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Lentivirus-mediated bifunctional cell labeling for *in vivo* melanoma study

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SUMMARY

Lentiviral vectors (LVs) are capable of labeling a broad spectrum of cell types, achieving stable expression of transgenes. However, for *in vivo* studies, the duration of marker gene expression has been highly variable. We have developed a series of LVs harboring different promoters for expressing reporter gene in mouse cells. Long-term culture and colony formation of several LV-labeled mouse melanoma cells showed that promoters derived from mammalian house-keeping genes, especially those encoding RNA polymerase II (Pol2) and ferritin (FerH), provided the highest consistency for reporter expression. For *in vivo* studies, primary B16BL6 mouse melanoma were infected with LVs whose luciferase-GFP fusion gene (Luc/GFP) was driven by either Pol2 or FerH promoters. When transplanted into syngeneic C57BL/6 mice, Luc/GFP-labeled B16BL6 mouse melanoma cells can be monitored by bioluminescence imaging *in vivo*, and GFP-positive cells can be isolated from the tumors by FACS. Pol2-Luc/GFP labeling, while lower in activity, was more sustainable than FerH-Luc/GFP labeling in B16BL6 over consecutive passages into mice. We conclude that Pol-2-Luc/GFP labeling allows long-term *in vivo* monitoring and tumor cell isolation in immunocompetent mouse melanoma models.

SIGNIFICANCE—In this study we have developed and identified lentiviral vectors that allow labeled mouse melanoma cells to maintain long-term and consistent expression of a bifunctional luciferase-GFP marker gene, even in syngeneic mice with an intact immune function. This cell-labeling system can be used to build immunocompetent mouse melanoma models that permit both tumor monitoring and FACS-based tumor cell isolation from tissues, greatly facilitating the *in vivo* study of melanoma.

Keywords

Bioluminescence; cell labeling; FACS; GFP; lentiviral vectors; luciferase; syngeneic mouse model

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INTRODUCTION

Melanoma patients who are diagnosed with disseminated melanoma usually die within one year. Understanding the mechanisms that drive melanoma progression is therefore of paramount importance to researchers and clinicians alike. In this respect, relevant experimental animal models of melanoma progression provide a vital platform for molecular discovery, target validation and the preclinical testing or screening of anti-melanoma therapies (Larue and Beermann, 2007). Melanoma progression involves a series of complex tumor-host interactions, including stromal reactions (Gaggioli and Sahai, 2007), dissemination (Medic et al., 2007), and immune responses. Melanoma cells have been shown to develop the capacity to evade immune surveillance at a relatively early stage (Khong and Restifo, 2002). In late stages, they can exploit the immune response to enhance metastasis and tumor growth (Hussein, 2006). Therefore, relevant *in vivo* studies of melanoma progression will require models based on mice with intact immune function. Models in which murine melanoma cells are transplanted into syngeneic immunocompetent mice have been well utilized to this end (Talmadge *et al.*, 2007).

A desirable animal model of melanoma should allow tracking of melanoma growth and progression over time. For syngeneic transplantation models this can be achieved by expressing a marker gene in melanoma cells, allowing monitoring using bioimaging methods. Luciferase and fluorescent protein genes (such as green fluorescence protein, GFP) are two of the most commonly used markers in animal cancer models. Cells harboring the luciferase gene can be tracked *in vivo* by bioluminescence (BL) imaging in a quantitative manner, but BL imaging lacks the resolution for pinpointing individual cells (Gross and Piwnica-Worms, 2005). GFP-labeled cells can be distinguished or isolated by endoscopic fluorescence microscopy or FACS, respectively; however, the absorption of fluorescence by animal tissues makes monitoring of cells in deeper locations problematic (Hoffman, 2005). The functions of BL and fluorescence are actually complementary to each other, and the combination of both can greatly enhance the power of bioimaging to facilitate animal studies.

Lentiviral vectors (LVs) have proven to be excellent vehicles for delivery of marker genes for cell labeling. They are capable of efficiently infecting both static and cycling cells, and labeling a broad spectrum of cell types. The LV-delivered genes stably integrate into the genome of the infected cells without the need for drug selection (Klimatcheva *et al.*, 1999). However, several factors may affect the sustainability and consistency of transgene expression. For example, mammalian cells tend to silence proviral genes. This mechanism depends on the structure of the proviral gene and the nature of the cell type that is being transduced (He *et al.*, 2005). Overexpression of xenobiotic reporter genes such as GFP may cause cellular toxicity, resulting in the counter-selection of unlabeled cells (Liu et al., 1999). In animals, the expression of exogenous genes can induce an immune response, which may affect the survival of the labeled cells (Stripecke et al., 1999, Latta-Mahieu et al., 2002). This is a serious concern, especially for cancer models based on immunocompetent syngeneic mice. Therefore, optimization of the LVs and the reporter genes is necessary for their use in different types of mouse tumor models.

