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Identification of the Tctex-1 regulatory element that directs expression to neural stem/progenitor cells in developing and adult brain

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Abstract

Previous studies showed that Tctex-1 immunoreactivity is selectively enriched in the germinal zones of adult brain. In this report, we identify a regulatory region of the Tctex-1 gene that is capable of directing transgenic expression of green fluorescent protein (GFP) reporter that recapitulates the spatial and temporal expression pattern of endogenous Tctex-1. This construct specifically targeted expression to the nestin$^+$/Pax6$^+$/GLAST$^+$ radial glial cells and Tbr2$^+$ intermediate progenitors when the reporter construct was delivered to developing mouse neocortex via in utero electroporation. Characterization of mice transgenically expressing GFP under the same regulatory element showed that the GFP expression is faithful to endogenous Tctex-1 at the subgranular zone (SGZ) of dentate gyrus, ventricular/subventricular zone of lateral ventricles, and ependymal layer of 3rd ventricle of adult brains. Immunolocalization and bromodeoxyuridine incorporation studies of adult SGZ in four independent mouse lines showed that Tctex-1:GFP reporter selectively marks nestin$^+$/GFAP$^+$/Sox2$^+$ neural stemlike cells in two mouse lines (4 and 13). In two other mouse lines (17 and 18), Tctex-1:GFP is selectively expressed in type-2 and type-3 transient amplifying progenitors and a small subset of young neuronal progeny. The P/E-Tctex-1 reporter mouse studies independently confirmed the specific enrichment of Tctex-1 at adult SGZ stem/progenitor cells. Furthermore, these studies supported the notion that an analogous transcriptional program may be used to regulate neurogenesis in embryonic cerebral cortex and adult hippocampus. Finally, the genomic sequences and the reporter mouse lines described here provide useful experimental tools to advance adult neural stem cell research.

Keywords

Tctex-1; adult neurogenesis; neural stem/progenitor cells; regulatory element

INTRODUCTION

Stem cell research in the central nervous system holds great promise for the development of novel therapies for brain damage and other human diseases. Researchers are keenly interested in adult stem cells because they do not pose the ethical questions raised by embryonic stem cells. One of the ultimate goals in adult stem cell research is to better

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understand the biology of these cells so that scientists may develop strategies to directly manipulate them within the damaged brain. In the dentate gyrus (DG) of the hippocampus, new neurons continue to be born from resident stem/progenitor cells at the subgranular zone (SGZ) throughout our lives (Altman and Das, 1965; Cameron et al., 1993; Eriksson et al., 1998; Kornack and Rakic, 1999). Adult-generated granular neurons are indistinguishable from neighboring granular neurons in terms of their morphology, synaptic connections, and electrophysiological properties (Cameron et al., 1993; Hastings and Gould, 1999; Markakis and Gage, 1999; van Praag et al., 2002). Finally, the functional importance of the adult-generated hippocampal neurons in learning and memory has been experimentally confirmed (Saxe et al., 2006; Clelland et al., 2009; Saxe et al., 2007; Zhang et al., 2008).

At least three partially overlapping cell populations have been identified at adult SGZ based on their distinct morphologies and expression of molecular markers (Ehninger and Kempermann, 2008; Zhao et al., 2008). Type-1 progenitors (or neural stem-like cells, Type B cells (Seri et al., 2001)), which rarely divide, can be identified by their expression of glial fibrillary acidic protein (GFAP) and nestin (Fukuda et al., 2003) and by their radial processes with highly elaborated arbors branching into the molecular layers (Mignone et al., 2004). A nuclear transcription protein Sox2 has also been recognized as a marker for mainly Type-1 cells (Steiner et al., 2006). There are at least two types of transient amplifying progenitors. Type-2 cells (or Type D cells (Seri et al., 2001)), are most highly proliferative cells at SGZ, have short horizontal processes; they express transcription factor Tbr2 (T-box brain gene 2) (Hodge et al., 2008) and low-level neuronal-lineage markers such as doublecortin (DCX). Type-3 progenitors represent cells committed to the neural fate, and during their transit to exit cell cycle to become postmitotic neurons. These cells are highly variable in morphology and often have vertically orientated processes displaying DCX, polysialylated neural cell adhesion molecule, and TuJ1. Whereas the identity of the neural stem cells in the adult DG has been under debate (Garcia et al., 2004; Palmer et al., 1997; Seaberg and van der Kooy, 2002; Seri et al., 2001), the prevailing model has been that Type-1 progenitors represent the primary precursors that give rise to transient amplifying progenitors, which subsequently differentiate into granule neurons (Seri et al., 2004; Seri et al., 2001).

It has been recently discovered that Tctex-1 (or DYNLT1 (Pfister et al., 2005)), previously recognized as a light chain of cytoplasmic dynein (King et al., 1996), is selectively enriched in stem-like cells and cycling progenitors, but not in mature granule cells and astrocytes, in the adult dentate gyrus (DG) (Chuang et al., 2001; Dedesma et al., 2006). The SGZ-enriched Tctex-1 expression pattern was confirmed by in situ hybridization, suggesting that Tctex-1 expression is primarily regulated at the transcriptional level. In the present study, we identify the genomic sequences specifying Tctex-1 expression in the dentate progenitors of adult hippocampus and generate Tctex-1:GFP reporter mice in which the adult hippocampal stem-like and granular neuron progenitors were genetically marked.

MATERIALS AND METHODS

Plasmids and generation of transgenic mice

Chicken actin-CMV (CAG) promoter-directed HcRed was obtained from Dr. Connie Cepko (Harvard). E/nestin:hGFP (Roy et al., 2000a; Roy et al., 2000b; Wang et al., 2000) was obtained from Dr. Neeta Roy (Weill Medical College). pGLAST-DsRed2 and pTα-1-DsRed2 (Ever and Gaiano, 2005) were obtained from Dr. Nicholas Gaiano (Johns Hopkins University School of Medicine). To generate the reporter constructs P/E-Tctex-1:GFP and P/E-Tctex-1:DsRed, an EcoR1 fragment (spanning −5,873 NT to 2861NT of Tctex-1 genomic sequence) was taken from a mouse Tctex-1 bacterial artificial chromosome contig (clone RP23-122p23, CHORE). The sequences between NT 155 and NT165 were replaced by GFP

or DsRed coding sequence (Fig. 1A). Finally, ~9.5 kb fragments containing the entire Tctex-1 regulatory domain and reporter gene of the above constructs were transferred and inserted into the backbone of pCAGIG vector (gift of Connie Cepko (Matsuda and Cepko, 2004)) to produce the transgenes P/E-Tctex-1:GFP (Fig. 1A) and P/E-Tctex-1:DsRed.

