from single and double transgenic mice and prepared dissociated cells for fluorescence-activated cell sorting (FACS) analyses. GFP (Figure 1E) or RFP (Figure 1F) expressing cells derived from single reporter mice showed a distinct pattern relative to negative control cells (Figure 1D) and could be isolated by FACS. While GFP and RFP cells derived from single transgenic mice could be effectively separated when artificially mixed together (Figure 1G), confirming the two fluorescent reporters do not overlap in optical spectrum, most cortical cells derived from the double transgenic mice co-expressed GFP and RFP (Figure 1H). These results were also observed using a different Nestin-GFP line, suggesting the expression overlap was not due to strain difference. As the overall RFP and GFP expression in the brain appeared to correlate with endogenous patterns of DCX and nestin expression (Figure 1A and 1B), the observed extensive co-existence of GFP and RFP was likely caused by a transient temporal overlap of nestin and DCX expression during differentiation (for example, DCX expression may have started at the time when nestin expression was tapering down but not completely gone), or retention of progenitor reporter GFP into RFP expressing progeny, or both. This phenomenon indicates that isolation based solely on the expression of a neural progenitor cell specific reporter (Figure 1E) results in a mixed population. This highlights the importance of the use of a progeny specific promoter for distinguishing progeny from progenitor cells.

Should the co-expression of GFP and RFP be caused by a temporal overlap of the two reporters and/or GFP retention, we reasoned that among GFP⁺ cells, the fraction of GFP⁺RFP⁻ cells would be neural progenitor cells. Therefore, our strategy for obtaining cortical neural progenitor cells would be to sort for GFP⁺RFP⁻ cells by depleting the GFP⁺RFP⁺ cells within the population of GFP⁺ cells derived from the double transgenic embryos. We found that GFP⁺RFP⁺ cells obtained from double transgenic mice and RFP⁺ cells obtained from single transgenic mice were indistinguishable, for example they both expressed similar sets of neuronal markers but not progenitor markers and they did not form neurospheres. Therefore, to achieve a better neural progenitor cell purification using FACS approach, in our practice, we bred heterozygous Nestin-GFP mice with homozygous DCX-RFP mice to yield both GFP/RFP double positive and RFP single positive littermate embryos, which were used for isolating GFP⁺RFP⁻ cells and RFP⁺ cells, respectively. Purified GFP⁺RFP⁻ and RFP⁺ cells were used for comparative biochemical and functional analyses. Example of FACS analysis of the purified GFP⁺RFP⁻ cells or RFP⁺ cells was shown in Figure 1I and 1J, respectively. The fraction of GFP⁺RFP⁻ cells accounts for approximately 5-6% of the total dissociated cortical cells from the E15.5 cortices.

We have characterized the purified GFP⁺RFP⁻ cells in comparison to RFP⁺ cells and found that, as expected, GFP⁺RFP⁻ cells behaved as neural stem/progenitor cells *in vitro*. This was supported by the following observations. First, GFP⁺RFP⁻ cells showed enriched RNA expression of neural stem/progenitor cell markers as determined by real-time PCR (Figure

2A). Second, GFP⁺RFP⁻ cells, but not RFP⁺ cells, could form neurospheres (Figure 2B), a neural stem/progenitor cell feature under *in vitro* culture²⁰. The primary neurospheres, when dissociated and re-plated in culture, yielded more secondary spheres (Figure 2B), suggesting an ability of these cells to self-renew. Finally, GFP⁺RFP⁻ cells were able to differentiate when cultured under a differentiation-promoting condition in the absence of growth factors (Figure 2C), becoming neurons (DCX⁺), astrocytes (GFAP⁺), or oligodendrocytes (O4⁺). These data thus indicate that the purified GFP⁺RFP⁻ cells display *in vitro* features of neural stem/progenitor cells.

