HHS Public Access

Author manuscript

Mol Biotechnol. Author manuscript; available in PMC 2024 May 20.

Published in final edited form as:

Mol Biotechnol. 2013 June ; 54(2): 350–360. doi:10.1007/s12033-012-9570-3.

Targeting of the Enhanced Green Fluorescent Protein Reporter to Adrenergic Cells in Mice

Jixiang Xia,
Burnett School of Biomedical Sciences, College of Medicine, University of Central Florida, 6900 Lake Nona Blvd, Orlando, FL 32827-7047, USA

Namita Varudkar,
Burnett School of Biomedical Sciences, College of Medicine, University of Central Florida, 6900 Lake Nona Blvd, Orlando, FL 32827-7047, USA

Candice N. Baker,
Burnett School of Biomedical Sciences, College of Medicine, University of Central Florida, 6900 Lake Nona Blvd, Orlando, FL 32827-7047, USA

Ibrahim Abukenda,
Burnett School of Biomedical Sciences, College of Medicine, University of Central Florida, 6900 Lake Nona Blvd, Orlando, FL 32827-7047, USA

Celines Martinez,
Burnett School of Biomedical Sciences, College of Medicine, University of Central Florida, 6900 Lake Nona Blvd, Orlando, FL 32827-7047, USA

Aruna Natarajan,
Department of Pediatrics, Medstar-Georgetown University Hospital, Washington, DC, USA

Alexander Grinberg,
Program in Genomics of Differentiation, Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD, USA

Karl Pfeifer,
Program in Genomics of Differentiation, Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD, USA

Steven N. Ebert
Burnett School of Biomedical Sciences, College of Medicine, University of Central Florida, 6900 Lake Nona Blvd, Orlando, FL 32827-7047, USA

Abstract

Adrenaline and noradrenaline are important neurotransmitter hormones that mediate physiological stress responses in adult mammals, and are essential for cardiovascular function during a critical

steven.ebert@ucf.edu .

Electronic supplementary material The online version of this article (doi:10.1007/s12033-012-9570-3) contains supplementary material, which is available to authorized users.

period of embryonic/fetal development. In this study, we describe a novel mouse model system for identifying and characterizing adrenergic cells. Specifically, we generated a reporter mouse strain in which a nuclear-localized enhanced green fluorescent protein gene (*nEGFP*) was inserted into exon 1 of the gene encoding *Phenylethanolamine n-methyltransferase* (*Pnmt*), the enzyme responsible for production of adrenaline from noradrenaline. Our analysis demonstrates that this knock-in mutation effectively marks adrenergic cells in embryonic and adult mice. We see expression of nEGFP in Pnmt-expressing cells of the adrenal medulla in adult animals. We also note that nEGFP expression recapitulates the restricted expression of Pnmt in the embryonic heart. Finally, we show that nEGFP and Pnmt expressions are each induced in parallel during the in vitro differentiation of pluripotent mouse embryonic stem cells into beating cardiomyocytes. Thus, this new mouse genetic model should be useful for the identification and functional characterization of adrenergic cells in vitro and in vivo.

Keywords

Phenylethanolamine-*N*-methyltransferase; Adrenal medulla; Chromaffin cells; Green fluorescent protein; Knock-in

Introduction

Adrenergic hormones are key molecular modulators of physiological stress responses and are essential for cardiac function during fetal and early neonatal development [1–3]. The principal adrenergic hormones in mammals are adrenaline and noradrenaline. In adults, they are primarily produced in the chromaffin cells of the adrenal medulla where they can then be secreted directly into the circulation during periods of stress [3]. Mice that cannot produce adrenaline due to targeted disruption of the *Pnmt* gene show impaired blood pressure regulation (high) and cardiac output (low) responses to stress, though heart rate and baseline cardiac parameters were not significantly altered [4].

Although there is no requirement for Pnmt in the developing embryo, disruption of precursor enzymes in the catecholamine biosynthesis pathway, tyrosine hydroxylase (Th) and dopamine *β*-hydroxylase (Dbh), result in embryonic lethality from apparent heart failure due primarily to the absence of noradrenaline [1, 2]. Interestingly, the earliest site of adrenergic hormone production is the heart [5–7], beginning around embryonic day 8.5 (E8.5) and continuing well after birth in mice [8]. In addition, adrenergic hormones are produced by neurons in the central and peripheral nervous systems and also by several other cell types including lymphocytes and cells in the retina, kidney, spleen, and testes [9–18]. The role of adrenergic hormones in these non-neuronal tissues is poorly understood at present.

Adrenergic cells can be identified by expression of the biosynthetic enzymes responsible for production of adrenaline and noradrenaline. Dbh converts dopamine to noradrenaline, and phenylethanolamine *n*-methyltransferase (Pnmt) converts noradrenaline to adrenaline. Thus, Pnmt expression generally serves as a marker for adrenaline-producing cells while Dbh expression in the absence of Pnmt expression generally serves as a marker for noradrenaline-producing cells. Using targeted genetic strategies in mice, we previously described a model

where we inserted the *Cre-recombinase* (*Cre*) gene into exon 1 of the Pnmt gene [8]. By inter-breeding these mice with ROSA26-β-galactosidase reporter animals, we showed that the β-galactosidase reporter was specifically expressed in the medulla of the adult adrenal gland and in the developing embryonic heart in patterns consistent with those observed for endogenous Pnmt expression in those tissues [6–8, 19]. The Cre/ROSA strategy marks cells that not only actively express Pnmt, but also those that expressed it sometime earlier in development, thus identifying cells within a tissue derived from a Pnmt-expressing (Pnmt+) cell lineage. A limitation of this approach, however, is that it is impossible to distinguish cells that are actively adrenergic versus those that are not now synthesizing hormones but are descendants of precursor cells that were adrenergic.

To overcome this limitation, we developed a new model by targeting a *nEGFP* reporter gene to the mouse *Pnmt* locus to create $Pnmt^{+/nEGFP}$ and $Pnmt^{nEGFP/nEGFP}$ reporter mice. This study describes the initial characterization of these mice, including verification of genetic targeting and analysis of reporter gene expression in the adult adrenal gland and developing heart. We also characterize the activation of *nEGFP* expression and of endogenous Pnmt in mouse embryonic stem cells (mESCc) as they differentiate into beating cardiomyocytes.

Materials and Methods

Materials

Andwin Scientific Tissue-Tek* CRYO-OCT compound was obtained from Fisher Scientific (Pittsburgh, PA). Sheep anti-Pnmt primary antibody was from Chemicon, Inc. (now Millipore). Texas Red conjugated anti-sheep IgG secondary antibody was obtained from Jackson Immuno-research (West-Grove, PA). Rabbit anti-GFP antibody and Alexafluor 594-conjugated donkey anti-rabbit secondary antibody were obtained from Invitrogen, Inc. (Carlsbad, CA). Vectashield mounting medium for fluorescence (H-1000) and mounting medium with propidium iodide (PI) (H-1300) were obtained® from Vector Laboratories (Burlingame, CA). Cell culture media (Dulbecco's Modified Eagle Medium) and supplements were from Invitrogen (Carlsbad, CA). Fetal bovine serum was from Hyclone (Logan, UT). TRIzol® and MuLV Reverse Transcriptase were also from Invitrogen. All other chemicals and reagents used in this study were obtained from Sigma-Aldrich (St. Louis, MO).