For transgenic mouse production, the ~10 kb SfoI/StuI fragment from P/E-Tctex-1:GFP construct was gel purified and used for pronuclear injection (Hogan et al., 1986). DNA was injected into fertilized F_2 eggs obtained from matings of C57BL/6JxCBA/J F1 mice. Sixteen founders out of a total of 47 live-born mice were identified to be positive for transgene by genomic Southern blot assay and polymerase chain reaction (PCR) using primers recognizing GFP (5′-GAGGAGCTGTTCACCGGGGTG-3′ and 5′-GTGGTTGTCGGGCAGCAGCAC-3′). Adult animals of four independent lines (4, 13, 17 and 18) were used for experiments. Lines were propagated through backcrossing with CD1 mice; all studies were carried out with F_2-F_4 mice. No detectable change in the GFP expression pattern was found as the breeding progressed. All animal procedures were approved by the Institutional Animal Care and Use Committee of Weill Cornell Medical College.

Antibody (Ab) characterization

Tctex-1—This Ab recognized a single band of ~13 kD on immunoblots containing lysates of rat brains and human embryonic (HEK) cells (Chuang et al., 2001; Sachdev et al., 2007; Tai et al., 1998); this band was largely diminished in Tctex-1-shRNA treated cells (Chuang et al., 2005). Tctex-1 Ab immunostaining on brain sections match the in situ hybridization data (Dedesma et al., 2006).

NeuN—This Ab recognized two bands in the 46-48 kDa range on immunoblots containing whole brain extracts or protein isolated from purified nuclei (Mullen et al., 1992). The NeuN antibody is reported to label most classes of neurons, and negative controls run by the manufacturer to test specificity include nonneuronal cells such as fibroblasts (manufacturer's technical information). We foud that the antibody does not label any dividing cells in the adult SGZ ((Dedesma et al., 2006); current study).

BrdU—This Ab did not generate any signal in tissue from an animal that has not been injected with BrdU.

DCX—This Ab recognized a doublet of ~40 kDa on immunoblots of HEK cell lysate ectopically expressing DCX, but not in non-transfected HEK cells (Manufacture's data sheet). Immunoreactivity obtained with these antibodies was completely abolished through preadsorption with peptide corresponding to the immunogen (Noctor et al., 2008).

SOX-2—In the current study, this Ab labeled the nuclei of $Nestin^+/GFAP^+$ Type-1 like cells in adult SGZ, consistent with the previous reports (Komitova and Eriksson, 2004; Steiner et al., 2006),

GFAP—This Ab recognized a single band of ~52 kDa on immunoblots containing astrocytes (Debus et al., 1983). In the current study, this Ab stained radial glia-like stem cells in subventricular zone (SVZ) and DG, consistent with the previous reports (Doetsch et al., 1999; Seri et al., 2001).

Nestin—This Ab recognized a doublet band of ~200 Kda on immunoblots containing E15 rat spinal cord extracts (Hockfield and McKay, 1985). In the current study, this Ab stained

radial glia-like neural progenitors in adult mouse SVZ and DG, consistent with the previous report (Seri et al., 2001).

GFP—No immunofluorescence was detected on brain sections lacking GFP transgene. Western blot analysis on lysates of cells expressing GFP demonstrates a single band at ~30 kDa.

DsRed—No immunofluorescence was detected on brain sections lacking HcRed or DsRed transgene.

Tbr1—Tbr1 Ab immunostaining on brain sections matched in situ hybridization data; this Ab did not stain Tbr1 knockout brain section, and did not generate any signal on immunoblots using brain protein extracts of Tbr1 knockout mice (Englund et al., 2005).

Tbr2—This Ab recognized a single 73-kD band, matching the molecular weight of Tbr2 (Quinn et al., 2007). The Tbr2 Ab did not label mature neurons and showed very little colocalization with Pax6-expressing cells in the embryonic VZ ((Englund et al., 2005); Fig. 2 in the current study).

Ki67—A double band at 245-395 kDa is detected by Western blot analysis (Key et al., 1993). In the current study all Ki67-labeled cells were found to be colabeled with short-pulse BrdU-labeled cells, as expected for specific staining of proliferating cells.

Pax6—This Ab recognized two bands of 47 and 39 kDa (corresponding to two alternatively spliced PAX6 isoforms) on immunoblots containing chicken retinal lysates (Ziman et al., 2003). Staining with this Ab coincided with the mRNA expression pattern of mouse Pax6 (Stoykova and Gruss, 1994).

Alexa dye-conjugated (Invitrogen), biotinylated-conjugated secondary Ab (Vector), and Cy5-conjugated (Jackson) secondary Ab were also used.

In utero electroporation

In utero electroporation (IUE) was performed exactly as described (Saito and Nakatsuji, 2001; Tabata and Nakajima, 2001). Briefly, CD1 mice (gestational day 13.5) are anesthetized with an i.p. injection of ketamine/xylazine mixture. Endotoxin-free plasmid DNA (1 μl total volume containing either 2 μg of a single plasmid or 1 μg each of two plasmids) was injected through the uterus into the targeted brain regions of embryos using pulled glass capillaries (Drummond Scientific). Voltage pulses (37V, 50 msec; BTX Square Wave gene pulser) are generally delivered into the embryo by holding the embryo in parallel along its anterior-posterior axis through the uterus with forceps-type electrodes.

BrdU treatment and immunohistochemistry

Embryonic brains positively transfected based on the fluorescent protein expression were harvested 40 h later and fixed in 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH 7.4) overnight at 4°C. For adult brain harvest, animals were perfused with 4% PFA (Dedesma et al., 2006). Coronal sections of 40 μm were cut on a Leica vibratome (Leica, Nussloch, Germany). Immunostaining was carried out by using a free floating method previously described (Dedesma et al., 2006) except an antigen retrieval procedure was incorporated for Tbr1 and Tbr2 detection (Hevner et al., 2001). Immunolabeling of GFP and DsRed were used routinely to detect the reporter proteins in electroporated brain slices. Negative controls (e.g., omitting primary antibodies) did not give rise to specific signal in all studies. All immunolabeled samples were examined on a Leica confocal microscope. At

least 3 mice were analyzed for each experiment. Statistical analysis was performed using GraphPad Prism 4.0b software (GraphPad Software, Inc).