To further evaluate the effectiveness of the dual reporter strategy, we compared neural progenitor cells isolated using the double tags (GFP⁺RFP⁻ cells, Figure 1I) and those isolated using a single tag (GFP⁺ cells, Figure 1E) in parallel for their specific expression of cellular markers or for their ability to form neurospheres. In a quantitative PCR analysis, our data showed that, in reference to RFP⁺ cells, GFP⁺RFP⁻ cells more effectively enriched neural progenitor cell specific markers than GFP⁺ cells (Figure 2A). Similarly, in a neurosphere formation analysis, GFP⁺RFP⁻ cells were capable of generating neurospheres much more efficiently than GFP⁺ cells, in reference to mock sorted total cortical cells (Figure 2D). These data further suggest that the use of two genetic tags is both necessary and effective for isolation of neural progenitor cells.

Profiling of gene expression in purified neural progenitor cells vs. progeny

We next performed a gene expression profiling comparing the purified GFP⁺RFP⁻ and RFP⁺ cortical cells using Affymetrix microarray. The profiling data further revealed molecular distinctions between GFP⁺RFP⁻ and RFP⁺ cells. First, the data showed that GFP⁺RFP⁻ and RFP⁺ cells enriched known markers appropriate for neural progenitor cells and neurons, such as neural progenitor cell markers Nestin, Pax6, Musashi, ephrin-B1 and Tbr2 in GFP⁺RFP⁻ cells, and neuron markers DCX, β III-tubulin, Sox5, and Tbr1 in RFP⁺ cells. Second, assigning a 2-fold difference in RNA ratio as an artificial cut-off, the microarray profiling identified 1853 and 1979 probe sets that are differentially expressed between GFP⁺RFP⁻ and RFP⁺ cells, respectively, representing 1606 and 1454 unique genes in each cell type. Molecular and functional annotations of these differentially expressed genes (a gene list could be found in the Supplementary Figure S2 and S3) showed that the two populations of cells displayed distinct features of proliferating cells and neurons, respectively. For example, analyses using the PANTHER classification system showed that the top biological process in genes differentially expressed in GFP⁺RFP⁻ cells include cell cycle control, DNA metabolism, nucleic acid metabolism, or cell proliferation, while those of RFP⁺ cell-specific genes include neurotransmitter release, ion transport, regulated exocytosis, or synaptic transmission (Figure 3A). Classification of molecular function in differentially expressed genes showed enrichment of transcription factors or histones in GFP⁺RFP⁻ cell-specific genes and enrichment of various types of channels or traffic proteins in RFP⁺ cell-specific genes (Figure 3B). Gene family or pathway analyses of GFP⁺RFP⁻ cell-specific genes using DAVID or Ingenuity bioinformatics resources identified many novel genes or gene families potentially critical for neural progenitor cell regulation (e.g. Tables 1 and 2B), and revealed extensive enrichment of cancer-related signaling pathways (data not shown). Finally, in many cases, the array data revealed co-enrichment of multiple members of a gene family in one cell type but not the other. For instance, amongst genes known to have a function in neural stem/progenitor cell maintenance, four members of the Notch family and three of the Gli family of transcription factors, were seen highly enriched in GFP⁺RFP⁻ cell population (Table 2A). We found a number of gene families of signaling molecules, receptors, transcription factors or enzymes, not previously known for a particular function in neural stem/progenitor cells, showing multiple members differentially expressed in one cell type and suggesting potential

functions in the regulation of the state of self-renewal and differentiation or in other capacities in progenitor cells. Table 2B listed some examples of different types of the identified genes, including membrane receptors, transcription factors, extracellular matrix proteins, etc. These profiling results provide additional support to validate the dual reporter strategy for neural progenitor cell purification, and document a transcriptome of cortical neural progenitor cells.

Expression analyses of candidate neural progenitor cell specific genes

To further characterize the profiling data, we performed expression analyses to examine candidate genes revealed in microarray showing neural progenitor cell specific enrichment. Real-time PCR of several candidate genes identified by microarray (Table 2B) confirmed that the mRNAs of these genes were enriched in GFP⁺RFP⁻ cells (Figure 3C). RNA *in situ* hybridization analyses of representative genes listed in Table 2B showed that their mRNAs were selectively expressed in the cortical VZ which harbors neural progenitor cells (Figure 3D). This pattern of RNA expression is similar to that of many genes known for a specific function in neural progenitor cell regulation, such as Pax6, the Notch pathway genes, the Wnt pathway genes, or the ephrin-B pathway genes. This expression pattern presents an alternative verification of the neural progenitor cell purification strategy and provides a validation of the transcriptome data based on microarray analysis.