In this study, we aimed to develop cell-labeling LVs for use in immunocompetent mouse models of melanoma. We hypothesized that the type of promoters in the LV was critical for sustainable and consistent reporter activity in melanoma cells of syngeneic mouse models. To test this notion, we designed a series of ubiquitous promoters to drive reporters in LVs. We observed that promoters derived from mammalian house-keeping genes were capable of maintaining more long-term and consistent reporter activity in melanoma cells. We further demonstrated that the bifunctional luciferase-GFP fusion reporter (Luc/GFP) driven by those

promoters identified as superior allowed both the tracking of the labeled cells *in vivo* and their analysis by FACS. Moreover, the reporter activity of these labeled melanoma cells was consistent over several passages in immunocompetent syngeneic mice.

RESULTS

Development of lentiviral vectors for long-term cell labeling

pSico LV, which contains an H1 promoter and a GFP gene driven by the CMV promoter located between two lox-P sites, was originally designed for shRNA-mediated gene targeting, and has been reported to yield high transduction efficiency for both human and mouse cells (Ventura *et al.*, 2004). We removed the lox-P sites and H1 promoter and kept its CMV-driven GFP reporter, renamed it pSico-CMV-GFP, and employed this as a backbone vector for further modification. Reasoning that the promoter is crucial for reporter consistency, we replaced the CMV promoter in pSico with one of the following: hybrid SV40 enhancer-human ferritin promoter (FerH), RNA polymerase gene promoter (Pol2), hybrid SV40 enhancer-glucose regulation protein 78 gene promoter (GRP78), hybrid SV40 enhancer-glucose regulation protein 94 gene promoter (GRP94), and CMV enhancer- β -actin promoter-globulin intron enhancer hybrid promoter (CAG). They can be categorized according to the genes that the core promoters were derived from: housekeeping genes (FerH, Pol2, and CAG), stress-responsive genes (GRP78 and GRP94), and a viral gene (CMV). The constructed vectors were named based on the promoter and reporter (for example, FerH-GFP represents GFP driven by the FerH promoter in the pSico-based vector). Their structures are illustrated in Fig. 1A.

Next, the functions of these six LVs were evaluated in HeLa cells and three melanoma cell lines of different origins. The B16 cell line is derived from a spontaneous C57BL/6 mouse melanoma (Fidler, 1975). The HGF-Mel cell line is derived from a UV-induced melanoma of a HGF-transgenic FVB/N mouse (Recio *et al.*, 2002). p19NR-Mel cells are p19-null mouse melanocytes (Ha *et al.*, 2007) transformed by N-Ras (see Methods). These cell lines were infected with various titers of the six LVs containing the GFP reporter. At the condition that yielded 1% to 10% GFP-positive cells, the median fluorescence intensity (FI) was the index of the promoter activity (Fig. 1B). In general, FerH, GRP78, and CAG can be clustered in a high-activity group, and Pol2, GRP94, and CMV in a low-activity group. Pol2 was the weakest promoter in all the tested cell lines. Notably, the relative promoter activities among the tested LVs were similar in all four cell lines, corroborating their activity as ubiquitous promoters.

Sustainability and consistency of the reporters in labeled melanoma cells

As the next step, the B16, HGF-Mel, and p19NR-Mel cells were labeled with the six GFP-containing LVs, sorted, and returned to culture for further characterization. The GFP-positive ratio of each of the labeled cell lines was monitored periodically by FACS for 32 to 35 days, as shown in Fig. 2. For p19NR-Mel cells, the GFP-positive ratio of FerH-, Pol2-, GRP94-, and CAG-GFP-labeled cells was consistently maintained, while those of GRP98-GFP and CMV-GFP decreased over time (Fig. 2A, left panel). For HGF-Mel cells, the GFP-positive ratios of all the LV-labeled cells were consistently maintained, except those labeled with CMV-GFP (Fig. 2A, middle panel). For B16 cells, most changes occurred in the first five days. The GFP-positive ratios of FerH-, GRP78-, and GRP94-GFP-labeled cells did not significantly change, that of Pol2-GFP-labeled cells increased, and those of CAG- and CMV-GFP-labeled cells decreased. Thereafter, all the GFP-positive ratios were constantly maintained (Fig. 2A, right panel). Overall, the FerH- and Pol2-GFP labeling ratios were among the most consistent for all three melanoma cell lines, while the CMV-GFP labeling ratio was the most inconsistent among all the reporters.

The changes in the distribution of the labeled cells can be more accurately revealed by the FACS profile of the GFP intensity, as shown in Fig. 2B. In general accordance with the GFP-positive ratios, the profiles of p19NR-Mel cells labeled with FerH- and Pol2-GFP, along with CAG-GFP, did not change significantly over time. In contrast, in the profiles of GRP78-, GRP94- and CMV-GFP-labeled p19NR-Mel cells, a shift of the whole population toward the low-GFP direction was observed (Fig. 2B, upper panels). The peak shift and profile change in these cells suggested that the reporter activity decreased in various subpopulations during long-term culture. In the profile of the HGF-Mel cells, the range of GFP intensity distribution did not change significantly for any of the labeled cells, except for those labeled with CMV-GFP, whose peak in the profile shifted toward the GFP-negative direction (Fig. 2B, middle panels). The profiles of the FerH- and Pol2-GFP-labeled B16 cells did not significantly change over time. Inexplicably, the peaks in the profiles of GRP78-, GRP94- and CAG-GFP-labeled B16 seemed to shift toward the higher GFP direction. Similar to the cases of p19NR-Mel and HGF-Mel, the peak in the profile of CMV-GFP-labeled B16 cells shifted toward the GFP-negative direction, indicating the reporter activity diminished early during culturing (Fig. 2B, lower panels).