Pnmt-nEGFP Knock-in Construction

The mutagenesis construct was generated essentially as described [8] but replacing the *Cre-recombinase* gene with the cDNA for enhanced green fluorescent protein (EGFP) tagged with nuclear leading sequence (nEGFP). Thus, this construct fused Pnmt 5′ non-coding sequences and the Pnmt ATG to sequences encoding EGFP. A *frt*-flanked neomycin cassette was inserted behind nEGFP for positive selection. The diphtheria toxin A (DT-A) cassette was placed outside of the homologous arms as a negative selection marker. The linearized Pnmt-nEGFP plasmid was electroporated into R1 mESC line and G418 resistant clones were isolated and their DNAs were analyzed by Southern blotting using probes external to the targeting construct. The correct targeted mESC lines were injected into blastocysts of mouse embryos. Chimeric founder animals were crossed with Jackson

Laboratories strain 003946. These animals are transgenic for the *FLP1 recombinase* gene [20], thus allowing us to remove the NeoR cassette. Subsequently, mice were backcrossed and maintained in a 129X1/SvJ genetic background. Genotyping was accomplished by PCR using a three primer system (5′-CAGGCGCCTCATCCCTCAGCAGCC-3′; 5′-Pnmt-CTGGCCAGCGTCGGAGTCAGGGTC-3′; 5′-GCTCGA CCAGGATGGGCACC-3′) to identify bands of 200 and 181 bp specific for the wild-type *Pnmt* and the *Pnmt::GFP* insertion alleles, respectively.

Fluorescence and Immunofluorescence (IF) Histology

All animal work was done according to NIH and to PHS policy and was approved by the Animal Care and Use Committees of the NICHD and University of Central Florida. Embryos from wild-type ($Pnmt^{+/+}$), heterogeneous ($Pnmt^{+/nEGFP}$), and homozygous mutant ($Pnmt^{nEGFP/nEGFP}$) were isolated and dissected at developmental stages described in text with noon of the day of the vaginal plug designated as embryonic day 0.5 (E0.5). The protocols for dissection and IF staining are as described [8].

Quantification of nEGFP+ cells

For adult adrenal gland, positive fluorescent cells were counted between heterozygous and homozygous strains. For this purpose, pictures were taken from adrenal gland sections using a 20× objective lens. The exposure time and compensations were set same for all the samples in order for comparison and quantification. In order to exclude disturbance from autofluorescence, image under red fluorescence filter from each sample was also captured. A cell with nuclear-localized green fluorescence without red fluorescence was designated as nEGFP+. Counting was performed by separate people who were blinded as to genotype of each sample.

Differentiation of mESCs into Beating Cardiomyocytes

Both R1 and Pnmt-nEGFP pluripotent mESCs were maintained on mouse embryonic fibroblasts in DMEM medium supplemented with 15 % fetal bovine serum plus 1,000 units/ml leukemia inhibitory factor (LIF or ES-GRO®, Millipore). mESCs were differentiated by the hanging-drop as described previously [21, 22].

RT-PCR

Total RNA from mESCs at different differentiation stages was extracted by TRIzol® reagent, and cDNA was synthesized with MuLV Reverse Transcriptase. RT-PCR for assessing endogenous Pnmt mRNA expression was performed with the following primers: forward, 5′-GGTGGCTCAGACCTG AAG-3′ and reverse, 5′-GCCATCAGGGTTGCTCAG-3′. The PCR product was detected under agarose gel electrophoresis. RNA from adult adrenal glands was used as a positive control for Pnmt expression, while the absence of input RNA was used as a negative control.

Statistical Analysis

For the quantification of nEGFP+ cells from adrenal glands, the average from five samples of each genotype was calculated and expressed as mean ± SEM. Student's *t* test was used for

comparison between heterozygous and homozygous group, with $p < 0.05$ required to reject the null hypothesis.

Results

Genetic Targeting of the nEGFP Reporter Gene to the Pnmt Locus

To identifyand evaluate adrenergic cells in vivo, we inserted the nEGFP reporter gene with a nuclear localization sequence (nEGFP) into exon 1 of the endogenous mouse Pnmt gene as outlined in the schematic shown in Fig. 1a. Correct targeting was confirmed by Southern blotting (Fig. 1b) using 5′ and 3′ probes external to the genomic sequences used to direct the homologous recombination. A correctly targeted clone was used to make the knock-in mice. Through successive breeding, we removed the Flp-flanked NeoR cassette and obtained $Pnmt^{+/+}$, $Pnmt^{+/nEGFP}$, and $Pnmt^{nEGFP/nEGFP}$ littermates for analysis. Note that even though the Pnmt insertion completely abrogates expression of Pnmt and thus eliminates adrenaline, $Pnmt^{nEGFP/nEGFP}$ animals are viable, fertile, and show no obvious differences in health, weight, or appearance (data not shown). Similar results were already reported for the $Pnmt^{Cre/Cre}$ animals [8].

Characterization of nEGFP Reporter Expression in Mouse Adrenal Glands

The chromaffin cells of the adrenal medulla represent the single most abundant location of adrenaline production and, hence, of Pnmt expression in adult animals. Thus, we examined reporter gene expression in adult mouse adrenal gland tissue sections to determine if nEGFP expression was present as expected. In the mouse adrenal gland, the medulla and surrounding cortex are easily distinguishable from each other. As shown in Fig. 2, nEGFP expression was observed in the medulla of mice with the targeted $Pnmt^{+/nEGFP}$ allele, but was absent in wild-type ($Pnmt^{+/+}$) controls. In contrast, the low levels of fluorescence in the cortex are indistinguishable in wild-type and in $Pnmt^{+/nEGFP}$ animals indicating that nEGFP expression is specific to the medulla and thus follows the patterns of the endogenous *Pnmt* gene.

To evaluate the specificity of the nEGFP reporter gene expression in more detail, we performed IF staining for endogenous Pnmt protein expression in adrenal gland sections from $Pnmt^{+/nEGFP}$ mice using a red fluorescent secondary antibody tag so that we could visualize both Pnmt (Fig. 3a, b) and nEGFP (Fig. 3c, d) expression simultaneously in the same sections. Endogenous Pnmt expression is mainly cytoplasmic while nEGFP expression was restricted to the nucleus of fluorescently labeled cells. Thus, overlay of the red and green fluorescent images shows co-expression in red fluorescent cells containing green nuclei, indicating that nEGFP was expressed in the nuclei of Pnmt-expressing adrenal medullary cells (Fig. 3e, f).

Confirmation that EGFP expression was principally localized to nuclei was obtained by co-staining the sections with the red fluorescent nuclear stain, PI. Within EGFP+ cells of the adrenal medulla, PI labeled the cells in a pattern nearly identical to that observed for EGFP (Fig. 4, arrows). Altogether these data demonstrate that EGFP expression is localized to nuclei of adrenal medullary cells in $Pnmt^{+/nEGFP}$ mice.

Expression of the nEGFP reporter was confined to cells that also expressed endogenous Pnmt. It is clear, however, that the nEGFP expression was found in only a subset of Pnmt-expressing cells. We observed that the intensity of nEGFP fluorescence was variable in nEGFP+ cells (Figs. 2, 3, 4), possibly indicating that expression may be limited in some cells. To estimate the percentage of Pnmt+ cells represented by nEGFP+ cells in these adrenal sections, we counted each type of cell (red or green fluorescence) in several representative sections such as those shown in Figs. 2, 3, and 4, and found that ~15–20 % of the total number of Pnmt+ cells were also clearly nEGFP+.