For BrdU labeling, animals were injected with a single pulse of BrdU (100 μg/g body weight). The injections were repeated 3 times with 2-h intervals. Animals were perfused with 4% PFA at survival time points of 1 h, 24 h, and 72 h after the last BrdU administration. Brains were sectioned, stained, and scored as described (Dedesma et al., 2006). To prepare the images for publication, the original confocal TIFF files were imported into Adobe Photoshop (Adobe Systems, Mountain View, CA); all images were processed, adjusted for brightness and contrast, and resized to 300 dpi by using this application.

RESULTS

Tctex-1 genomic sequences specifying GFP reporter expression in neural stem and progenitor cells in developing neocortex

A recent report suggested that a similar transcriptional program may control neurogenesis in adult SGZ and in embryonic cerebral cortex (Hodge et al., 2008). This finding prompted us to search for the genomic region of Tctex-1 that can direct its selective adult SGZ expression by using developing neocortex as an expression model system because the IUE method permits rapid delivery of reporter genes into neocortex. Based on the alignment of Tctex-1 genes of different species, we designed and generated a GFP reporter construct consisting of the GFP cDNA driven by an evolutionally conserved ~6-kb 5′-upstream, ~2-kb intron 1 and ~0.2 kb intron 2 segment of mouse Tctex-1 (i.e., P/E-Tctex-1:GFP; Fig. 1 A).

In these experiments, the P/E-Tctex-1:GFP plasmid, along with a control reporter plasmid (CAG promoter-directed HcRed), were co-transfected into embryonic day 13.5 mouse neocrotices via IUE. Confocal examination of the cortical sections harvested 40 h after transfection showed that a large fraction of HcRed transfected cells with strong red fluorescence resemble post-mitotic young neurons; they had developed multi-polar processes and migrated into the intermediate zone (IZ). The remainder of the $HcRed^+$ cells at the ventricular zone (VZ) and SVZ exhibited much weaker red fluorescence, indicating attenuated promoter activity (Fig. 1 C). In contrast, the cell bodies of the large majority of GFP-expressing cells were distributed throughout the VZ and SVZ zones (Fig. 1 B). Many of these cells bore features of radial glial (RG) cells, displaying extended radial processes with their endfeet contacting both the ventricular and pial surfaces (Fig. 1 B, arrows).

Immunostaining of several transcription factors (e.g., Pax6, Tbr2, Tbr1) that mark cells at different stages during neocortical development was carried out to further characterize the types of cells targeted by the Tctex-1 promoter/enhancer (Fig. 2). Pax6 was specially expressed in RG cells whose cell bodies are enriched at the VZ (Englund et al., 2005;Gotz et al., 1998). Tbr2 was expressed in the intermediate progenitors that are distributed predominantly in the SVZ (Englund et al., 2005;Hodge et al., 2008). Tbr1 has been previously found to be expressed by postmitotic neurons that are distributed throughout the IZ, subplate, and cortical plate (Englund et al., 2005;Hevner et al., 2001). The immunostaining showed that 76% ± 3% and 33% ± 1% (300 cells in 3 independent experiments) of Tctex-1:GFP^+ cells also expressed Pax6 (Fig. 2 A-C) and Tbr2 (Fig. 2 D-F), respectively, and practically no Tctex-1:GFP^+ cells were $Tbr1^+$ (Fig. 2 G-I).

Several cell-type specific reporters have been shown to be able to target distinct populations of neocortical cells (Gal et al., 2006; Wang et al., 1998; Wang et al., 2007). For example, Tα-1 promoter preferentially targets intermediate progenitors and post-mitotic young neurons (Wang et al., 2000). The GLAST promoter preferentially targets RG cells (Gal et al., 2006). To independently analyze the cell types that the Tctex-1 promoter targeted, we

co-electroporated P/E-Tctex-1:GFP plasmid with pGLAST:DsRed2 or pTα-1:DsRed into neocorticies and examined the coincidence of cells expressing both GFP and DsRed. Confocal microscopic examination of brain sections harvested 40 h post-transfection showed that 80% ± 4% and 31 ± 3% of GFP^+ cells also expressed GLAST- (Fig. 3 A-C) and Tα-1-promoter-directed DsRed2 (Fig. 3 D-F).

Finally, reporter constructs driven by the enhancer element of nestin (e.g., E/nestin:hGFP) have been reported to be useful for targeting both RG as well as intermediate progenitors in embryonic brains (Gal et al., 2006). We co-electroporated the E/nestin:hGFP reporter construct and the reporter plasmid in which DsRed gene was under the control of P/E-Tctex-1 (i.e. P/E-Tctex-1:DsRed) into developing neocortex and compared their expression patterns. Interestingly, the transfected cortical slices showed that almost 100% of $DsRed^+$ cells also expressed GFP (Fig. 3 G-I). Taken together, the promoter profiling and immunostaining studies suggested that our identified Tctex-1 promoter/enhancer directed specific gene expression in both RG and intermediate progenitors in developing neocortex.

Generation and characterization of P/E-Tctex-1:GFP transgenic mice

To evaluate whether the genomic fragment of Tctex-1 that directed specific expression in the neocortical progenitors also had transcription activity in adult neural stem/progenitors, we generated transgenic mice carrying P/E-Tctex-1: GFP. We obtained a total of 16 independent lines of such mice. Among these, all four independent founder mice we tested generated progeny stably expressing the transgene. Examination of the GFP immunoreactivity of adult (i.e. 8-week-old or older) mouse brains of all 4 lines (4, 13, 17 18) showed that cells expressing Tctex-1:GFP, like those that expressed endogenous Tctex-1 (Dedesma et al., 2006), were enriched at the SGZ of DG and the lateral ventricle (LV) (see below). Nevertheless, close examination suggested that the GFP expression patterns in these 4 mouse lines fell into two groups and our characterization of the natures of the GFP^+ cells were described separately below.

