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Analysis of neurogenesis using transgenic mice expressing GFP with nestin gene regulatory regions

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Introduction

The complex structure of mammalian central nervous system (CNS) originates from multipotent neural stem cells. Even in the adult brain neural stem cells persist and neurogenesis continues in some regions (Alvarez-Buylla and Garcia-Verdugo, 2002; Kempermann *et al.*, 2004). Furthermore, recent observations are revealing a potential of non-neurogenic CNS regions to generate neurons in pathological conditions (Lie *et al.*, 2004). Unraveling the mechanisms of neurogenesis is therefore important to understand the development, remodeling, and restoration of CNS structure and function.

To facilitate experimental analysis of neurogenesis in the mammalian CNS, effective identification of stem or progenitor cells is crucial. Several groups have generated transgenic mice expressing a marker protein such as green fluorescent protein (GFP) under the control of nestin gene regulatory regions (Yamaguchi *et al.*, 2000; Kawaguchi *et al.*, 2001; Beech *et al.*, 2004; Mignone *et al.*, 2004). Nestin is a class IV intermediate filament expressed in stem cells and progenitor cells of the nervous system (Lendahl *et al.*, 1990). This review aims to summarize how the transgenic mice have been utilized and discuss potentials and reservations of using this animal system.

For the transgene construction, we utilized the second intron of nestin gene, which was known to drive the expression in neural stem and progenitor cells (Zimmerman *et al.*, 1994). We also included the 5′ upstream region (promoter region) in the transgene construct, whose regulatory function is still unclear.

Embryonic period

At embryonic day 11.5, GFP expression was observed in the neuroepithelium, with the strong expression in the ventricular zone. Kawaguchi *et al.* (2001) generated transgenic mice that express GFP under the control of the second intron of nestin gene and Hsp 68 minimal promoter, in which strong GFP expression was similarly observed in the ventricular zone. By sorting GFP-fluorescent cells, they successfully enriched the stem cell population from embryonic forebrain as well as from the periventricular area of adult forebrain. Likewise, GFP-fluorescent cells were isolated from the mesencephalic region of embryos and expanded *in vitro*. These cells were transplanted to Parkinson's disease model mice, which restored the animals from the abnormal movement (Sawamoto *et al.*, 2001). Yoshida *et al.* (2003) sorted GFP-fluorescent progenitor cells from telencephalic regions and showed the correlation between their differentiation and integrin expression. These results indicate the usefulness of the animal system for the isolation of a stem cell population that is applicable to functional restoration of CNS diseases.

Importantly, GFP in the transgenic mice could be observed not only in authentic neuroepithelial cells but also in radial glia (Yamaguchi *et al.*, 2000; Kawaguchi *et al.*, 2001). Radial glia is now considered to be the neural stem cell in various brain regions (Anthony *et al.*, 2004). GFP expression in radial glia would be useful for analyzing the most initial events of neurogenesis.

Neonate and adult

At postnatal day 7, strong GFP expression was observed in the dentate gyrus of hippocampus and olfactory system that extends from periventricular zone to the olfactory bulb (OB) via the specific pathway called rostral migratory stream (RMS) (Figure 1A). In addition, GFP expression was observed in the cerebellum, where massive neurogenesis occurs in postnatal period. In the adult animals (Figure 1B), strong GFP expression continued in the two neurogenic regions, the dentate gyrus of hippocampus and the olfactory system, but extinguished from the cerebellum. These results indicate that GFP expression generally occurs when and where neurogenesis is undergoing.

Stem cells in the RMS are considered to be GFAP positive (Alvarez-Buylla and Garcia-Verdugo, 2002). In the RMS of our transgenic mice, GFAP-expressing cells were GFP-positive, suggesting that RMS stem cells were visualized by GFP fluorescence. Migrating neuroblasts that express PSA-NCAM (Alvarez-Buylla and Garcia-Verdugo, 2002) were also GFP-positive in our transgenic mice. In the OB, GFP-positive cells were scattered in the granule cell layer and glomerular layer (Figure 1C). BrdU labeling showed that some of newly generated neurons that migrate into the OB were GFP-positive (Figure 1D). Some GFP-expressing cells in the granule cell layer extended radial processes, which might correspond to radially migrating 'class 2' granule cells (Petreanu and Alvarez-Buylla, 2002). Thus GFP expression appears to visualize cells from their multipotent precursor stage to the early stage of the maturation into neuronal cells.