We speculated that the observed variable nEGFP+ expression in a subset of Pnmt+ cells may have been due, in part, to the sensitivity of fluorescence detection for identification of nEGFP+ cells. If this was the cause, then we should, in theory, be able to increase sensitivity of detection by amplifying the fluorescence via indirect IF staining techniques using an anti-GFP antibody and fluorescently labeled secondary antibody. To this end, we performed IF staining of adrenal sections of $Pnmt^{+/nEGFP}$ mice with anti-GFP primary antibody and a red fluorescent secondary antibody. Our results show that anti-GFP IF was detected in a subset of nEGFP+ cells in the adrenal sections (Fig. 5). The staining was restricted to nuclei as indicated by co-localization with DAPI, as also shown in Fig. 5. In wild-type control adrenal sections ($Pnmt^{+/+}$), no specific anti-GFP IF staining or nEGFP+ expression was observed (Supplemental Fig. 1). Thus, anti-GFP IF appears to specifically identify nEGFP+ cells. Instead of seeing an increased number of nEGFP+ cells with this technique, however, it appears that we were only able to identify a subset of the total (~40–50 %) number of nEGFP+ cells using this method.

To further investigate the issue of why nEGFP+ cells were not found in all Pnmt+ cells, we compared nEGFP expression in adrenal sections from mice with one nEGFP copy ($Pnmt^{+/nEGFP}$) versus those that had two copies ($Pnmt^{nEGFP/nEGFP}$). Representative images of adrenal sections expressing nEGFP are shown in Fig. 6a, b. Quantitative assessment of the number of nEGFP-expressing (nEGFP+) cells per section revealed that there was a significant twofold increase in the number of nEGFP+ cells found in the homozygous *nEGFP/nEGFP* condition compared with those from mice with only one copy (+/*nEGFP*) (Fig. 6c). Despite this increase in the number of nEGFP+ cells in homozygous animals, there was still considerable variability in expression intensity in different cells, including many Pnmt+ cells that did not display detectable nEGFP expression. Thus, while we certainly observed significantly more nEGFP+ cells in the adrenal sections from mice homozygous for this allele, the total number of nEGFP+ cells still only represented about 30–40 % of the total number of Pnmt+ cells observed. Altogether, these results indicate that the amount of nEGFP stably synthesized in *Pnmt::nEGFP* insertion cells is variable from cell to cell.

Evaluation of nEGFP Expression During Heart Development In Vivo and In Vitro

In the heart, Pnmt expression is first detected during early embryonic development at about the time that the heart first starts to beat at ~E8.0–8.5 in mice [4, 5]. Pnmt expression increases over the next several days, reaching peak levels at ~E10–11 [7, 8]. To determine if nEGFP expression could be identified in cardiac adrenergic cells, we examined E10.5 heart sections from $Pnmt^{nEGFP/nEGFP}$ mice for the presence of nEGFP+ cells. This was

more difficult than anticipated due to the relatively high background autofluorescence in the green spectrum. At low magnification, it was extremely difficult to distinguish nEGFP+ cells from non-expressing cells (Fig. 7a). At higher magnification, however, characteristic green fluorescent nuclei were evident in cardiac cells (Fig. 7b, arrows) near the atrioventricular junction, a region that has been associated with strong Pnmt expression in the developing rat and mouse embryonic heart from previous studies [4, 13]. Note that the nEGFP expression was not visible in the red spectrum, whereas nearby autofluorescent cells showed similar patterns of fluorescence in both the red and green spectra (Fig. 7b, c). These data show that nEGFP expression could be identified in presumptive adrenergic cells from *Pnmt*$^{nEGFP/nEGFP}$ embryonic heart sections.

As an alternative approach, we examined endogenous Pnmt and nEGFP reporter expression in *Pnmt*$^{+/nEGFP}$ mESCs before and after inducing their differentiation into beating cardiomyocytes. As shown in Fig. 8a, Pnmt mRNA was not detected in undifferentiated pluripotent mESCs (lane 1), but appeared subsequent to the onset of contractile activity at 7 + 3d (lane 2), and continued to be associated with beating cardiac-differentiated mESCs at 7 + 5d (lane 3). Thus, endogenous Pnmt mRNA is restricted to mESCs that had been induced to differentiate into beating cardiomyocytes, and is not detectable in undifferentiated mESCs.

Evaluation of nEGFP expression in these mESCs uncovered small clusters of nEGFP+ cells at 7 + 5d (Fig. 8b–g). We used the same criteria as above (green fluorescence in cell nuclei showing no autofluorescence in the red spectrum), and found no clear nEGFP staining in undifferentiated mESCs (not shown). Here, we confirm nEGFP+ expression by showing that the green fluorescence (Fig. 8b) colocalized with anti-GFP IF red fluorescence (Fig. 8c) and DAPI nuclear blue fluorescence (Fig. 8d) in the overlay images shown in Fig. 8e, g. From this same image, we can see that in this case, only one of 3 or 4 cells in this small cluster was fluorescently labeled (compare Fig. 8f, g). When undifferentiated (pluripotent control) mESCs were similarly stained, we did not detect any nEGFP+ cells (Supplemental Fig. 2). The staining that was observed after induction of cardiac differentiation was observed in a relatively small number of cells, and was often difficult to find. Typically, we observed only single cells (like in Fig. 8b–g) or small clusters as shown for the three-cell cluster depicted in Supplemental Fig. 3. Based on these results, we estimate that the relative number of nEGFP+ cells in cardiac-differentiated mESCs at 7 + 5d to represent only 18 confirmed nEGFP+ cells out of a total of ~72,000 cells analyzed, or about 0.025 % of the total number of mESCs evaluated after 7 + 5d of cardiac differentiation where maximal expression of Pnmt mRNA was observed (Fig. 8a). These results show that nEGFP expression was induced in association with heart development in *Pnmt*$^{+/nEGFP}$ embryonic cells both in vitro and in vivo.

Discussion

In this study, we have described the initial characterization of a new mouse model where a *nEGFP* reporter gene was inserted into the endogenous mouse *Pnmt* gene locus to create heterozygous *Pnmt*$^{+/nEGFP}$ and homozygous *Pnmt*$^{nEGFP/nEGFP}$ mice. Our results showed that *nEGFP* reporter gene expression was specifically expressed in adrenergic chromaffin

cells of the adrenal medulla based on co-expression with endogenous Pnmt protein in these cells. Nuclear localization of the signal was confirmed by co-labeling the cells with the nuclear dyes, PI and DAPI. We also demonstrated expression of nEGFP near the atrioventricular junction region of the developing mouse heart in vivo, and in association with endogenous Pnmt expression in mESCs after they were induced to differentiate into beating cardiomyocytes in vitro. These initial analyses indicate that the *nEGFP* reporter gene is correctly targeted and expressed in adrenergic cells in the adrenal glands as well as those associated with embryonic heart development. Although further characterization is needed to evaluate reporter gene expression in more detail at different developmental time points and in other tissues known to express endogenous Pnmt, such as retinal neurons, brainstem neurons, lymphocytes, lungs, liver, spleen, kidney, and testes [9–18], these initial results suggest that nEGFP expression successfully identifies Pnmt-expressing cells in mice.

The $Pnmt^{+/nEGFP}$ mouse model provides two distinct advantages compared with similar knock-in models that inserted the *Cre-recombinase* gene into the mouse Pnmt locus [8, 19]. First, the *nEGFP* knock-in marks cells actively expressing Pnmt while the *Cre* knock-in marks cells that had a history of expressing *Pnmt* at some point in their development in addition to cells actively expressing *Pnmt*. Second, nEGFP can potentially be used to identify and isolate viable adrenergic cells directly using fluorescence imaging and collection strategies such as fluorescence-activated cell sorting (FACS). When comparing the staining results from these models, it appears that the *Pnmt*-driven *nEGFP* expression is restricted to a subset of cells that showed positive expression of *Pnmt*-driven *Cre-recombinase* from previous studies [8, 19]. To identify cells that had expressed *Cre-recombinase*, the mice were crossed with ROSA26 reporter mice containing the *β-galactosidase* reporter gene. Within the adrenal glands, β-galactosidase staining was restricted to the adrenal medulla. In the heart, β-galactosidase staining was initially found in the muscle walls of the cardiac chambers of the developing embryonic heart, similar to the nEGFP staining patterns observed in this study.