P/E-Tctex-1:GFP is expressed in the $GFAP^+$ primary precursors of adult SGZ and SVZ

In two independent lines (i.e., line 4 and line 13; referred to as line 4 for simplicity), Tctex-1:GFP expressing cells at DG exhibited long processes that radiated through granular layer and branched out at molecular layer (Fig. 4 A, R). These cells were morphologically similar to that of neural stem-like cells described for this brain region (Mignone et al., 2004). Indeed, a large subset of GFP expressing cells was positive for Sox2 (arrows in Fig. 4F, G), a transcription factor primarily associated with the DG neural stem-like cells (Komitova and Eriksson, 2004). Furthermore, the radial processes of $GFP^+/Sox2^+$ cells were immunolabeled with GFAP (arrowheads in Fig. 4 F, H) as well as nestin (arrowheads in Fig. 4 J, L). Endogenous Tctex-1 was also detected in both the cell bodies and processes of these GFP^+ cells (Fig. 4 N, O). Only a subset of the SGZ Tctex-1:GFP^+ cells were positive for Ki67, a marker for cells in the cell cycle (arrowheads in Fig. 4 A-C, R-T). However, using a protocol that involved three BrdU injections at 2-h intervals and harvest 1 h after the last injection, ~25% of the $BrdU^+$ SGZ cells were GFP^+ (Fig. 4 A, B). These $BrdU^+$ cells most likely represent the transient-amplifying cells, consistent with the presence of horizontal processes (Fig. 4 A, B, arrowhead). These results collectively suggested that GFP in these two transgenic lines was predominantly expressed in $Sox2^+/GFAP^+/nestin^+$ neural stem-like cells and early amplifying progenitors of adult SGZ.

In the lateral wall of the LV, the majority of Tctex-1:GFP cells exhibited $GFAP^+$ processes that intercalated extensively between other cells, thus most likely to be type-B cells (Fig. 4 V-Z). GFAP Ab also selectively immunolabeled striatial astrocytes, Tctex-1:GFP was, however, undetectable in these cells (data not shown). Although rare, GFP was also detected

in the $BrdU^+/Ki67^+$ proliferating cells (SD Fig. 1A-J). These cells were often solitary (SD Fig. 1F-J, arrow) and $GFAP^-$ (data not shown), most likely representing type-C cells. Prominent GFP signal was also found in cells along anterior medial walls overlying the septum, which were also $GFAP^+$ (data not shown).

Finally, prominent GFP signal was found in a large subset of cells (both ependymal cells and process-bearing tanycytes) along the ependymal layer of the 3rd ventricle, where we confirmed that endogenous Tctex-1 was also selectively enriched (SD Fig. 1K-M). None of the GFP^+ cells in the 3rd ventricle were labeled by Ki67 or BrdU (SD Fig. 1N-R).

P/E-Tctex-1:GFP is predominantly expressed in the transient amplifying progenitors of adult SGZ and SVZ

Examination of the DG regions of mouse lines 17 and 18 (hereby referred to as line 17) showed that Tctex-1:GFP^+ cells and $NeuN^+$ mature granule neurons were mutually exclusive from each other (Fig. 5E-H). In contrast to the Tctex-1:GFP cells in mouse line 4, a significant fraction (~ 48%) of Tctex-1:GFP cells were Ki67-positive; Fig. 5 I-L). We then characterized the proliferation ability of Tctex-1:GFP^+ cells by using BrdU incorporation assay. In these experiments, the animals received three consecutive injections of BrdU within 6 h and were then allowed to survive for an additional 1 h, 24 h, or 72 h after the last injection. About 26 ± 4% of 1-h BrdU-incorporated early progenitors were also Tctex-1:GFP^+ (Fig. 5 Q-T). Consistently, these cells tended to have tangentially oriented cell bodies and short horizontal processes (Fig. 5T, arrows). Longer survival time resulted in an increase in the fraction of Tctex-1:$GFP^+/BrdU^+$ cells out of total $BrdU^+$ cells (i.e., 55 ± 6% at 24-h harvest and 70 ± 3.3% at 72-h harvest), indicating that Tctex-1:GFP was also expressed in the later progenitors.

We further analyzed the identities of Tctex-1:GFP SGZ cells in these mouse lines by colocalization study using previously described Abs that mark type-2 or type-3 progenitors. Nuclear Tbr2 labeling primarily highlighted the type-2 progenitors and a subset of type-3 progenitors (Hodge et al., 2008). In contrast, DCX-derived process labeling was distributed primarily in type-3 progenitors as well as post-mitotic young neurons. Under low-power survey, it appeared at the SGZ, that the DCX^+ cells outnumbered the Tctex-1:GFP^+ cells, which outnumbered the $Tbr2^+$ cells. Detailed confocal examination showed that essentially all Tbr2-immunoreactive cells were also Tctex-1:GFP^+ (Fig. 6 A-C, arrows). However, a population of $GFP^+/Tbr2^-$ cells also existed (Fig. 6A-C, arrowheads). The DCX co-localization studies showed that all three populations — DCX^+/GFP^+ cells (Fig. 6 E, arrows), DCX^+/GFP^- cells (Fig.6 D-F, open arrow), and DCX^-/GFP^+ cells (Fig. 6 D-F, arrowhead) — were detected. Finally, very few, if any, GFP-expressing SGZ cells were positive for Sox2 (data not shown), nestin (Fig. 6 J-L), or GFAP (Fig. 6 G-I). Although the GFAP-labeled processes were often in close proximity with the GFP^+ processes, most of them were not coincidental. These results, collectively, suggested that Tctex-1:GFP expressing cells in these mice primarily represented type-2 and type-3 progenitors.

In the LV of transgenic line 17, intense GFP signal was present in a large fraction of ependymal cells (SD Fig. 2A-E, arrowheads) and SVZ cells, many cells in the latter were also labeled with Ki67 and BrdU (Fig. 5 U-Y). The $GFP^+/Ki67^+/BrdU^+$ cells most likely represented transient-amplifying type-C cells (open arrow, Fig. 5 V), which typically had evaginated nuclei (Doetsch et al., 1997), or type-A neuroblasts. The majority of GFP^+ cells with $GFAP^-$ (arrowheads, SD Fig.2 A-E), nevertheless, $GFAP^+/GFP^+$ cells were also detected (arrow, SD Fig. 2A-E). Finally, almost all cells (both ependymal and tanycytes) along the ependymal layer of the 3rd ventricle throughout the entire rostral-caudal neuroaxis had strong GFP signal (SD Fig.2 F-J).

