Chapter 2

Nestin-Based Reporter Transgenic Mouse Lines

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Abstract

Nestin expression marks stem and progenitor cells of the neural lineage. Transgenic mouse lines, originally generated to identify neural stem cells, can also help to identify, track, and isolate stem and progenitor cells in a range of tissues of the ectodermal, endodermal, and mesodermal origin. Here, we describe the generation of transgenic mouse lines expressing fluorescent proteins (FP) under the control of critical regulatory elements of the nestin gene and their use for identifying and analyzing adult stem and progenitor cells in various tissues.

Key words Nestin, Hair-follicle-associated pluripotent (HAP), Stem cells, Transgenic animals, Reporter lines, Immunocytochemistry, Nestin-GFP

1 Introduction

Nestin is an intermediate filament protein, which became a valuable reporter of stem cells. First identified over 20 years ago [1], it gradually became a standard marker of stem and progenitor cells of the neural lineage and, more recently, of several other lineages as well. The first forays that characterize the gene were made when monoclonal antibodies against embryonic neural tissue were raised [2] and one of the clones (Rat-401) was shown to recognize an epitope of a novel type IV intermediate filament protein. This was followed by the cloning of the gene, designated as nestin, for its expression in neuroepithelial stem cells [1]. Nestin is expressed in proliferative zones of the embryonic and adult mammalian brain and is not detected in differentiated cells of neural tissue.

The gene for nestin has been thoroughly studied and several crucial regulatory elements that are responsible for expression in embryonic (and, later, in adult) tissues have been identified [3–5]. The most important element controlling nestin expression in cells of neuroepithelial and neural stem lineage is a strong transcriptional enhancer located in the second intron of the mouse, rat, and human nestin gene. This enhancer contains POU/Pbx binding sites that

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are crucial for nestin expression in neural stem cells. The neural enhancer is so strong and specific that it can reliably direct expression of heterologous genes, even when combined with unrelated promoters. These properties of the nestin neural enhancer have been used to generate lines of transgenic animals expressing fluorescent proteins (GFP or other colors) in the developing and adult nervous system [6–8]. Several lines of evidence demonstrate that FP-positive cells in these nestin-based reporter transgenic animals accurately represent neural stem and early progenitor cells and cover the vast majority of such cells [7, 9–12]. These reporter animals can be used to identify neural stem and early progenitor cells, track them in vivo, isolate them for profiling or culturing, determine quantitative changes, and detect their proliferation, differentiation, or death.

Remarkably, after the nestin-based transgenic mouse lines have been shown to reliably highlight stem cells of the neural lineage, it gradually became apparent that their utility is broader and that nestin-driven transgenes can report the location of stem and progenitor cells in other tissues as well. The panel of tissues in which expression of nestin-FPs reveals cells with stem-like properties is continuously growing, but even an incomplete list includes liver (oval cells; [13]), anterior pituitary (multipotent adult stem cells; [14]), bone marrow (mesenchymal stem cells; [15]), testes (precursors of testosterone-producing Leydig cells; [16]), muscle (satellite cells; [17]), skin [18], and hair-follicle-associated pluripotent (HAP) stem cells [19, 20, 26–30]. This provides the ability to identify and analyze stem and progenitor cells of these tissues in the same animal, thus facilitating experimental design and helping to reveal potential coordinated changes in the stem cell compartments of various tissues of the same reporter animal. Here, we describe the generation of nestin-FP transgenic animals and their use for detecting and counting tissue-specific stem and progenitor cells.

2 Materials

2.1 Cloning, Isolation of Tail DNA, PCR

Standard reagents and procedures are described in [21, 22] and in paragraphs below. Restriction enzymes, DNA ligase, and DNA polymerase are purchased from New England Biolabs (Beverly, MA). Plasmid isolation columns and QIAquick gel extraction kit are purchased from Qiagen (Santa Clarita, CA). AmpliTaq© enzyme and 1× AmpliTaq© PCR buffer are purchased from Boehringer-Mannheim (Indianapolis, IN) and pBluescript vector (pBSM13+, Stratagene, La Jolla, CA).

2.2 Immunocytochemistry

1. Polyclonal antibody to GFP (host—chicken; Aves Laboratories, Tigard, OR) and is used at a working dilution 1:400.
2. Monoclonal antibody to GFP (host—mouse; Developmental Studies, Hybridoma Bank, Iowa City, IA) and is used at working dilution of 1:1000.

3. Polyclonal antibodies to 5-bromo-2-deoxyuridine (BrdU) (host—rat; Accurate Chemicals, Westbury, NY).
4. AlexaFluor 488 goat anti-chicken antibody (Molecular Probes, Eugene, OR). Secondary antibodies are used at a working dilution 1:500.
5. AlexaFluor 594 goat anti-mouse antibody (Molecular Probes, Eugene, OR). Secondary antibodies are used at a working dilution 1:500.
6. AlexaFluor 568 goat anti-rat antibody (Molecular Probes, Eugene, OR). Secondary antibodies are used at a working dilution 1:500.
7. BrdU, paraformaldehyde, Triton X-100, sodium azide, PBS, and goat serum (Sigma, St. Louis, MO).