It should be noted that the targeting vectors and strategies were similar for both models (*nEGFP* and *Cre*), so we expected some similarity in expression patterns that also matched those observed for endogenous Pnmt staining. The more limited staining observed in the *nEGFP* knock-in model likely reflects, in part, the fact that only active *Pnmt*-expressing cells show nEGFP expression compared with the active plus historical expression demarcated by the *β-galactosidase* reporter generated by *Cre*-activation of the *ROSA26* locus [8]. When compared to endogenous Pnmt expression using anti-Pnmt IF staining techniques, we found that only a subset of active *Pnmt*-expressing cells also displayed *nEGFP* reporter expression. Our analysis suggested that this may be a sensitivity issue. At any given time, some adrenergic cells may not have expressed enough nEGFP to effectively mark the nucleus above the fluorescent background. In this regard, the variability in nEGFP fluorescence between cells is quite interesting as it suggests that the activity of the medullary cells is not uniform. We hope to use our system to characterize this cell to cell variability in future studies.

Study Limitations

Although EGFP is widely employed as reporter gene, recent studies show several disadvantages for its application in mESCs and transgenic animals [23, 24]. The spectrum of EGFP fluorescence overlaps with background autofluorescence, which poses challenges for distinguishing EGFP+ cells from non-expressing cells. This is especially true when reporter gene expression is relatively weak or at low abundance, as was the case in the embryonic heart. Improved fluorescence imaging techniques and equipment should help resolve the autofluorescence challenges, which were also partially overcome in this study by comparing fluorescence images in the red and green spectra. Specific EGFP+ fluorescence was only observed in the green spectrum, whereas autofluorescence has a much broader bandwidth and is evident in both the green and red spectra on our microscope. Specificity of EGFP+ expression was confirmed in several examples with anti-GFP IF staining.

In addition to the challenges posed by autofluorescence, we also observed considerable variation in EGFP+ cell staining within tissues that express Pnmt. This was most clearly seen in the adrenal medullae where many Pnmt+ cells did not display detectable EGFP expression. Other studies have also noted variable expression for the EGFP reporter in mammalian cells. For example, Swenson did a systematic analysis of GFP transgenic mouse using three different types of GFP mice strains with “ubiquitous” promoters and various methodologies for data acquisition, such as direct fluorescence detection, IF, and immunohistochemistry with diaminobenzidine (DAB) deposition. GFP expression characteristics were also determined in various organs [24]. Notably, GFP expression in different organs varied widely between the three genotypes tested, and the differences from same organs were also significant. No matter which promoter was used to drive GFP expression, not all the cells from examined tissues were GFP+ in any of the three transgenic lines. There may be inherent limitations in the nEGFP reporter gene itself, perhaps due to toxicity, turnover, nuclear reprogramming, and/or other effects of GFP and its derivatives that have yet to be fully defined. Alternatively, the nEGFP cDNA sequence itself may influence expression from the Pnmt locus. Other explanations for the non-uniform nature of nEGFP expression could involve stochastic or allele-specific expression [25, 26]. These questions remain unresolved, and will likely be the focus of future investigations. Regardless of the specific underlying mechanisms, the main limitations of the model primarily concern the variability of the nEGFP reporter, and difficulties in distinguishing its expression from background autofluorescence when in low abundance, such as the case observed during embryonic heart development.

Conclusion

Despite the limitations of the EGFP reporter indicated above, we have nevertheless shown that this reporter gene was correctly targeted to the mouse *Pnmt* gene locus. Further, we successfully created a new mouse genetic knock-in *Pnmt::nEGFP* model that expresses nEGFP exclusively in Pnmt+ cells. In no cases did we find nEGFP expression that was inconsistent with endogenous Pnmt expression. Expression was verified in adult adrenal chromaffin cells as well as embryonic cardiac cells. This model should be useful for identification and characterization of adrenergic cells in a variety of other tissues/cells also

known to express Pnmt, such as retina, brainstem, lung, testes, lymphocytes, and others [9–18].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This study was supported by funding from the NIH (HL78716) to S.N.E., and intramural NICHD funds to K.P.

References

1. Thomas SA, Matsumoto AM, & Palmiter RD (1995). Noradrenaline is essential for mouse fetal development. Nature, 374, 643–646. [PubMed: 7715704]
2. Zhou QY, Quaife CJ, & Palmiter RD (1995). Targeted disruption of the tyrosine hydroxylase gene reveals that catecholamines are required for mouse fetal development. Nature, 374, 640–643. [PubMed: 7715703]
3. Kvetnansky R, Sabban EL, & Palkovits M (2009). Catecholaminergic systems in stress: Structural and molecular genetic approaches. Physiological Reviews, 89, 535–606. [PubMed: 19342614]
4. Bao X, Lu CM, Liu F, Gu Y, Dalton ND, Zhu BQ, et al. (2007). Epinephrine is required for normal cardiovascular responses to stress in the phenylethanolamine *N*-methyltransferase knockout mouse. Circulation, 116, 1024–1031. [PubMed: 17698731]
5. Ignarro LJ, & Shideman FE (1968). Appearance and concentrations of catecholamines and their biosynthesis in the embryonic and developing chick. Journal of Pharmacology and Experimental Therapeutics, 159, 38–48. [PubMed: 5643949]
6. Ebert SN, Baden JM, Mathers LH, Siddall BJ, & Wong DL (1996). Expression of phenylethanolamine *n*-methyltransferase in the embryonic rat heart. Journal of Molecular and Cellular Cardiology, 28, 1653–1658. [PubMed: 8877775]
7. Ebert SN, & Thompson RP (2001). Embryonic epinephrine synthesis in the rat heart before innervation: Association with pacemaking and conduction tissue development. Circulation Research, 88, 117–124. [PubMed: 11139483]
8. Ebert SN, Rong Q, Boe S, Thompson RP, Grinberg A, & Pfeifer K (2004). Targeted insertion of the Cre-recombinase gene at the phenylethanolamine *n*-methyltransferase locus: A new model for studying the developmental distribution of adrenergic cells. Developmental Dynamics, 231, 849–858. [PubMed: 15517585]
9. Bohn MC, Goldstein M, & Black IB (1982). Expression of phenylethanolamine *N*-methyltransferase in rat sympathetic ganglia and extra-adrenal chromaffin tissue. Developmental Biology, 89, 299–308. [PubMed: 7035256]
10. Hammang JP, Bohn MC, & Messing A (1992). Phenylethanolamine N-methyltransferase (PNMT)-expressing horizontal cells in the rat retina: A study employing double-label immunohistochemistry. The Journal of Comparative Neurology, 316, 383–389. [PubMed: 1577991]
11. Ziegler MG, Bao X, Kennedy BP, Joyner A, & Enns R(2002). Location, development, control, and function of extra-adrenal phenylethanolamine *N*-methyltransferase. Annals of the New York Academy of Sciences, 971, 76–82. [PubMed: 12438093]
12. Ziegler MG, Kennedy BP, & Houts FW (1998). Extra-adrenal nonneuronal epinephrine and phenylethanolamine-*N*-methyltransferase. Advances in Pharmacology, 42, 843–846. [PubMed: 9328029]
13. Davidoff MS, Ungefroren H, Middendorff R, Koeva Y,Bakalska M, Atanassova N, et al. (2005). Catecholamine-synthesizing enzymes in the adult and prenatal human testis. Histochemistry and Cell Biology, 124, 313–323. [PubMed: 16052322]