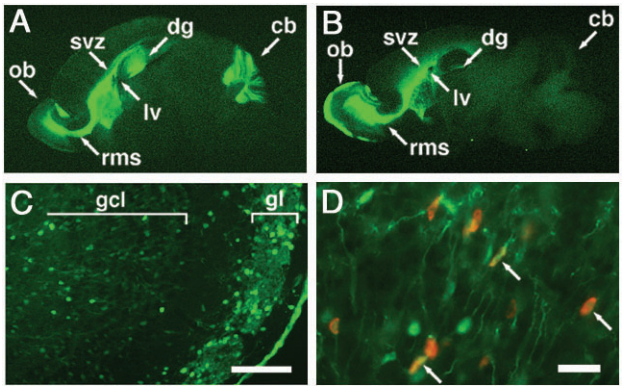

Figure 1 GFP expression in the transgenic mice utilizing the second intron and the 5′ promoter region of the nestin gene. **(A, B)** Parasagittal sections of P7 brain (A) and adult brain (B, 12 weeks after birth). ob, olfactory bulb; rms, rostral migratory stream; svz, subventricular zone, lv, lateral ventricle; dg, dentate gyrus; cb, cerebellum. **(C)** Adult OB. gcl, granule cell layer; gl, glomerular layer. **(D)** A coronal view of the granule cell layer of the adult OB after 7days of BrdU injection. Arrows indicate double positive cells for GFP (green) and BrdU (red). Scale bars, 100 μm (C); 20 μm (D). Modified from Yamaguchi *et al*. (2000) with permission.

In the hippocampal dentate gyrus, GFP-expressing cells were mostly confined to the subgranular layer. Detailed morphological, molecular, and electrophysiological analysis revealed that there were two types of GFP-expressing cells (Filippov *et al.*, 2003; Fukuda *et al.*, 2003). One type of cells had astrocytic features, while the other type of cells showed phenotypes of neuronal lineage. It was proposed that the latter cells were produced from the former ones. Interestingly, exposure of animals to environmental complexity and voluntary physical activity differentially affected the population of GFP-expressing cells (Kronenberg *et al.*, 2003). These results shed light on the mechanisms of morphological and functional differentiation during early steps of neurogenesis.

Implications of GFP expression in various transgenic mouse lines

Mignone *et al.* (2004) generated transgenic mice expressing GFP using the second intron of nestin gene and the 5′ promoter region (5.8 kb) which is longer than that in our mice (2.5 kb). GFP expression pattern in the mice appeared quite similar to our mice. In the adult animals they divided GFP-expressing cells into GFP-bright and GFP-dim cells, and showed that GFP-bright cells express GFAP while GFP-dim cells express a neuronal marker, βIII-tubulin. Kawaguchi *et al.* (2001) divided GFP-expressing cells into GFP weakly-positive and strongly-positive cells, and demonstrated that cells with stem cell-like properties were enriched in the strongly positive cell population of embryos. These results indicate that GFP fluorescence level can be utilized to segregate distinct populations of precursor cells.

It should be cared that GFP expression might not faithfully reproduce endogenous nestin expression. In the mice raised by Mignone *et al.* (2004), most GFP-positive periglomerular cells in the OB are negative for nestin expression. In our transgenic mice also, some GFP-expressing cells in the OB are negative for nestin protein (unpublished observation). Implication of GFP expression should be confirmed in individual types of cells and brain regions, in individual animal lines using different transgene constructs.

Beech *et al.* (2004) have generated transgenic mice expressing a marker protein using the 5.8 kb 5′ promoter region and the 5.4 kb downstream region that contains all three introns and adjacent exon sequences of the nestin gene. Intriguingly, the marker expression in the OB of the mice was observed only in periglomerular cells but not in granule cells. They suggest that the transgene expression represents a subset of cells which differentiates exclusively into periglomerular cells.

Conclusion

Utilizing GFP fluorescence in the nestin gene regulatory region–GFP transgenic mice, isolation, morphological analysis and functional analysis for neural stem and precursor cells have been facilitated. However, the profile of GFP expression might not always be faithful to the endogenous nestin expression, and the expression appears to vary among animal lines with different transgene constructs. Thus the phenotypes of GFP-expressing cells need to be carefully examined in every case of analysis. Detailed analysis would make such heterogeneity advantageous for dissecting the mechanisms of neurogenesis.

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