3 Methods

3.1 Generation of Nestin-FP Transgenic Animals

Here, we describe the production of reporter transgenes and transgenic animals. Procedures are described using the original nestin-GFP line [7] as an example.

3.1.1 Nestin-ZGF and Nestin-GFP Expression Constructs

To generate the expression vector, we used plasmids containing the nestin promoter and the second intron from the rat nestin gene that were generously provided by Drs. R. McKay and L. Zimmerman. We also used constructs containing an enhanced version of GFP, a polyadenylation sequence from the genome of simian virus 40 (SV40), and a pBluescript vector. The major steps for generating the nestin-ZGF expression vector and nestin-GFP constructs are as follows:

1. A cloned SV40 fragment containing a polyadenylation sequence (polyA) was digested with XbaI and BamHI restrictases and the resulting 0.25 kb long fragment was subcloned into pBSM13+ vector. The Xbal site of the resulting polyA-pBSM13+ plasmid was then blunt-ended by treatment with Klenow DNA polymerase. A 5′-AGGCGCGCCT-3′ linker containing a recognition sequence for AscI was cloned into this site, reestablishing the Xbal sites on either side of the now-present AscI restriction site.
2. A 1.8 kb fragment containing the second intron of the rat nestin gene was isolated using BamHI and SmaI restrictases and then inserted into the polyA-pBSM13+ plasmid near the 3′-end of the polyA sequence (polyA-intron2-pBSM13+ plasmid). Then the HindIII site of the plasmid was cleaved, blunt-ended, and re-ligated, thus creating an NheI site.
3. A 5.8 kb fragment containing the promoter of the rat nestin gene was isolated using SpeI and SalI restrictases and inserted

into the polyA-intron2-pBSM13+ plasmid opened by NheI and SalI enzymes (the SpeI restriction site is compatible with the NheI site), placing the nestin promoter near the 5′-end of the polyA sequence. The resulting nestin promoter-polyA-intron2-pBSM13+ plasmid (nestin-ZGF) was used as a basic vector for cloning genes of interest (e.g., GFP).

4. An 0.82 kb fragment containing EGFP was isolated from the pEGFP-N1 plasmid (Clontech) using the NotI enzyme, blunt-ended by Klenow DNA polymerase, and then ligated to an AscI linker (as above). This created an AscI restriction site in place of the NotI site. The XmaI restriction site of the EGFP-N1 5′ polylinker was blunt-ended and religated in order to destroy the SmaI site.
5. The resulting eGFP fragment was then digested with SalI and AscI enzymes, creating a 780 bp DNA fragment which was ligated into the nestin-ZGF plasmid digested with SalI and AscI, positioning it between the nestin promoter and polyA site (nestin-GFP plasmid). The same strategy was used for inserting other genes of interest (e.g., CFPnuc, dTimer, mCherry).

3.1.2 Generation of Transgenic Mice

1. The nestin-GFP plasmid was purified using cesium chloride centrifugation or a Qiagen plasmid isolation column (Qiagen, Santa Clarita, CA).
2. 10 μg of the purified plasmid was digested with the restriction enzyme SmaI.
3. The DNA fragment containing the nestin promoter-EGFP-polyA-intron2 sequences was separated from the pBSM13+ on an 0.8% agarose gel.
4. The band was cut from the agar and purified using the Qiagen QIAquick gel extraction kit.
5. 3 μg of the isolated and purified fragment was used for generating transgenic mice.
6. DNA was injected into the pronuclei of ~500 oocytes of the C5BL/6xBalb/cBy hybrid mouse strain (or other appropriate mouse strain) using established procedures [21].
7. The injected oocytes were then transferred to 12 pseudo-pregnant female mice. 80–100 pups were born in a typical experiment and transgenic animals were determined using PCR.

3.1.3 Genotyping of Nestin-GFP Transgenic Mice

1. DNA was isolated from tails using established procedures [21].
2. The sequences of the primers used for PCR were 5′-GATCACTCTCGGCATGGACGAGC-3′ (corresponding to the last 40 bases of the EGFP sequence) and 5′-GGAGCTGCA CACAACCCATTGCC-3′ (corresponding to 225 bases into the nestin second intron).

3. PCR was performed in 30 μl containing 1.5 mM $MgCl_2$, 1× AmpliTaq© PCR buffer (Boehringer-Mannheim, Indianapolis, IN), 0.2 nM each of dNTP, 0.4 μM of each primer, and 1 U AmpliTaq© (Boehringer Mannheim).
4. Thirty-five cycles of PCR with an annealing temperature of 65° (30 s) and an extension temperature of 72° (1 min) were used.
5. The expected fragment of 510 bp was detected in eight out of the 86 F-0 mice (in the experiment described in [7]. Of these eight transgenic mice, three were male and five were female.
6. Seven transgenic animals produced progeny, which was analyzed using flow cytometry and immunohistochemistry. All seven substrains demonstrated the expected pattern of EGFP expression. Choice of strain was eventually limited to two after demonstrating high sensitivity and specificity for EGFP cell labeling.