14. Pendleton RG, Gessner G, & Sawyer J (1978). Studies onthe distribution of phenylethanolamine *N*-methyltransferase and epinephrine in the rat. Research Communications in Chemical Pathology and Pharmacology, 21, 315–325. [PubMed: 694228]
15. Andreassi JL, Eggleston WB, & Stewart JK (1998). Phenylethanolamine *N*-methyltransferase mRNA in rat spleen and thymus. Neuroscience Letters, 241, 75–78. [PubMed: 9507924]
16. Andreassi JL, Eggleston WB, Fu G, & Stewart JK (1998). Phenylethanolamine *N*-methyltransferase mRNA in rat hypothalamus and cerebellum. Brain Research, 779, 289–291. [PubMed: 9473698]
17. Bergquist J, Tarkowski A, Ekman R, & Ewing A (1994). Discovery of endogenous catecholamines in lymphocytes and evidence for catecholamine regulation of lymphocyte function via an autocrine loop. Proceedings of the National Academy of Sciences of the United States of America, 91, 12912–12916. [PubMed: 7809145]
18. Josefsson E, Bergquist J, Ekman R, & Tarkowski A (1996). Catecholamines are synthesized by mouse lymphocytes and regulate function of these cells by induction of apoptosis. Immunology, 88, 140–146. [PubMed: 8707341]
19. Ebert SN, Rong Q, Boe S, & Pfeifer K (2008). Catecholamine-synthesizing cells in the embryonic mouse heart. Annals of the New York Academy of Sciences, 1148, 317–324. [PubMed: 19120124]
20. Farley FW, Soriano P, Steffen LS, & Dymecki SM (2000). Widespread recombinase expression using FLPeR (flipper) mice. Genesis, 28, 106–110. [PubMed: 11105051]
21. Maltsev VA, Wobus AM, Rohwedel J, Bader M, & Hescheler J (1994). Cardiomyocytes differentiated in vitro from embryonic stem cells developmentally express cardiac-specific genes and ionic currents. Circulation Research, 75, 233–244. [PubMed: 8033337]
22. Wobus AM, Wallukat G, & Hescheler J (1991). Pluripotentmouse embryonic stem cells are able to differentiate into cardiomyocytes expressing chronotropic responses to adrenergic and cholinergic agents and Ca^{2+} channel blockers. Differentiation, 48, 173–182. [PubMed: 1725163]
23. Van Overstraeten-Schlogel N, Delgaudine M, Beguin Y, & Gothot A (2006). Limitations of the use of GFP transgenic mice in bone marrow transplantation studies. Leukaemia & Lymphoma, 47, 1392–1393.
24. Swenson ES, Price JG, Brazelton T, & Krause DS (2007). Limitations of green fluorescent protein as a cell lineage marker. Stem Cells, 25, 2593–2600. [PubMed: 17615263]
25. Harper CV, Finkenstadt B, Woodcock DJ, Friedrichsen S, Semprini S, Ashall L, et al. (2011). Dynamic analysis of stochastic transcription cycles. PLoS Biology, 9, e1000607. [PubMed: 21532732]
26. Held W, & Kunz B (1998). An allele-specific, stochastic gene expression process controls the expression of multiple Ly49 family genes and generates a diverse, MHC-specific NK cell receptor repertoire. European Journal of Immunology, 28, 2407–2416. [PubMed: 9710218]

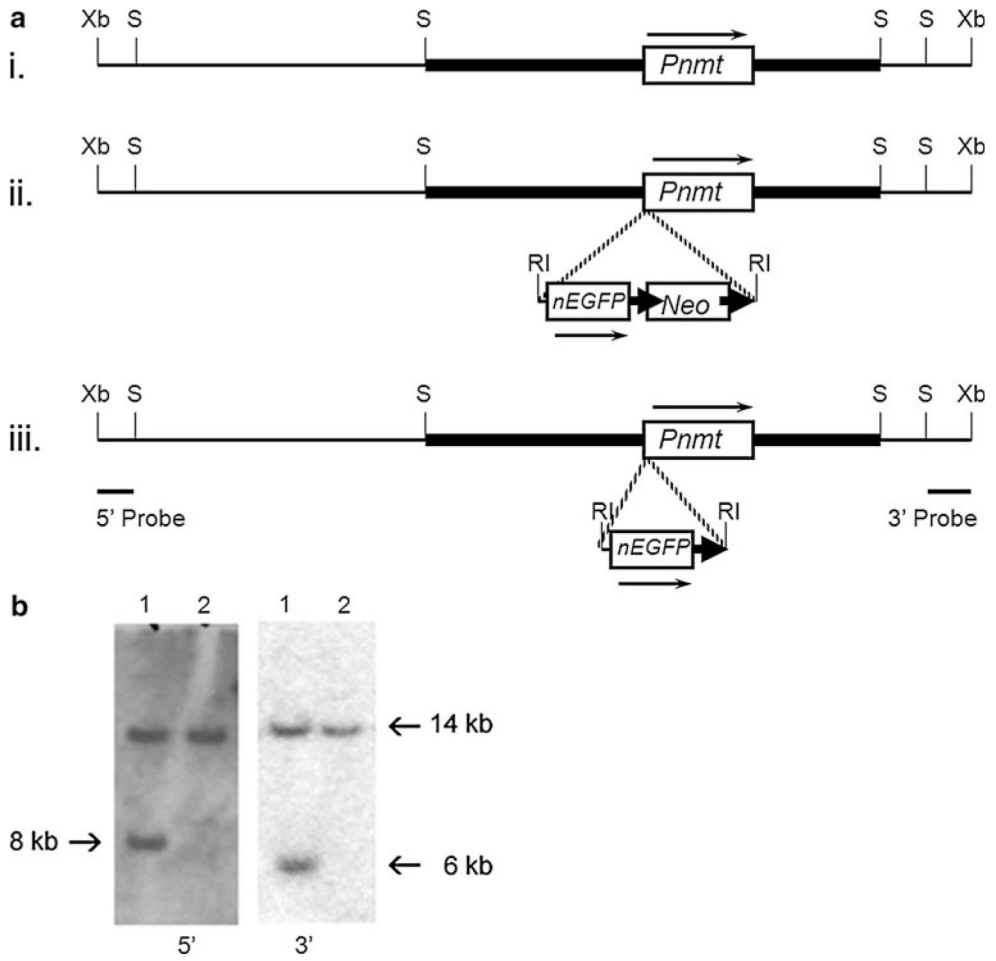

Fig. 1.

