GFP Expression in the Mammary Gland for Imaging of Mammary Tumor Cells in Transgenic Mice

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Abstract

To examine the behavior of tumor cells in tumors developing directly from mammary tissue in transgenic models, we have evaluated transgenic mice expressing GFP3 and using MMTV-Cre or WAP-Cre in combination with the Cre-activatable CAG-CAT-EGFP construct, we find stronger expression of GFP that is still tissue specific. These animals provide a range of expression of GFP that is suitable for analysis of transgenic mammary tumors and metastases in vivo at the single cell level of resolution.

Introduction

Critical to an understanding of the process of metastasis is examination of the behavior of tumor cells that are metastasizing from the primary tumor. Metastasis involves a complex interaction between tumor cells, the extracellular matrix, the adjacent stromal cells, and blood and/or lymphatic vessels (1–3). Studies with xenograft models expressing GFP and using in vivo imaging have become increasingly important in providing new insights into the mechanisms of metastasis at the cellular level (3–6). Increased metastatic ability in xenograft models can correlate with cell polarization toward blood vessels, reduced cell fragmentation, and higher levels of motile host cells (7). However, transgenic models of tumorigenesis and metastasis provide a number of important advantages over xenograft models including the following: tumors that develop directly from the mammary epithelium; an intact immune system; and opportunities for genetic manipulation of the host environment (8). We have therefore examined several methods for specific expression of GFP in the mammary gland and find that they provide a range of GFP expression that is useful for in vivo analysis of tumor cell behavior.

Materials and Methods

Generation of MMTV-GFP Transgenic Mice. The coding sequence of the enhanced GFP gene from pEGFPN1 was subcloned into p206 (9) as follows (Fig. 1A). EGFP N1 was digested with NotI, and p206 was digested with EcoRI. Both digested plasmids were then blunted with Klenow and then digested with HindIII. The EGFP N1 fragment was then ligated into the digested p206, resulting in the construct named p206-GFP shown in Fig. 1A. The MMTV long terminal repeat provides specific transcription in the mammary gland, whereas the SV40 splicing and polyadenylation fragment enhances export and translation.

For generation of transgenics, the p206-GFP plasmid was prepared using the Qiagen Endofree Plasmid kit (Qiagen, Valencia, CA). DNA was prepared for microinjection by digestion of p206 plasmid with SalI and SpeI, and the expression construct was used to generate potential founder mice in the Einstein Transgenic Mouse Facility using standard methods.

Genotyping. To identify founders and transgenic progeny via Southern blot, genomic DNA was extracted from 1 cm of tail using the Promega DNA extraction kit (Promega, Madison, WI). The nucleic acid pellet was dissolved in 100 µl of DNA Rehybridization Solution (10 mM Tris and 1 mM EDTA). Genomic DNA (5 µg) was cut with BamHI overnight at 37°C. After gel electrophoresis and Southern transfer, the nylon filters were hybridized with GFP probe labeled with the AlkPhos DNA labeling system (Amersham).

For genotyping by PCR, about 0.5–1 cm of tail was digested in tail lysis buffer together with 10 mg/ml proteinase K, and then the DNA was precipitated with isopropanol, rinsed with ethanol, and resuspended in 200 µl of water. One µl was then used for genotyping by PCR. Primers (Table 1) were used at a final concentration of 0.5 µM in a total of 12 µl with 30–35 cycles of the following sequence: 94°C for 30 s; 60°C for 1 min (65°C for WAP-Cre and MMTV-Cre); and 72°C for 1 min.

RT-PCR. Total RNA was isolated from tissues of adult FVB mice (negative control), MMTV-EGFP mice, and MMTV-EGFP-pyMT mice using Trizol (Life Technologies, Inc.). One µg of total RNA was reverse transcribed using superscript II reverse transcriptase (Life Technologies, Inc.) and an oligo(dT)12–18 primer. An aliquot of 5 µl of the reverse transcription reaction was used in a 50-µl PCR reaction. Thirty cycles of PCR were performed in the presence of two pairs of specific primers (Table 1). The RT-PCR products were separated on a 2% agarose gel. A 475-bp band represented the EGFP mRNA, and a 230-bp band indicated β-actin as internal control.