TAM receptor tyrosine kinases in neural progenitor maintenance

Neural progenitor cell transcriptome (GFP⁺RFP⁻ cell specific genes) could be a useful resource for systematic characterization of molecular factors involved in neural progenitor cell regulation. As a start to identify novel factors, we reasoned that data showing multiple members of a gene family selectively enriched in progenitors (Table 2B and data not shown) might serve as a strong indicator for predicting a link of the gene family to certain function in neural progenitor cell regulation. As a test, we explored the potential function of the TAM receptor tyrosine kinase (RTK) family in neural progenitor cells, as all three known TAM receptors (Axl, Tyro-3, and Mer) and one ligand (protein S) were revealed by our data to be selectively enriched in neural progenitor cells (Table 2B and Figure 3C and 3D). Unlike many other RTKs, the TAM receptors are found to be more prominently expressed postnatally extending into adulthood, but not widely or highly expressed during development. The TAM receptors were initially discovered for their role in promoting cell survival, and later were found to play a central role in immune regulation²¹. They are also over-expressed in many types of human cancers, for example, over-expression of Axl is seen in gliomas²², suggesting that TAM receptors can play a role in cell proliferation. Consistent with RNA *in situ* hybridization result, we found that Axl protein was enriched in the apical area of the cortical VZ, co-expressing with the proliferating marker Ki67 or the neural progenitor cell marker Pax6 (Figure 4A).

Similar to other RTKs, the TAM receptors can signal through homo- and hetero-dimerization²¹. To probe the possible function of TAM receptors in neural progenitor cells, we employed a dominant negative (DN) inhibition approach, which could achieve a more effective inhibition of RTK activation when multiple family members were co-expressed. It was previously shown that expression of a dominant negative Axl (Axl-DN, receptor lack of cytoplasmic domain) could suppress glioma cell growth and invasion²². We introduced Axl-DN expression plasmid into the embryonic mouse cortex via *in utero* electroporation. Expression of Axl-DN mutant caused more cortical cells to translocate from the VZ into the intermediate zone (IZ) and cortical plate (CP) (Figure 4B), an indication of differentiation of the affected neural progenitor cells^{16, 23}. This was supported by the observation that cells having moved into the CP were positive for neuronal markers β III-tubulin (Figure 4C), suggesting that the accumulation of more cells outside the VZ area was a result of induced

differentiation but not due to premature neural progenitor cell migration. Consistent with this, expression of Axl-DN mutant in the cortex led to an increase in the number of β III-tubulin positive neurons and a concomitant loss of nestin positive progenitor cells, as determined using acutely dissociated cell culture derived from the electroporated brains (Figure 4D). Correlating with the loss of nestin positive progenitors, analyses of Pax6⁺ or Tbr2⁺ cells in the cortex showed that expression of Axl-DN mutant caused a reduction in the number of both Pax6⁺ and Tbr2⁺ progenitor cell types (% of Pax6⁺ or Tbr2⁺ cells in the total population of the transfected cells comparing Axl-DN to EGFP control, Figure 4E), and this effect was not due to elevated cell death as determined by expression of cleaved caspase 3 (data not shown). We also tested a soluble Axl receptor, the extracellular domain of Axl receptor (Axl-ECD). Secretion of the Axl-ECD would be expected to compete with the interaction between endogenous TAM receptors and their ligands, Gas6 and protein S, both of which are expressed in the cortex, thereby blocking normal function of the three TAM receptors in progenitor cells. We found that expression of Axl-ECD in the cortex similarly caused more cortical cells to move from the VZ into the intermediate zone (IZ) and cortical plate (CP) (Figure 4B).