Construction of knock-in *Pnmt::nEGFP* allele. **a** Schematic depiction of (*i*) wild-type, (*ii*) Pnmt-GFP-Neo, and (*iii*) Pnmt-GFP alleles. The *rectangles* represent the coding sequences and the *thickened lines* show the DNA sequences used to direct homologous recombination to introduce GFP and NeoR into the first exon of Pnmt. **b** Representative Southern blot analyses of genomic DNAs from G418-resistant ES clones. After digestion with *Xba*I and hybridization with the 5′ external probe described in a, correctly targeted chromosomes display an 8 kb band in addition to the 14 kb band characteristic of the wild-type chromosome. The 3′ external probe identifies 6 and 14 kb bands from the targeted and wild-type chromosomes, respectively. Thus, *lane 1* is from a correctly targeted cell line, while *lane 2* shows the pattern for a non-targeted wild-type cell. *R1 Eco*RI; *S Sac*I; *Xb Xba*I

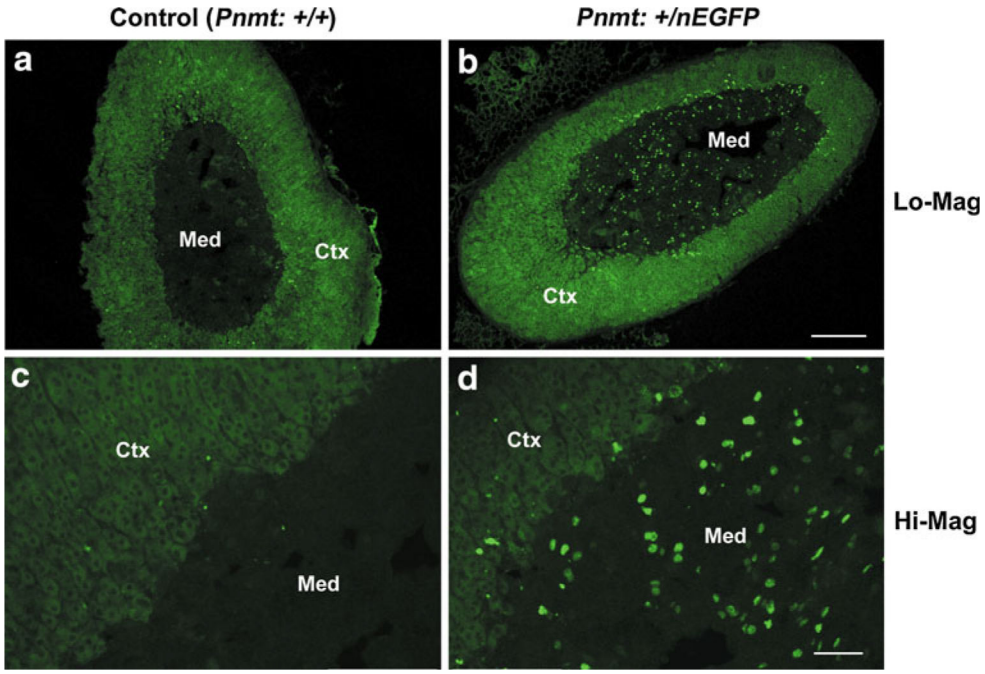

Fig. 2.

nEGFP expression in mouse adrenal glands. **a**, **b** Low-magnification views of fluorescent imaging of adrenal gland sections from wild-type control and *Pnmt*$^{+/nEGFP}$ mice, respectively. Scale bar 200 μm. **c, d** Higher magnification of same image. *Scale bar* 50 μm. *Ctx* cortex, *Med* medulla

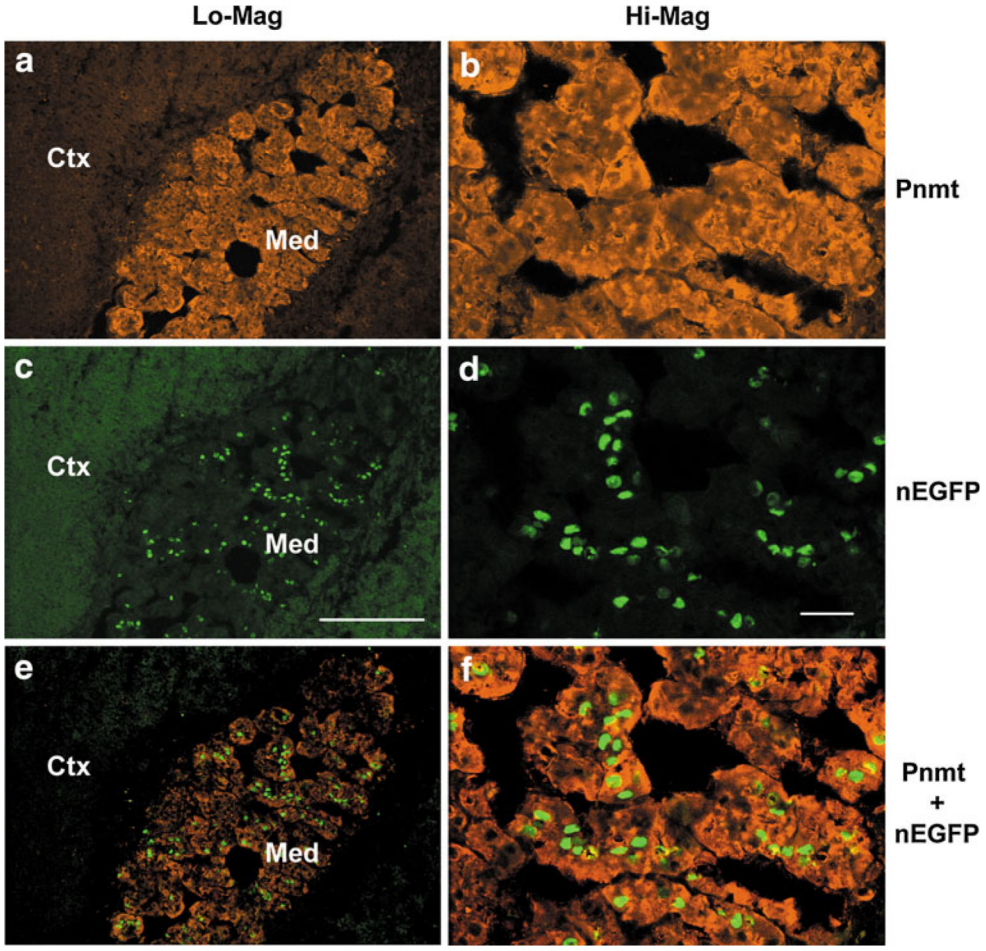

Fig. 3.

Identification of nEGFP and endogenous Pnmt in adrenal chromaffin cells. **a, b** Pnmt IF histochemical staining in adult mouse adrenal gland sections as visualized for red fluorescence (Texas Red filter). **c, d** EGFP expression in the same adrenal sections but visualized for green fluorescence (GFP filter). **e, f** Overlay of Pnmt and nEGFP staining for each section. **a, c, e** *Lo-mag* low-magnification: scale bar 200 μm. **b, d, f** Hi-Mag high-magnification: scale bar 30 μm

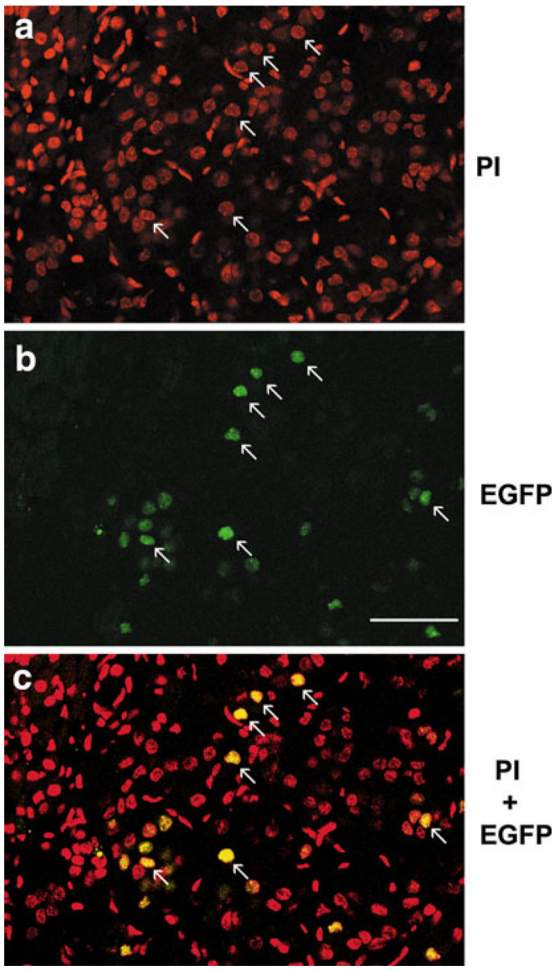

Fig. 4.