Multiphoton Microscopy. A 10W Millenium Xs laser (Spectra Physics) was used to run a Radiance 2000 multiphoton system (Bio-Rad) that gives an output of 850 mW at 960 nm. For GFP fluorescence, 960 nm is the optimal imaging wavelength. Time-lapse sequences were taken at 60-s intervals for 30 min. The images were collected using Bio-Rad Lasersharp 2000 software at 50 lines/s. Images were processed using NIH Image 1.61/ppc and Adobe Photoshop for live imaging, a mouse was put under 5% isoflurane anesthesia, and a skin flap was cut so as to expose the tumor without disrupting blood flow to the tumor. The animal was then placed on the microscope, and the isoflurane was maintained through the duration of the experiment. The isoflurane was lowered from 2% to 0.75% as necessary to maintain even breathing. No viewing window was required to obtain images of single cells in mammary tissue.

<table>
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<th>Table 1 Primer sequences used for PCR analysis</th>
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3 The abbreviations used are: GFP, green fluorescent protein; MMTV, mouse mammary tumor virus; pyMT, polyoma middle T; RT-PCR, reverse transcription-PCR.
Results and Discussion

For directed expression of GFP by the MMTV promoter in the mammary epithelium, we inserted the GFP gene into the p206 expression vector as shown in Fig. 1A. Transgenic mouse lines stably expressing GFP were prepared in the Einstein Transgenic Mouse Facility using standard methods. Founder animals were identified by PCR and Southern blotting, and stable transmission of the transgene was established for two separate founders (termed 25 and 57 lines). Animal colonies were genotyped by Southern analysis, and RT-PCR was used to confirm expression of GFP. RT-PCR indicated expression in the mammary epithelium and salivary gland with slight expression in the liver in some animals (Fig. 1). Animals expressing GFP from the MMTV-GFP transgene were prepared in the Einstein Transgenic Mouse Facility using standard methods. Founder animals were identified by expressing GFP were prepared in the Einstein Transgenic Mouse Facility using standard methods. Founder animals were identified by expressing GFP. RT-PCR analysis of expression in control (FVB) and MMTV-GFP transgenic (25 line) mice. Organs from control and MMTV-GFP transgenic animals were removed, RNA was isolated, and RT-PCR analysis was performed as described in “Materials and Methods.” PCR for MMTV-GFP and beta-actin (positive control) was performed on each sample. Samples labeled “tumor” were from MMTV-PyMT transgenic tumors from control FVB animals or from animals also carrying the MMTV-GFP transgene. C, image from mammary fat pad from a MMTV-GFP female not carrying a tumor. Image shows a ductal structure that is fluorescent in the green channel only. Scale bar, 25 μm.

We also examined the use of combinations of transgenes to express higher levels of GFP in the mammary gland. We used a Cre-activatable promoter, CAG-CAT-EGFP (16, 17), in combination with Cre driven by the whey acidic protein promoter (WAP-Cre) or the MMTV promoter [MMTV-Cre (18, 19)]. Female mice carrying Cre and CAG-CAT-EGFP transgenes showed brightly fluorescent cells in the mammary gland in virgin animals (Fig. 3A). These were significantly brighter than the corresponding MMTV-GFP-expressing cells shown in Fig. 1C. WAP-Cre/CAG-CAT-EGFP transgenic animals had a relatively small fraction of cells that were fluorescent, which increased significantly during lactation (Fig. 3B, left panel), consistent with activation of the WAP promoter during pregnancy and lactation. Surprisingly, expression of GFP was also seen in a portion of the salivary gland (Fig. 3B, right panel). However, expression was not detected by fluorescence or immunohistochemistry in lung, liver, or muscle (data not shown). As demonstrated in Fig. 3A, right panel, expression of GFP in virgin MMTV-Cre/CAG-CAT-EGFP transgenic animals was more uniform than that in virgin WAP-Cre/CAG-CAT-EGFP transgenic animals, although immunohistochemical analysis showed that GFP was still not present in every epithelial cell, consistent with the known mosaic expression pattern of this promoter.