To further assess the TAM receptor function, we next analyzed TAM receptor knockout mice¹⁸. Should TAM receptors be required for promoting neural progenitor cell state, loss of function of TAM receptors would be expected to cause early neuronal differentiation in the cortex, which would lead to more migrating neurons streaming out of the proliferating zone (the VZ/SVZ area)^{17, 24}. We thus looked at BrdU/Ki67 co-staining in the E15.5 mutant brains, which were labeled with BrdU for 24 hours. We found that in comparison to wild-type brains, the Axl/Mer double knockout mutant brains displayed more BrdU⁺Ki67⁻ cells (progenitor cells undergoing differentiation) above the proliferating VZ/SVZ area (Figure 5A), suggesting early differentiation of neural progenitor cells due to deletion of the Axl and Mer genes. The Axl single knockout mutant brains did not show obvious difference from the wild-type brains (Figure 5A), perhaps due to compensatory response by the remaining Tyro-3 and Mer in the mutant brains. Cell marker staining revealed that the BrdU⁺Ki67⁻ cells outside the VZ/SVZ in the Axl/Mer double mutant brains were positive for β III-tubulin (Figure 5B), suggesting that they are differentiating neurons. Further analysis of cell cycle reentry and exit²⁵ showed that knockout of Axl and Mer caused more progenitor cells to leave the cell cycle (Figure 5C), which led to the accumulation of more migrating neurons beyond the VZ/SVZ. Together, our combined data from expression pattern analyses, *in utero* electroporation assays, and genetic mutant studies collectively suggest that TAM receptor function is important for the maintenance of cortical neural progenitor cells.

Discussion

Purification of somatic stem/progenitor cells will enable comprehensive analyses of biological regulation using combined molecular, cellular and system biological technologies, which will provide insights on the mechanisms that govern self-renewal and differentiation. Due to the lack of reliable surface markers, direct purification from primary tissues remains a challenging task. Genetic tagging offers unique advantage to achieving this goal, however, as our results demonstrated, a single genetic tag for marking stem/progenitor cells is defective for this purpose. Our observations indicate that while the reporter gene, controlled by a progenitor cell-specific promoter, may be faithfully expressed in progenitor cells, the reporter protein may persist into a progeny, thus compromising the specificity of progenitor cell labeling. In this study we have designed and validated a dual labeling strategy based on linearly-related expression of two reporters for specific isolation of endogenous progenitor cells. We have shown that by differential labeling employing a neural progenitor cell specific promoter in conjunction with a progeny specific early promoter, prospective isolation of neural progenitor cells can be achieved. With particular relevance to genome-

wide studies of neural progenitor cells, our data demonstrated that the dual reporter strategy could achieve prospective isolation of both progenitor cells and their progeny from the same animals thereby providing physiologically related father-daughter cells suitable for comparative global analyses. In principle, this strategy should be generally applicable for the purification of stem/progenitor cells in other tissues or organs.

The necessity of using two genetic tags for specific stem/progenitor cell labeling was evident in several aspects. First, FACS sorting of reporter expressing cells revealed that progenitor cells isolated based on one tag were prominently mixed with progeny. Second, quantitative PCR of marker expression or assay for self-renewability in neurosphere formation showed that genetic dual labeling could achieve significantly better enrichment of progenitor cells compared to using a single tag. Finally, with the purification of neural progenitor cells and their neuronal progeny, we were able to obtain a neural progenitor cell transcriptome. Analyses of this new transcriptome in comparison with neural progenitor cell genes characterized previously using a single tag also revealed that genetic dual labeling could achieve a better purity in neural progenitor cell isolation. For example, using a FACS sorting method based on expression of Sox1-GFP or Sox2-GFP reporter, Aubert et al.¹¹ and D'Amour et al.¹² explored neural progenitor cell genes and identified 15 and 158 candidates, respectively. Comparison between our data and these published datasets revealed that, while there were many genes commonly identified for progenitor specific expression, some of the genes reported in the previous studies, for example *Nhlh2*, *Lrrn1*, *Rtn1*, *Khdrbs3* identified in the study of Aubert et al., were enriched in differentiating (RFP⁺) cell population, and *Nhlh2* and *Lrrn1* were shown to be expressed in the cortical neuron layer²⁶. Impurity using a single genetic tag was also cautioned in these studies^{11, 12}, and this was similarly thought to be caused by “perdurance of GFP after differentiation”¹¹. Therefore, these data collectively suggest that, regardless of the progenitor specific promoter being employed (Nestin, Sox1, or Sox2), using a single genetic tag can not achieve specific labeling of neural progenitor cells. Adding a companion tag to mark differentiation is a necessary and effective strategy for achieving progenitor cell isolation. These data also suggest that caution should be taken to interpret *in situ* identification data when a stem/progenitor cell or an intermediate precursor is marked by a single tag. Co-staining of a differentiation marker will help verify the identity of the intended primitive cell type.