DISCUSSION

Neural progenitors in developing and adult brains share a similar gene regulation pathway

In this report, we identified a non-coding sequence of Tctex-1 that drives GFP reporter gene expression to neural precursors in developing and adult brain. IUE-based targeting experiments suggested that P/E-Tctex-1-directed GFP expression is predominantly present in the undifferentiated neuroepithelium, but down-regulated in cells undergoing differentiation, thus becoming confined to the VZ/SVZ of developing neocortex. Recent evidence suggested that the Nestin^{+}/Pax6^{+}/GLAST^{+} radial glials and Tbr2^{+} intermediate progenitors of developing neocortex are analogous to the GFAP^{+} type-B cells, and type-A/type-C cells, respectively, of adult SVZ (Kriegstein and Alvarez-Buylla, 2009). This is in agreement with our finding that the same regulatory element directs the specific expression of the reporter to the corresponding cell types of both embryonic and adult VZ/SVZ. However, interestingly, the same regulatory element also specifies the expression to the neural stem/progenitors at the adult SGZ. Although the relationship between embryonic cortical and adult SGZ neural progenitors remains unclear, our data strongly imply that these cells share common machineries for gene expression regulation. This conjecture is consistent with the recent notion that a parallel cascade of transcription factors (e.g., Pax6, Tbr2) exists in the developing neocorticies and the adult DG (Hodge et al., 2008). While this conjecture awaits experimental testing, it is also tempting for us to predict that the neural progenitors of different origins and/or of different developmental stages may exhibit overlapping cellular properties.

Although transgenic mice are the conventional method for screening a regulatory element for gene expression, it is nevertheless time-, labor- and cost-consuming. A methodology which could combine the power and certitude of the transgenic approach together with a rapid screening technique would allow researchers to more quickly define regions of DNA involved in the spatial regulation of a given gene. In this report, we showed that a genomic fragment of the Tctex-1 gene that faithfully directs reporter expression in adult brain could be identified based on IUE-mediated gene expression in developing brain. Matsuda and Cepko (Matsuda and Cepko, 2004) have previously found that “tissue transfection” provides a convenient and rapid method for the in vivo characterization of a promoter/enhancer among several types of retinal cells. The present study suggested that this approach has a broad application in other tissues, including brain.

Identification of a novel regulatory element specifying adult SGZ neural progenitors

In all four P/E-Tctex-1:GFP mouse lines we examined, practically none of the GFP^{+} cells expressed the mature neuronal marker NeuN in the DG, SVZ, or 3rd ventricle (Fig. 5; data not shown). However, examination of the adult SGZ cells expressing GFP reporter in these four mouse lines showed that one group of mice (i.e., lines 4 and 13) had GFP predominantly marking type-1 (GFAP^{+}/Sox2^{+}/ Nestin^{+}) neural-stem like cells whereas the other group of mice (i.e., lines 17 and 18) had GFP predominantly marking type-2/type-3 neural progenitors (Fig. 7). Previous studies showed that the majority of short-lived BrdU-labeled type-2 or 3 progenitors are Tctex-1^{+}, yet, a small fraction (~9.6%) of 4-week-old BrdU+ cells remaining at the SGZ (vs. migrating into the granular cell layer) are Tctex-1^{+}/TuJ1^{-}/NeuN^{-} (Dedesma et al., 2006). The latter may represent the type-1 GFP^{+} cells detected in lines 4 and 13. It is not entirely clear why in these mouse lines the GFP protein preferably marks a somewhat distinct population of adult progenitors; one simple explanation for this difference is a subtle positional effect imposed upon the inserted transgenes.

Available evidence has suggested that the adult SGZ and SVZ adopt parallel cell lineages; in both lineages, the GFAP-expressing astrocyte-like cells represent the primary precursors that give rise to transient amplifying progenitors, which subsequently differentiate into neuroblasts and then mature neurons (Kriegstein and Alvarez-Buylla, 2009)). Thus, it is of interest to observe that the Tctex-1:GFP in line 4 is predominantly expressed in the primary precursors of both SGZ and SVZ, whereas in line 17, Tctex-1:GFP is preferably expressed in the actively self-renewing progenitors of both germinal regions. In the latter case, although the distribution patterns of Tctex-1:GFP and endogenous Tctex-1 were qualitatively identical at the adult SGZ, the BrdU birth-dating experiments suggested that the expression of GFP-Tctex-1 had a slight delay compared to that of the endogenous Tctex-1. In particular, our present study showed less than 30% of 1-h BrdU-labeled cells were Tctex-1:GFP positive. Instead, a higher fraction of 72-h BrdU labeled, type-3 progenitors expressed Tctex-1:GFP.

The 3rd ventricle has been proposed to be another site exhibiting neurogenic activity outside the SGZ and SVZ of the lateral ventricle (Xu et al., 2005). Although the numbers are small, mitotic progenitor cells appear to reside in the ependymal layer of the adult rat 3rd ventricle, and they could differentiate into neurons that could migrate and integrate into the hypothalamus. The neurogenic activity of this area could readily be upregulated upon various stimuli (Xu et al., 2005). Interestingly, GFP driven by the Sox2 promoter has recently been shown to be enriched in 3rd ventricle ependymal cells (Brazel et al., 2005). Our coincidental observation that the 3rd ventricle ependymal cells had a high level of Tctex-1 promoter/enhancer activity (in all 4 independent lines) as well as endogenous Tctex-1 further implies that the 3rd ventricle cells may possess intrinsic characteristics similar to those of SGZ and SVZ progenitors; however, a yet-to-be-identified suppressor may inhibit their mitogenic activity under normal physiological conditions.