To examine the GFP expression of these transgene combinations in mammary tumors, we created transgenic animals expressing GFP under these promoter combinations together with expression of PyMT or HER2/neu in the mammary gland. Tumors generated by either oncogene were brightly fluorescent in animals with WAP-CRE/CAG-CAT-EGFP (Fig. 3C). In comparison, the MMTV-GFP × MMTV-PyMT 25 and 57 lines were much dimmer. The increased GFP expression of the Cre-activated CAG-CAT-EGFP construct enabled more detailed and rapid data acquisition. By placing a mouse under isoflurane anesthesia on the multiphoton microscope, motile cells could be seen within the living tumor. Time-lapse images, taken every minute, showed a cell moving toward a blood vessel (Fig. 3D). In comparison with the matrix surrounding the vessel and the other cells in the image, the cell marked by an arrow moved to the border of the matrix and then rapidly disappeared, suggesting that it has intravasated.

In summary, different expression strategies provide varying levels of expression of GFP in the mammary gland. The MMTV-GFP animals provide a relatively low level of expression of GFP and are mosaic, which might be advantageous for analysis of solitary cells. The CAG-CAT-EGFP transgene utilizes the high expression induced by the beta-actin promoter to provide high levels of expression of GFP. Second harmonic imaging of the matrix around these tumors indicates that most of the matrix signal is around the periphery of the tumor, with relatively little matrix within the tumor mass. This pattern of matrix is similar to what was seen with the metastatic MTLn3 xenograft tumors (20). The expression of GFP in primary tumors of animals carrying the WAP-Cre transgene indicates that differentiation of mammary epithelial cells to the extent required for activation of the WAP promoter does not block formation of mammary tumors. These constructs allow in vivo imaging for visual analysis of metastatic properties in the living tumor. As we have shown earlier in an
Fig. 2. PyMT-induced mammary tumor production in MMTV-GFP transgenic mice. A, comparative histology between breast tumors generated in transgenic mice carrying only the MMTV-PyMT gene (left panels) and those carrying both MMTV-GFP and MMTV-PyMT (right panels). The images show adenoma (top row) and late carcinoma (bottom row). B, imaging GFP-expressing carcinoma cells in tumors from a MMTV-PyMT × MMTV-GFP transgenic mouse up to 200 μm into the tissue. A cluster of GFP-expressing tumor cells surrounded by matrix (purple) was imaged using multiphoton microscopy. A z-series was taken at 5-μm intervals. The first image shown is 65 μm into the tumor, and each step between images represents 15 μm. Scale bar, 25 μm. C, GFP-expressing carcinoma cells in tumors of MMTV-GFP × MMTV-PyMT transgenic mice. Carcinoma cells expressing GFP (green) were imaged using a multiphoton microscope. Left image is just the GFP (green) channel, whereas the right image shows another tumor with the tumor cells in green and the extracellular matrix in blue.

Fig. 3. GFP expression in WAP-Cre or MMTV-Cre × CAG-CAT-EGFP mice. A, GFP fluorescence (top panels) and immunohistochemical expression (bottom panels) in the mammary fat pads of WAP-Cre/CAG-CAT-EGFP (left panels) and MMTV-Cre/CAG-CAT-EGFP (right panels) mice at >8 weeks of age. The MMTV-Cre/CAG-CAT-EGFP image is a projection of a two-photon z-series showing GFP fluorescence in green and matrix fibers in blue. Reddish brown staining in the bottom images represents immunohistochemical labeling for GFP. Scale bar, 85 μm. B, GFP fluorescence of WAP-Cre/CAG-CAT-EGFP transgenic mice in the lactating mammary gland (left panel) and in the salivary gland (right panel). Scale bar, 200 μm. C, GFP fluorescence in mammary tumors of WAP-Cre/CAG-CAT-EGFP transgenic animals induced by PyMT (left panel) or Neu (right panel). GFP fluorescence is in green; matrix fibers are in purple. The images are 512 μm across. D, the locomotion of a cell in a MMTV-Neu, WAP-Cre, CAG-CAT-EGFP tumor is seen by in vivo multiphoton imaging, and images were captured every 60 s for 30 min. In this figure, the time between sequences is 10 min. The blood vessel is seen as a nonfluorescent region on the left (the border of the blood vessel is marked in a with a white line). The arrow points to the cell exhibiting motility. Scale bar, 25 μm.
orthotopically injected rat model, orientation toward vessels, cell polarization, cell fragmentation (7, 21), and cell/matrix interactions (20) are all important behavioral factors associated with intravasation and cell motility in vivo. The ability to image tumor cells in transgenic animals enables, for the first time, evaluation of tumor cell behavior in tumors that have developed directly from the mammary epithelium.

Acknowledgments

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References

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