In the developing cerebral cortex, neural progenitor cells show heterogeneity²⁷⁻³¹. Previous studies have identified two lineally related subtypes³²⁻³⁴, the Pax6⁺ apical radial glia progenitor cells and the Tbr2⁺ basal intermediate progenitor cells³⁵, as well as a population preferentially expressing tubulin alpha-1 promoter²⁸. More recently, further lineage analysis revealed an early presence of neuron-restricted and bipotent (neuron-glia) progenitors in the cortex³⁶. Purification of these subtypes of neural progenitor cells will be an obligatory step towards understanding how development of neural progenitor cells is regulated in neurogenesis and gliogenesis. We anticipate that the dual labeling strategy should also be useful for this purpose. For example, in the case of two lineally-related subtypes, a genetic differential labeling system directed by combining Pax6- and Tbr2-specific expression (Pax6⁺Tbr2⁻) or by pairing Tbr2- and DCX-specific expression (Tbr2⁺DCX⁻) should in principle allow isolation of apical progenitor cells and basal intermediate progenitor cells, respectively. In this regard, combining Pax6 and DCX, or Nestin and DCX (as in this study), would not allow separation of radial glia and intermediate progenitors, and indeed our microarray data showed that both Pax6 and Tbr2 transcripts were highly enriched in GFP⁺RFP⁻ cells. Interestingly, however, our data also showed that candidate progenitor cell-specific genes examined (e.g. Figure 3D and 4A) appeared to show expression patterns similar to Pax6 but differ from Tbr2, indicating that candidate neural progenitor cell specific genes revealed in our microarray analysis were predominantly apically expressed genes, perhaps reflecting the abundance of apical radial glia progenitors over basal intermediate

progenitors that were present in the mouse cortex as well as depletion of intermediate progenitors that show temporal overlap in Tbr2 and DCX expression (Tbr2⁺DCX⁺ cells). Thus, although the GFP⁺RFP⁻ cells isolated using a Nestin/DCX marker combination were a mixed progenitor cell population, the neural progenitor cell transcriptome we obtained in the current study appears largely representative for radial glia progenitor cells.

The neural progenitor cell transcriptome obtained in this study may aid future identification of causal factors for neural progenitor cell state, as one major possible role of a neural progenitor cell specific gene would be to participate in the decision either to self-renew or differentiate^{17, 24, 37-39}. As one example, the selective expression of TAM family RTKs in this transcriptome led to the identification of an important role of TAM receptors in cortical neural progenitor cell maintenance. It would be interesting to further investigate whether TAM receptors also play a role in other areas of active neurogenesis such as in the adult SVZ. On the other hand, some of these candidate genes may have functions not directly related to the cell fate specification or not commonly shared by different types of neural progenitor cells. For example, our profiling data revealed specific enrichment of nidogens (Nid1 and Nid2), tight junction proteins (Tjp1 and Tjp2), or gap junction proteins (Gapalpha1 and Gapalpha4) in neural progenitor cells. These molecules may not be directly involved in the regulation of self-renewal vs. differentiation, for example, previous genetic studies have found that basement membrane attachment does not appear to be linked to progenitor cell fate⁴⁰. These genes are likely to be more specifically associated with various other aspects of radial glia functions, such as cell polarity^{41, 42}, interkinetic nuclear migration⁴³, radial unit formation and maintenance⁴⁴, and/or guiding the migration of daughter neurons⁴⁵. The neural progenitor cell transcriptome may also aid identification of potential biomarkers and targets for brain cancer treatment. Recent studies have implicated that brain cancers may be derived from neural stem/progenitor cells through dysregulation of normally tightly controlled self-renewal⁴⁶⁻⁴⁹. Molecules that are critical for maintaining the self-renewal state of neural stem/progenitor cells are expected to also contribute to brain tumor initiation and/or progression. In this regard, our observation of a critical role of the TAM receptors in neural progenitor cell maintenance and the previous study reporting their role in glioma growth are intriguing with respect to the proposed link of brain tumors and neural stem/progenitor cells. In addition, it is interesting to note that some of the pleiomorphic adenoma gene family molecules (Plagl1, Plagl1, and Plagl2) are enriched in GFP⁺RFP⁻ cortical cells. One member of this family of molecules, Plagl2, was recently implicated in suppressing differentiation of neural stem cells and glioma-initiating cells via modulating Wnt pathway⁵⁰. Further characterization of the identified neural progenitor transcriptome may also provide novel insight into the mechanisms of carcinogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Dual reporter labeling for neural progenitor cell isolation