A number of genomic sequences that directed specific expression in adult born neural stem/progenitors cells have been isolated (Josephson et al., 1998; Yaworsky and Kappen, 1999; Zimmerman et al., 1994). However, our isolation of a relatively small genomic fragment with great specificity of targeting the stem/progenitors of adult brain adds an additional versatile tool for experimental manipulation of these cells. For example, this regulatory region could be employed for selective loss- or gain-of-function studies of genes of interest. In addition, tools that identify these cells may allow one to directly examine the development of these cells in vivo (i.e., fate mapping). These studies collectively may reveal the bona fide identity of these cells, their biology, and effective strategies to manipulate them.

Perspective function of Tctex-1 in adult stem/neural progenitors

The selective enrichment of Tctex-1 in the adult SGZ neural stem/progenitors cells was first described based on the immunohistology and in situ studies (Chuang et al., 2001; Dedesma et al., 2006). Similar expression pattern of the transgenic GFP reporter directed by the regulatory region of Tctex-1 independently confirmed the specific enrichment of Tctex-1 in these cells. Several cellular functions of Tctex-1, both cytoplasmic dynein-dependent and independent, have been reported (Chuang et al., 2005; Machado et al., 2003; Mueller et al., 2002; Nadano et al., 2002; Nagano et al., 1998; Schwarzer et al., 2002; Tai et al., 1999; Tai et al., 2001). However, the physiological roles of Tctex-1 in the adult-born neural stem/progenitors remains to be tested. Of great interest, Tctex-1 is also synonymous with activator of G protein signaling 2 (AGS2), it binds to the Gβ subunit and involved in non-canonical receptor-independent G protein signaling pathway (Sachdev et al., 2007; Takesono et al., 1999). It has been shown that non-canonical receptor-independent G protein signaling pathway plays a key role in the symmetry of cell division of fly neuroblasts and C. elegans embryoes (Gotta et al., 2003; Schaefer et al., 2001). A mammalian activator of G

protein signaling 3 (AGS3) has recently been suggested to participate in the cell fate determination of neural progenitors during neocortical development (Sanada and Tsai, 2005). It would be interesting to investigate Tctex-1's role in the cell fate choice of adult stem/neural progenitors in future studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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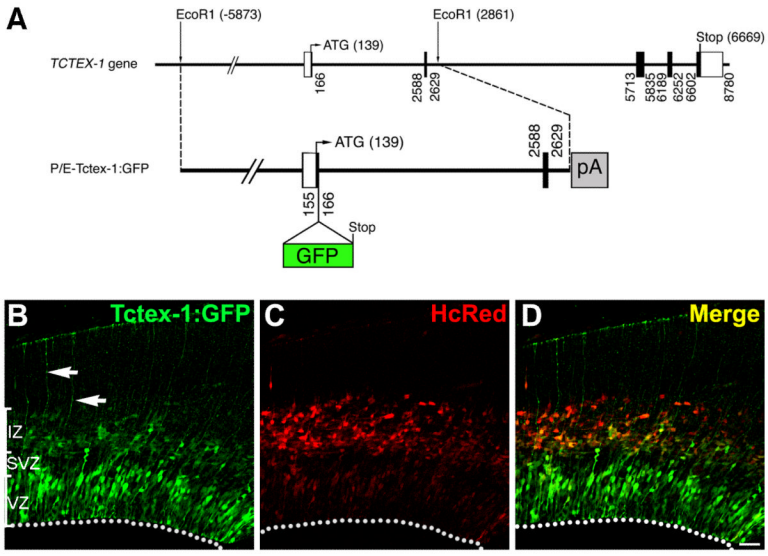

Fig. 1.

Identification of the regulatory element of the Tctex-1 gene that specifically targets the VZ/SVZ of developing neocortex. (A) Schematic diagrams of the mouse *TCTEX-1* gene (top) and P/E-Tctex-1:GFP report construct that encoded GFP fused 3′ to the first 5 residues of Tctex-1 (bottom). Exons are represented by boxes; the horizontal line represents introns; base 1 starts at the first nucleotide of the exon 1; ATG: start codon; pA: rabbit beta-globin polyA. (B-D) Confocal images of cortical slices cotransfected with P/E-Tctex-1:GFP (B) and pCAG-HcRed (C). Merged view is shown in (D). Dotted lines mark the ventricular borders. Arrows point to the RG fibers with their endfeet contacting both the ventricular and the pial surfaces. Bar= 50 μm. Supplementary Figure 3 is the magenta-green copy of this figure.

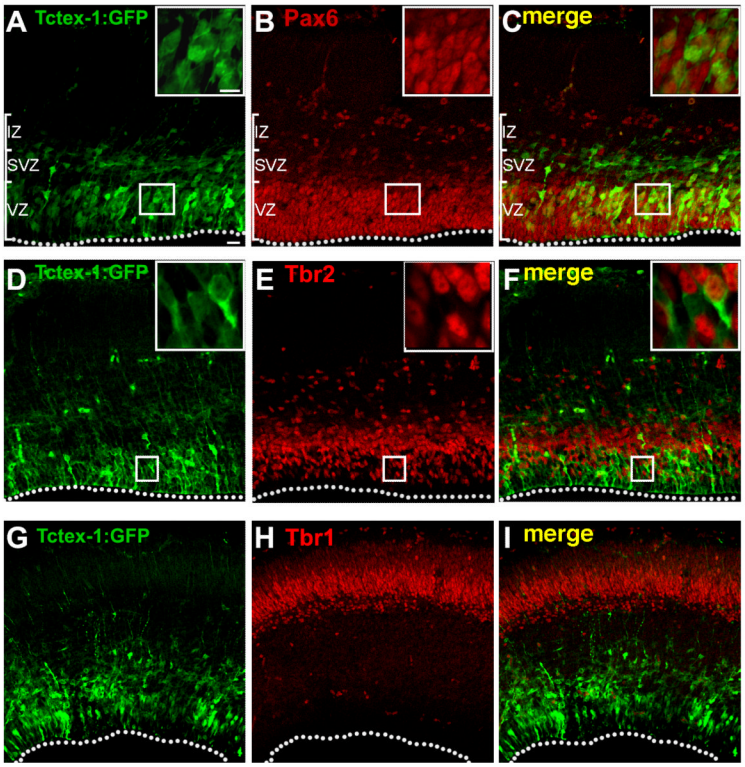

Fig. 2.