Nuclear localization of EGFP expression in adrenal chromaffin cells. **a** Propidium iodide (PI) staining of nuclei in adrenal chromaffin cells from an adult mouse adrenal gland section. **b** EGFP expression in the same section. **c** Overlay of PI and EGFP staining. *Yellow* staining indicates regions of overlap. *Scale bar* 50 μm (Color figure online)

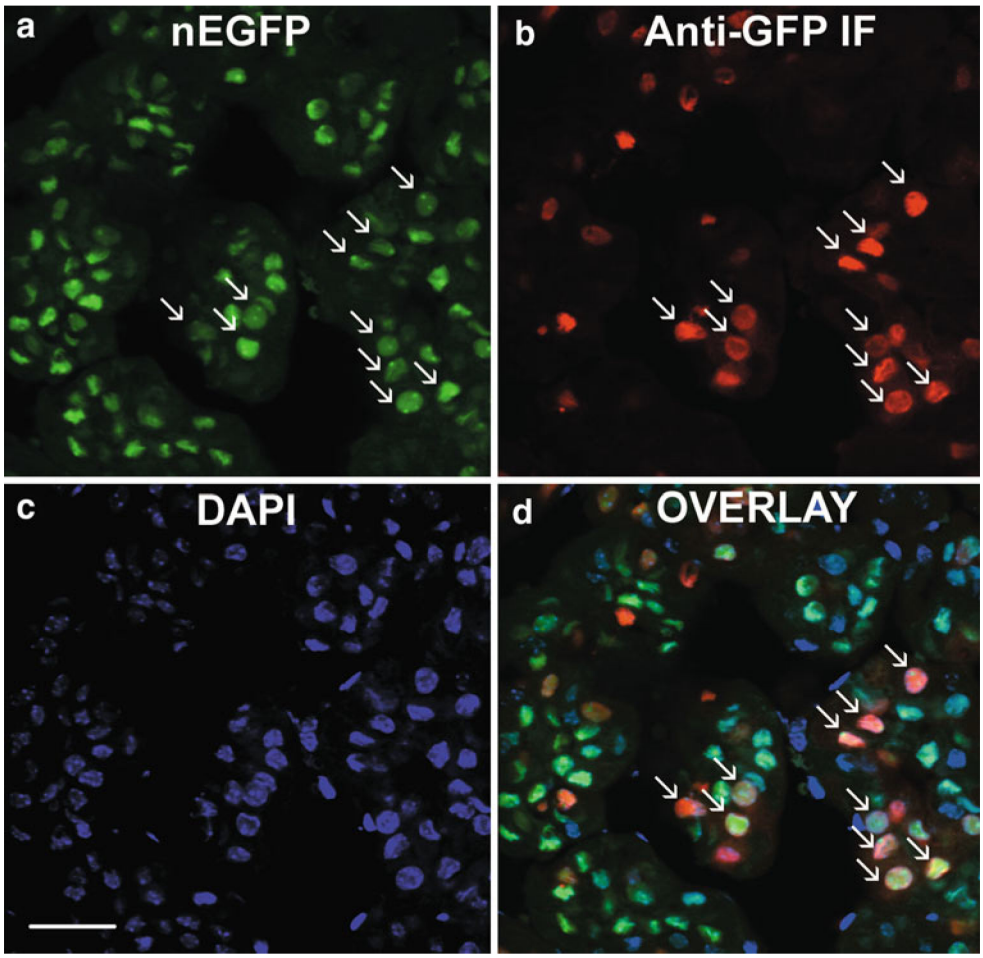

Fig. 5.

Co-localization of nEGFP and anti-GFP IF in nuclei of mouse adrenal gland sections. Positively stained cells were only found in the adrenal medulla. **a** nEGFP expression (*green*). **b** Anti-GFP IF staining (*red*). c DAPI nuclear stain (*blue*). **d** Overlay of images obtained in **a–c**. *Arrows* indicate examples of cells that were positively stained for nEGFP, anti-GFP IF, and DAPI. *Scale bar* 30 μm. Images were collected from heterozygous *Pnmt*$^{+/nEGFP}$ mice. Wild-type (*Pnmt*$^{+/+}$) control adrenal sections showed no nEGFP or anti-GFP staining (see Supplemental Fig. 1) (Color figure online)

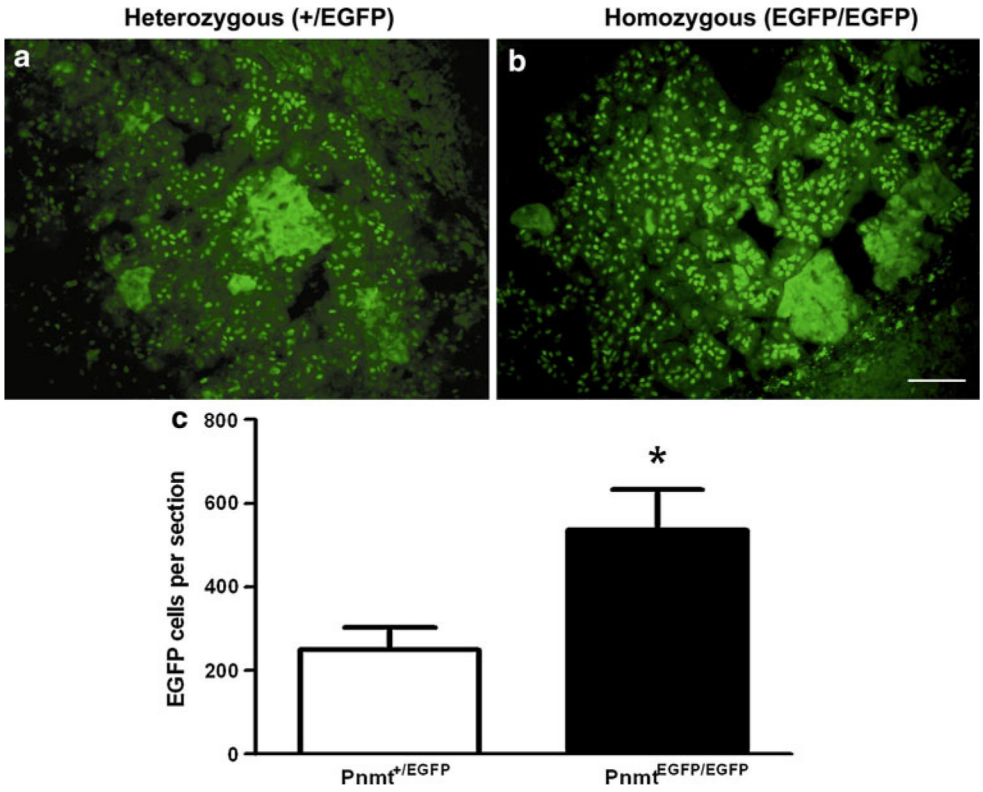

Fig. 6.