3.1.4 Genotyping of Nestin-GFP Transgenic Mice (Alternative Protocol)

1. Primers: 5′-ATCACATGGTCCTGCTGGAGTTC-3′ (GFP, GFP, or YFP) and 5′-GGAGCTGCACACAACCCATTGCC-3′ (nestin second intron).
2. PCR performed in 25 μl containing 2.5 mM $MgCl_2$, 1× AmpliTaq© PCR buffer, 0.2 nM of each dNTP, 0.5 μg of each primer, and 1 U AmpliTaq©.
3. Cycling conditions: step 1—94° for 3 min; step 2—94° for 30 s, 62° for 45 s, 72° for 45 s; repeat step 2 for a total of 32 cycles; step 3—72° for 2 min; step 4—hold at 4°. Product size—700 bp.

3.2 Immunocytochemical Detection of Nestin-FP-Expressing Stem and Progenitor Cells

In many cases, the fluorescence signal of nestin-FP cells can be detected directly using an epifluorescence or confocal microscope. However, in some tissues this signal is too weak to detect directly and requires amplification by immunocytochemistry. Furthermore, immunocytochemical detection of FPs is required when fluorescence is abolished by a particular procedure, for example, by treatment of samples with acid when fluorescent proteins are analyzed in conjunction with detection of BrdU-labeled proliferating cells. Here, we describe immunocytochemical detection of dividing (BrdU-labeled) cells expressing nestin-GFP, nestin-CFP, or nestin-YFP cells in sections of adult mouse brains. The same protocol can be used for most of the other (neural or non-neural) tissues. Further details for immunocytochemistry, microscopy, and cell quantitation can be found in [9, 12, 23–25].

1. Nestin-FP mice are injected with BrdU (150 mg/kg) 2 or 24 h before perfusion.
2. Mouse tissues are fixed by transcardial perfusion with 30 ml phosphate buffered saline (PBS) and 30 ml of 4% paraformaldehyde in PBS (pH 7.4). Brains are removed and further

post-fixed in 4% paraformaldehyde in PBS overnight at 4 °C; if necessary, samples are stored in PBS with 0.1% sodium azide at 4 °C until sectioning.

3. Brains are sagittally sectioned at a thickness of 50 μm with a vibratome. Sections are sequentially collected and subsets of sections at 300 μm intervals are taken for immunohistochemistry and analysis.
4. Sections are rinsed with PBS and denatured in 2 M HCl at 37 °C for 1 h for detection of BrdU-incorporating dividing stem and progenitor cells. The denatured sections are neutralized with 0.1 M borate (pH 8.0) twice for 20 min each.
5. Sections are rinsed with washing solution (PBS with 0.2% Triton X-100), and incubated for blocking and permeabilization in PBS with 2% Triton X-100 and 5% goat serum at room temperature for 2 h.
6. After rinses with washing solution, the sections are incubated at 4 °C overnight in antibody solution (PBS with 0.2% Triton X-100 and 3% goat serum) containing primary antibodies: chicken anti-GFP (1:400 dilution) and rat anti-BrdU (1:400 dilution). If necessary, mouse monoclonal anti-GFP antibody can be used.
7. The sections are rinsed with washing solution and incubated for 2 h at room temperature with Alexa Fluor-conjugated goat secondary antibodies (AlexaFluor 488 anti-chicken antibody and AlexaFluor 568 goat anti-rat antibody).
8. After rinses with washing solution, the sections are mounted on gelatin-coated slide glasses with DakoCytomation Fluorescent Mounting Medium and cover-slipped for microscopy.

4 Notes

1. In addition to PCR-based genotyping, nestin-GFP transgenic mice can be also identified by a characteristic green fluorescence in the brains of newborn mice, in the retina of adult live mice, or in the tails of adult live mice when analyzed at a minimum of 100× magnification.
2. Transgenic mice that are generated on a particular genetic background should be crossed to wild-type mice of a desired genotype (e.g., C57BL/6) for at least five generations.
3. Nestin-FP heterozygous transgenic mice can be bred to generate homozygous animals, which can then be crossed to other lines or to wild type mice without the need for further genotyping of the heterozygous progeny.

4. If additional antigens need to be analyzed by confocal microscopy (e.g., glial fibrillary acidic protein, GFAP), a rabbit polyclonal primary antibody (Sigma, cat. no. G9269) and AlexaFluor 594 goat anti-rabbit secondary antibody (cat. no. A11012) can be used. If the protocol necessitates the use of a mouse monoclonal antibody to GFP, Rat-401 antibody and AlexaFluor 594 goat anti-mouse antibody (cat. no. A11005) can be used.
5. The pattern of reporter expression in nestin-based transgenic lines is usually highly reliable in marking neuroepithelial cells in the developing embryo and neural stem and progenitor cells in the adult neurogenic zones (provided the construct contains the second intron enhancer element). However, the expression pattern can be different between different lines for non-neural tissues. This may reflect both the overall intensity of the signal (e.g., fluorescence failing to reach the threshold for detection in particular tissues) and subtle differences in the genomic integration site.

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