Colocalization studies demonstrating that P/E-Tctex:GFP targets a mixture of RG cells and intermediate progenitors in developing neocortex. Confocal images of P/E-Tctex-1:GFP transfected cortical slices that were co-labeled for Pax6 (A-C), Tbr2 (D-F), or Tbr1 (G-I). Inserts show an enlargement of the boxed areas. Bars=20 μm; 4μm (inserts). Supplementary Figure 4 is the magenta-green copy of this figure.

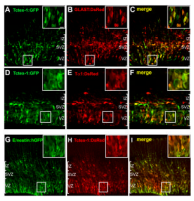

Fig. 3.

Promoter activity profiling assays demonstrating that P/E-Tctex:GFP targets a mixture of RG cells and intermediate progenitors in developing neocortex. Cortical slices harvested from brains co-transfected with P/E-Tctex-1:GFP and P/GLAST:DsRed (A-C) or P/Tα-1:DsRed (D-F) are shown. Cortical slices co-electroporated with P/E-Tctex-1:DsRed and E/nestin:hGFP constructs are shown in (C-I). Scale bars=20 μm; 4 μm (inserts). Supplementary Figure 5 is the magenta-green copy of this figure.

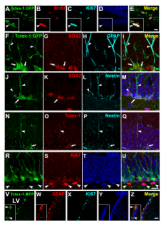

Fig. 4.

Expression pattern of P/E-Tctex-1:GFP cells in adult SGZ (A-U) and SVZ (V-Z) of mouse line 4. (A-E) A low-power confocal image of DG that was triple labeled with GFP, BrdU (1h after three BrdU injections at 2h intervals) and Ki67. (Insert) Enlarged view of a pair of $GFP^+/Ki67^+/BrdU^+$ cells displaying horizontally orientated processes, resembling type-2 cells. (F-I) Confocal images of SGZ triple labeled with GFP, Sox2, and GFAP. Note that the cell bodies of GFP^+ cells were often immunoreactive for Sox2 (arrows) and the processes of GFP^+ cells were positive for GFAP (arrowheads). (J-M) SGZ tripled labeled with GFP, Sox2, and nestin. Arrows point to the $GFP^+/Sox2^+$ cell body and arrowhead point to the $GFP^+/Nestin^+$ processes. (N-Q) Triple labeling of GFP, Tctex-1, and nestin showed that endogenous Tctex-1 is localized on both $nestin^+/GFP^+$ processes (arrowheads) and GFP^+ cell bodies (arrow). (R-U) Confocal images of showed that many Ki67-labeled SGZ cells did not expressed Tctex-1:GFP. (V-Z) Low-power confocal images of LV that was co-labeled with GFP (V), GFAP (W), Ki67 (X), and DAPI (Y). (Insert) Arrowheads point to $GFP^+/GFAP^+$ cells. Scale bars: 50 μm (A-E, V-Z); 20 μm (R-U), 10 μm (N-Q); 5 μm (J-M).

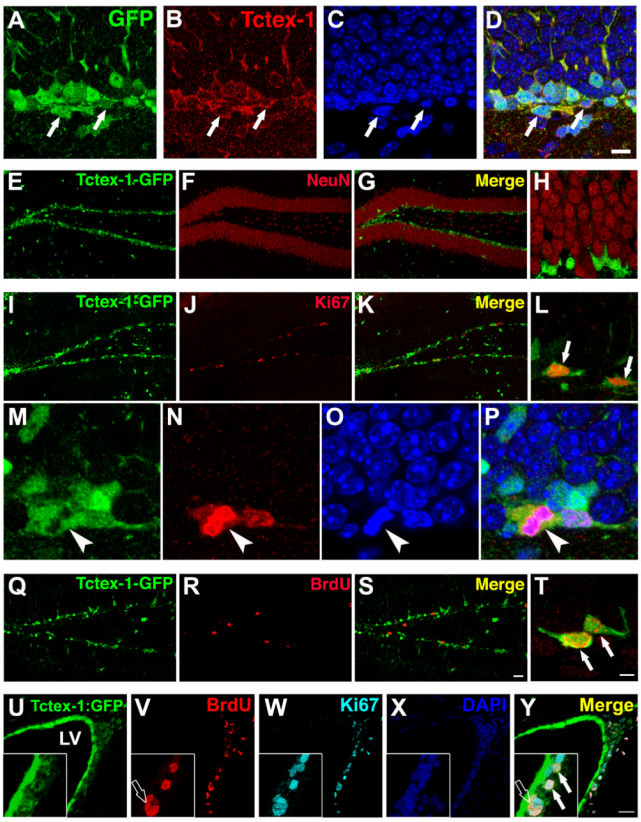

Fig. 5.

Transgenically expressed Tctex-1:GFP cells are active self-renewing progenitors at SGZ and SVZ (line 17). Confocal images of adult DG (A-T) and LV (U-Y) regions of transgenic mice carrying P/E-Tctex-1:GFP were co-labeled for GFP (green in all panels) along with Tctex-1- (A-D), NeuN (E-H), Ki67 (I-P), 1-h BrdU (Q-T). Many Tctex-1$^+$ SGZ cells had irregularly shaped nuclei and horizontally orientated processes (arrows in A-D). Tctex-1:GFP SGZ cell were often Ki67$^+$ (arrows in L) or BrdU$^+$ (arrow in T). A Ki67$^+$ / Tctex-1:GFP$^+$ cell under division is also shown (arrowhead in M-P). (U-Y) Low-power views showed Tctex-1:GFP expression marked a large subpopulation of SVZ cells and cells in the rostral migratory stream and many of them were BrdU$^+$/Ki67$^+$ (also see insert). An open arrow points to a cell resembling type-C which often had irregularly shaped nuclei with deep invaginations. Arrows pointed to cells resembling Type A cells. blue: DAPI. Scale bars= 4 μm (L, T); 20 μm (A-S); 50 μm (U-Y).