(A) Transgenic expression of monomeric RFP under the control of DCX promoter. RFP expression pattern in the developing forebrain is consistent with the endogenous DCX protein revealed by an anti-DCX antibody (green). RFP expressing cells are outside the ventricular zone in the cortex (ctx) and ganglionic eminence (ge), correlating with migrating neurons. (B) Transgenic expression of EGFP under the control of nestin promoter and enhancer. GFP expression pattern is similar to the endogenous nestin protein (red), showing restricted expression around ventricles. (C) Expression of RFP and GFP in the cortex of the double transgenic mice. VZ, ventricular zone; IZ, intermediate zone; CP, cortical plate. (D) FACS analysis of negative cortical cells from wild-type C57BL/6J mice. (E) Cortical cells from Nestin-EGFP single reporter mice. (F) Cortical cells from DCX-RFP single reporter mice. (G) Mixed cortical cells from B and C. (H) Cortical cells of RFP and GFP double reporter mice. (I) GFP⁺RFP⁻ cortical cells purified from double reporter mice. (J) RFP⁺ cortical cells purified from DCX-RFP reporter mice.

Figure 2. Characterization of purified GFP⁺RFP⁻ cortical cells

(A) Quantitative PCR analyses for enrichment of neural progenitor cell specific markers (in reference to RFP⁺ cells) in GFP⁺RFP⁻ cortical cells isolated from DCX-RFP/Nestin-GFP double transgenic mice and GFP⁺ cells derived from Nestin-GFP single reporter mice. (B) Purified GFP⁺RFP⁻ cortical cells formed both primary and secondary neurospheres, whereas RFP⁺ cortical cells do not. Initial cell plating density in neurosphere assays were at 1000 or 5000 cells/well (500μl in total volume) in a 24 well plate. (C) GFP⁺RFP⁻ cortical cells are multipotential *in vitro*, differentiating into neurons (DCX⁺), astrocytes (GFAP⁺), or oligodendrocytes (O4⁺). (D) Neurosphere formation analyses comparing purified GFP⁺RFP⁻ cells from DCX-RFP/Nestin-GFP double reporter mice and GFP⁺ cells from Nestin-GFP single reporter mice in reference to mock sorted total cortical cells (cortical cells that went through flow cytometer without GFP and RFP gating. We observed that cortical cells after sorting were less potent in generating neurospheres perhaps due to stress associated with sorting process).

Figure 3. Molecular/functional annotations and expression analyses of GFP⁺RFP⁻ or RFP⁺ cell-specific genes

Microarray profiling of cortical cells identified many genes differentially enriched (showing 2 fold or more difference in RNA ratio) in GFP⁺RFP⁻ cells or RFP⁺ cells. Profiling data are deposited with GEO, Gene Expression Omnibus (accession number GSE22946). (A) Annotation of top genes in the category of biological process. (B) Annotation in the category of molecular function. (C, D) Quantitative RT-PCR (C) and RNA *in situ* hybridization (D) analyses of candidate GFP⁺RFP⁻ cell-specific genes identified by microarray.