Comparison of nEGFP expression in heterozygous (*Pnmt*$^{+/nEGFP}$) and homozygous (*Pnmt*$^{nEGFP/nEGFP}$) mouse adrenal glands. **a, b** Representative adrenal gland sections with nEGFP expression from heterozygous (**a**) and homozygous (**b**) mice. **c** Quantitative analysis of average number of nEGFP+ cells per section for each genotype ($p < 0.05$, $n = 5$). *Scale bar* 100 μm

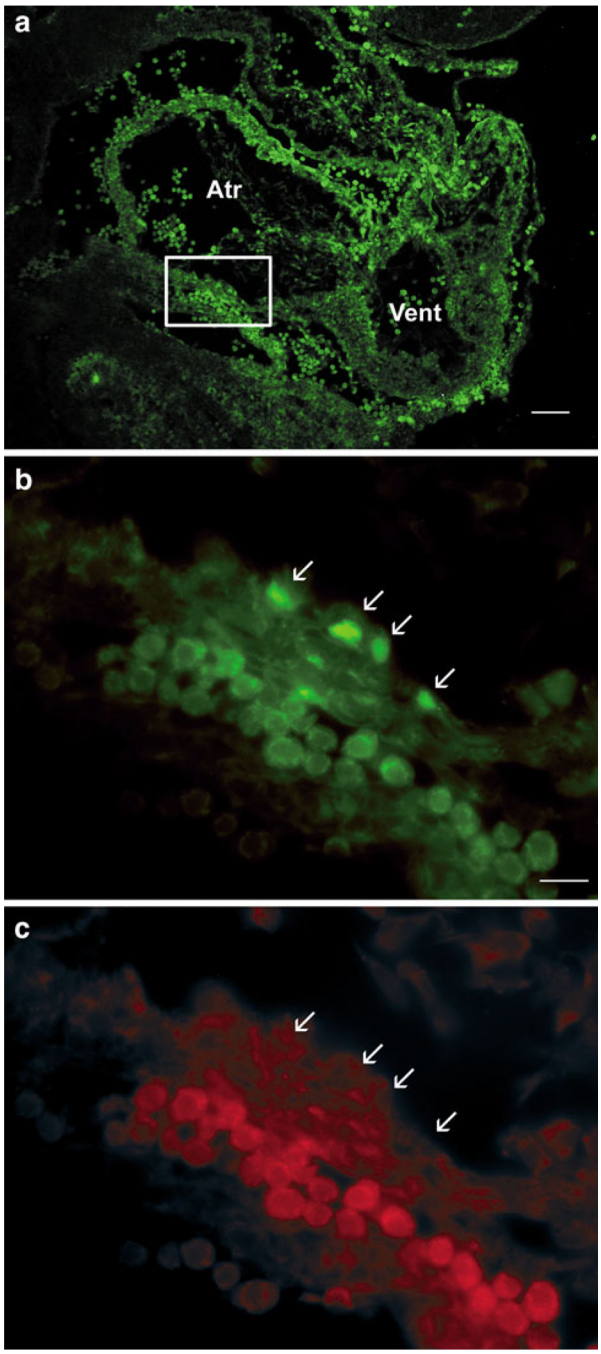

Fig. 7.

Detection of nEGFP expression from *Pnmt*$^{nEGFP/nEGFP}$ mouse hearts at E10.5. **a** Sagittal section of E10.5 mouse heart visualized in the green spectrum, with atrium (Atr) and ventricle (Vent) clearly distinguishable. *Scale bar* 100 μm. Although many cells appear to exhibit green fluorescence in this image, most of this fluorescence is due to background autofluorescence. This was determined by comparing fluorescent images in the green and red spectra. An example is shown in **b** and **c**, respectively. Note that the images shown in **b, c** represent an expanded view of the region of the E10.5 near the A-V junction (*boxed*

region from a) representing green and red fluorescent images, respectively. The nEGFP+ cells (*arrows*) were positively identified with the green fluorescence filter showing nEGFP expression to be exclusively evident when visualizing green but not red fluorescence. In contrast, autofluorescence was observed similarly in green and red spectra. *Scale bar* 20 μm

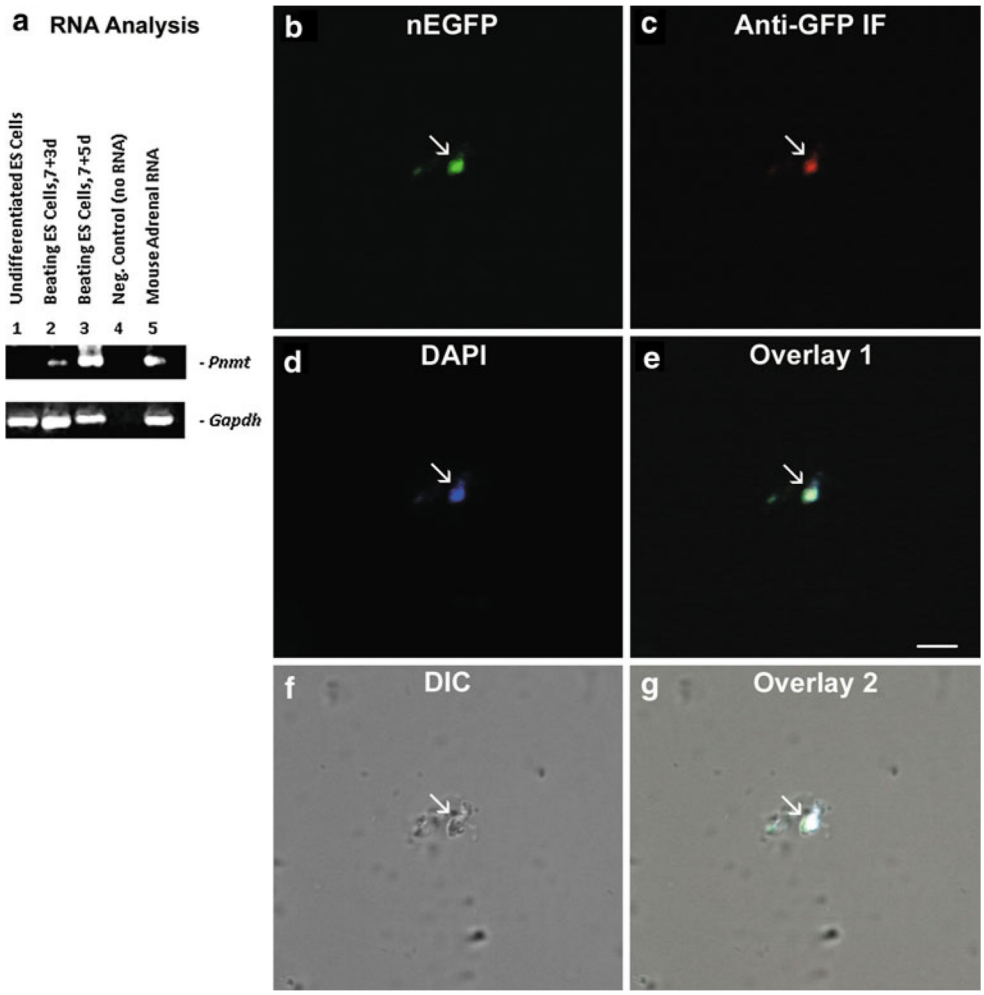

Fig. 8.

Induction of cardiac differentiation and activation of Pnmt and nEGFP expression in mESCs. **a** Endogenous mouse Pnmt gene expression as detected by RT-PCR before and after induction of cardiac differentiation in mESCs. This procedure takes (7 + *n*) days. **b–g** Images of nEGFP+ cells in 7 + 5d cultures of cardiac-differentiated mESCs as visualized in green fluorescence for nEGFP (**b**), red fluorescence for anti-GFP IF (c), blue fluorescence for DAPI (**d**), Overlay of **b–d** images (e), DIC (**f**), and overlay of b–d plus **f** (**g**). *Arrow* indicates the same positively stained mESC in **a–g**. *Scale bar* 10 μm