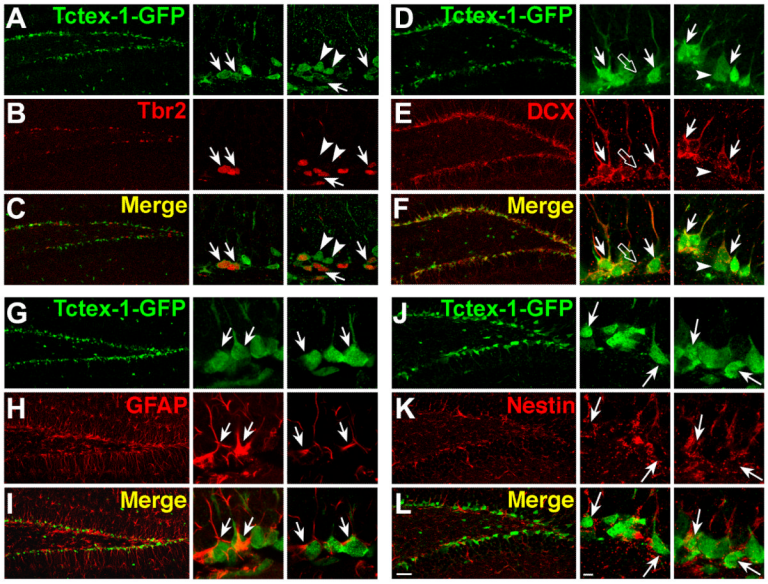

Fig. 6.

Adult SGZ Tctex-1:GFP cells represented type-2 and type-3 transient amplifying progenitors (line 17). Confocal images of adult DG slices of P/E-Tctex-1:GFP mice were co-labeled for Tbr2 (A-C), DCX (D-F), GFAP (G-I) or nestin (J-L). Inserts showed 2 sets of enlarged views. Arrows in (A-C) showed that all Tbr2^{+} cells are Tctex-1:GFP^{+}. Arrows, open arrows and arrowheads in (B-F) pointed to Tctex-1:GFP^{+}/DCX^{+}, Tctex-1:GFP^{-}/DCX^{+}, Tctex-1:GFP^{+}/DCX^{-} populations, respectively. Arrows in (G-I) showed that GFAP^{+} processes were often in close proximity to Tctex-1:GFP^{+} cells. However, Tctex-1:GFP cells did not express GFAP. Arrows in (J-L) showed that Tctex-1:GFP^{+} cells were occasionally surrounded by nestin-labeled fibers. Bar= 50 μm; 5 μm (inserts). Supplementary Figure 6 is the magenta-green copy of this figure.

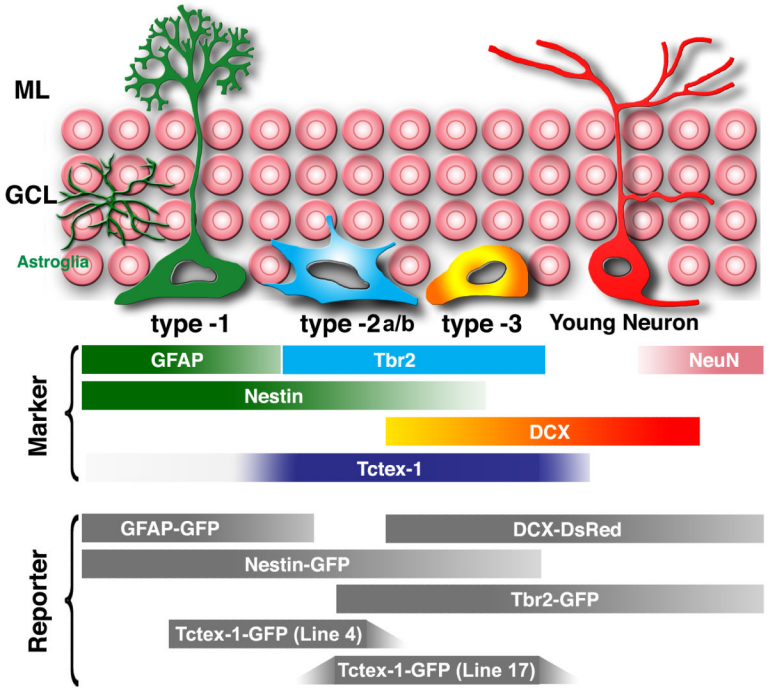

Fig. 7.

Cartoon depicting the estimated timeline of the expression of specific markers and reporter at adult SGZ (Couillard-Despres et al., 2006; Dedesma et al., 2006; Duan et al., 2007; Enikolopov and Overstreet-Wadiche, 2007; Filippov et al., 2003; Fukuda et al., 2003; Kempermann et al., 2004; Steiner et al., 2006).

Table 1

Primary Abs used in this study

Antibody	Species	Dilution	Source, catalog number	Immunogen
Tctex-1	Rabbit	1:100	C.-H. Sung	Recombinant full length bovine Tctex-1
NeuN	Mouse	1:400	Chemicon MAB377	Purified nuclei from mouse brains
GFAP	Mouse	1:400	Sigma G3893 (clone G-A-5)	GFAP isolated from pig spinal cord
BrdU	Rat	1:200	Harlan Sera-Lab Ltd MAS250C	BrdU
BrdU	Mouse	1:200	Roche 1170376 (clone BMC9318)	BrdU-BSA conjugate
DCX	Goat	1:500	Santa Cruz Biotechnology sc-8066	Human DCX peptide (amino acids 384-401)
Sox2	Rabbit	1:100	Stem Cell Technology 01438	Mouse Sox2 peptide (amino acids 113 – 127)
Nestin	Mouse	1:1000	BD Pharmingen 556309 (clone Rat401)	PFA fixed E15 rat spinal cord extracts
DsRed	Rabbit	1:500	Clontech 632496	*Discosoma sp*. red fluorescent protein
GFP	Rabbit	1:1000	Molecular Probes A6455	Purified GFP from *Aequorea victoria*
GFP	Chicken	1:1000	Abcam ab13970	Full-length recombinant GFP protein
Tbr1	Rabbit	1:1000	Gift from Dr. Robert Hevner	Tbr1 peptide (amino acids 1-20)
Tbr2	Rabbit	1:1000	Gift from Dr. Robert Hevner	Tbr2 peptide (EYSKDTSKGMGAYYAFYTSP)
Ki67	Rabbit	1:1000	Novacostra NCL-Ki67p	Recombinant fusion protein corresponding to a 1,086-bp Ki67 motif-containing cDNA fragment
Pax6	Mouse	1:200	Developmental Studies Hybridoma Bank PAX6	Recombinant protein corresp onding to the chicken PAX6 (amino acids 1-223)