Figure 4. Functional analysis of the TAM receptor tyrosine kinase Axl in the cortex
 (A) Immunohistochemistry showed enrichment of Axl protein in the apical area of the cortical VZ, co-localizing with the proliferation marker Ki67 or neural progenitor marker Pax6. (B) Expression of a dominant negative Axl receptor (Axl-DN) or a soluble Axl receptor (Axl-ECD) in the cortex caused more cells moving into the intermediate zone (IZ) and cortical plate (CP), suggesting induced differentiation and subsequent outward migration. (C) Cells that had left the VZ early due to expression of Axl-DN mutant protein were positive for neuronal marker β III-tubulin, consistent with induced differentiation by inhibition of TAM receptor function. Arrows show examples of Axl-DN expressing cells positive for β III-tubulin expression. (D) Acutely dissociated cell culture derived from electroporated brains (at E15.5) showed expression of Axl-DN caused neuronal differentiation of the affected cells, evidenced by an increase in the number of β III-tubulin positive neurons and a concomitant decrease in nestin positive progenitors. (E) Expression of Axl-DN mutant protein in the cortex caused a reduction in the number of Pax6⁺ or Tbr2⁺ neural progenitor cells, as a result of early differentiation. Scale bars indicate 50 μ m. Quantifications are represented as mean $\pm$ SEM. * $p < 0.01$ with cell distributions in the area of VZ/SVZ or the area of IZ plus CP. ** $p < 0.01$

Figure 5. Genetic analyses of TAM receptor function in neural progenitor cells

E15.5 brains were collected from wild-type, Axl single and Axl/Mer double knockout mice after labeling with BrdU for 24 hours and were analyzed for BrdU/Ki67 co-staining. (A) Axl/Mer double mutant brains displayed more BrdU⁺Ki67⁻ cells (differentiating cells) outside the proliferating zone (VZ/SVZ). Images were from two different brains of each genotype. Arrows mark the noticeable band of differentiating cells in the double mutant brains. (B) The BrdU⁺Ki67⁻ cells above the proliferating zone were positive for βIII-tubulin, indicating they were newly-generated neurons migrated out of the VZ/SVZ. The arrow shows one example of BrdU⁺βIII-tubulin⁺ cell. (C) BrdU⁺Ki67⁻ cells in the total BrdU positive cell population were documented in comparison between Axl/Mer double mutant brains and wild-type brains along the radial axis of respective images of the same magnification. Arrows mark examples of BrdU⁺Ki67⁻ cells. More progenitor cells in the Axl/Mer double mutant brains showed early cell cycle exit. Scale bars indicate 100μm. Quantifications are represented as mean ± SEM. **p* < 0.001

Table 1
Top protein domains enriched in GFP⁺RFP⁻ cells (DAVID analysis tool)

Term	Genes	%*	P-Value	Fold Enrichment**
EGF-LIKE DOMAIN PROTEIN	21	1.3	5.10E-08	4.2
KINESIN HEAVY CHAIN	16	1	3.30E-06	4.2
HISTONE H2B	8	0.5	5.90E-06	9.2
SOX TRANSCRIPTION FACTORS	10	0.6	7.10E-06	6.6
COLLAGEN ALPHA CHAIN	20	1.2	8.20E-06	3.2
DNA REPLICATION LICENSING FACTOR	7	0.4	9.90E-06	10.7
CYCLINS	9	0.6	1.00E-05	7.3
ZINC FINGER PROTEIN ZIC AND GLI	8	0.5	1.20E-05	8.5
FRIZZLED	8	0.5	6.60E-05	6.9
LAMININ	10	0.6	2.50E-04	4.5
ZINC FINGER PROTEIN	7	0.4	4.00E-04	6.4
BASIC HELIX-LOOP-HELIX TRANSCRIPTION FACTOR HES-RELATED	6	0.4	100E-03	6.9
LIM DOMAIN CONTAINING PROTEIN	14	0.9	1.10E-03	2.8
TYROSINE PROTEIN KINASE	17	1.1	1.20E-03	2.5
E2F	5	0.3	1.50E-03	8.6
HISTONE H2A	8	0.5	3.90E-03	3.8
BASIC HELIX-LOOP-HELIX PROTEIN NEUROGENIN-RELATED	6	0.4	4.40E-03	5.2
HISTONE H1/H5	5	0.3	6.00E-03	6.3
RHOMBOID-RELATED	4	0.2	6.40E-03	9.2
ETS	8	0.5	7.00E-03	3.4
SPECTRIN-LIKE CELL STRUCTURE PROTEIN	10	0.6	9.00E-03	2.8
LOW DENSITY LIPOPROTEIN RECEPTOR	6	0.4	9.80E-03	4.4
SIGNAL TRANSDUCER AND ACTIVATOR OF TRANSCRIPTION	4	0.2	1.10E-02	7.9
CYTOKINE RECEPTOR	7	0.4	1.10E-02	3.6
CTX-RELATED TYPE 1 TRANSMEMBRANE PROTEIN	5	0.3	1.20E-02	5.3
ATP-DEPENDENT HELICASE SMARCA (SWI/SNF-RELATED MATRIX-ASSOCIATED ACTIN-DEPENDENT REGULATOR OF CHP	8	0.5	1.30E-02	3.1
FIMBRIN/PLASTIN	3	0.2	1.50E-02	13.8
RHO-TYPE GTPASE ACTIVATING PROTEIN	3	0.2	1.50E-02	13.8
ANILLIN/RHOTEKIN (RTKN)	3	0.2	1.50E-02	13.8
MONOCARBOXYLATE TRANSPORTER	5	0.3	1.50E-02	4.9
RECA/RAD51/RADA DNA STRAND-PAIRING FAMILY MEMBER	4	0.2	1.60E-02	6.9
MARVEL-CONTAINING POTENTIAL LIPID RAFT-ASSOCIATED PROTEIN	5	0.3	2.50E-02	4.3
TRANSCRIPTIONAL ENHANCER FACTOR (TEF) RELATED	3	0.2	2.80E-02	10.3
XP-G/RAD2 DNA REPAIR ENDONUCLEASE FAMILY	3	0.2	2.80E-02	10.3
RAS GTPASE-ACTIVATING PROTEIN WITH IQ MOTIF	3	0.2	2.80E-02	10.3
METALLOTHIONEIN	3	0.2	2.80E-02	10.3

*
“%” indicates genes/total genes

**
“Fold enrichment” indicates enrichment fold change over background information. For example, for EGF-like domain protein, there is roughly 1.3% (in progenitor cell population) over 0.31% (background level) = 4.2 fold enrichment.

Table 2
Gene families selectively enriched in GFP⁺RFP⁻ cells

A. Examples of gene families known to function in neural progenitor cell maintenance

Gene family	Members enriched in GFP ⁺ RFP ⁻ cells
Notch ⁵¹	Notch1, 2, 3, 4
Hippo ⁵²	Yap1, Tead1, 2, 3
Gli ⁵³	Gli1, 2, 3
TGF beta ⁵⁴	TGF beta1, 2, 3

Table 2
Gene families selectively enriched in GFP⁺RFP⁻ cells

B. Examples of gene families not known for a particular function in neural progenitor cells

Gene family	Members enriched in GFP ⁺ RFP ⁻ cells	Fold Change (GFP ⁺ RFP ⁻ /RFP ⁺)	P-value
TAM receptor tyrosine kinases	Axl	8	2.29E-07
	Tyro-3	2.6	4.61 E-05
	Mer*	1.7	1.57E-02
	Protein S	3.9	2.75E-03
Sal-like	Sall1	7.1	9.14E-08
	Sall2	2.7	4.42E-04
	Sall3	5.5	3.57E-07
	Sall4	2.8	2.48E-04
Metallothionein	Mt1	7.3	1.35E-05
	Mt2	17.2	7.61E-07
	Mt3	9.6	1.92E-06
Nidogen	Nid1	11.2	5.42E-05
	Nid2	5.4	8.90E-05

* Fold enrichment in GFP⁺RFP⁻ cells less than the 2 fold artificial cut-off