In summary, the consistency of reporter gene expression in the labeled cells was fully dependent on the promoter type. In long-term culture, cells labeled with FerH- and Pol2-driven reporters were the most consistent, and those labeled with the CMV-driven reporter lost intensity. The expression of GRP78-, GRP94-, and CAG-driven reporters appeared to become diversified over time, resulting in the formation of subpopulations of various reporter activities. As expected, the nature of the transduced cell was also an important determinant.

Diversification of reporter activity during growth of melanoma cell colonies

The maintenance of a constant GFP-positive ratio in the FerH- and Pol2-GFP-labeled cell populations (Fig. 2B) suggested that little or no outgrowth of residual unlabeled cells was occurring. In fact, we have monitored the growth rate of unlabeled control and FerH-GFP-labeled cells in all three cell lines, and no change was found during the culturing period of 30 to 35 days (data not shown). To determine if the reporter activities were altered by intrinsic mechanisms, such as epigenetic silencing, GFP-positive labeled p19NR-Mel, HGF-Mel, and B16 cells were grown in soft agar. Since each colony is derived from a single cell, any change in the distribution patterns of GFP expression observed in the colony should be caused by intrinsic mechanisms occurring during cell proliferation. The GFP expression patterns of colonies whose diameters were bigger than 50 μm were examined under fluorescence microscopy (Fig. 3). The representative patterns of the GFP expression in p19NR-Mel colonies are illustrated in Fig. 3A. Colonies grown from FerH- and Pol2-GFP-labeled p19NR-Mel cells had relatively uniform distribution of GFP expression (Fig. 3A). However, the GFP intensity was diminishing in the peripheral area of colonies of GRP78- and GRP94-GFP-labeled p19NR-Mel cells. Moreover, one of the GRP78-GFP-labeled p19NR-Mel colonies exhibited a punctate or scattered pattern of GFP-positive cells (the smaller colony in GRP78 panels of Fig. 3A), highlighting the great alteration in reporter activity among cells. Significantly decreased GFP intensity and a punctate pattern were observed in the CMV-GFP-labeled p19NR-Mel colonies (Fig. 3A). For the CAG-GFP-labeled p19NR-Mel cells, a high level of GFP expression was maintained throughout the colonies, but a punctuate pattern was again observed (Fig. 3A). Interestingly, for cells labeled with each of the six reporters, the GFP distribution pattern in the colonies matched the GFP intensity distribution profile in Fig. 2B. For example, for FerH- and Pol2-GFP-labeled p19NR-Mel cells, the GFP intensity was distributed in a narrow range to form a major peak at day 32 (Fig. 2B), which corresponded to the uniform GFP distribution in the colonies with the same labeling (Fig. 3A). For GRP78-GFP-labeled cells in the upper panels

of Fig 2B, subpopulations of lower GFP intensity arose by day 32, making the distribution of GFP intensity more diverse. This trend corresponded to the uneven or punctate GFP distribution pattern observed in colonies with the same labeling shown in the GRP78 panel of Fig. 3A.

Representative GFP expression patterns within the HGF-Mel colonies are shown in Fig. 3B. The colonies of all the labeled HGF-Mel cells, except those labeled with CMV-GFP, showed fairly uniform distribution of GFP expression. The GFP expression in colonies of CMV-GFP-labeled cells was not only reduced but also exhibited a punctate pattern (Fig. 3B). Interestingly, subpopulations of B16 cells became pigmented during colony formation, resulting in the blockage of GFP fluorescence (Fig. 3C). However, it can still be observed that colonies of FerH-, Pol2-, and GRP78-GFP-labeled cells had maintained overall favorable GFP expression (Fig. 3C). Colonies of GRP94- and CAG-GFP-labeled cells also showed a partially punctate or uneven pattern (Fig. 3C). The GFP expression in the colonies of CMV-GFP-labeled cells appeared only in a scattered pattern with diminished intensity in most of the area (Fig. 3C).

Since each colony is derived from a single cell, the differential GFP expression among cells in the colonies, as shown in Fig. 3, must arise from reporter activity alterations within the individual cells. Thus, these results reflect the relative sustainability and consistency of the reporters in the six LVs. We conclude that FerH- and Pol2-driven reporters are more consistent than the other four reporters in the tested melanoma cells. They were therefore selected for LV-mediated cell labeling for the study of melanoma *in vivo*.

Development of bifunctional reporters

The abilities to both accurately monitor *in vivo* tumor progression and then purify the resulting cancer cells for detailed molecular analyses greatly enhance the value of mouse cancer models. To generate a bifunctional reporter gene for these purposes, we linked the luciferase and GFP genes to form a Luc/GFP fusion gene, in which the former domain allows tracking of cells in a semi-quantitative manner, and the latter allows cell isolation by FACS. This fusion gene was inserted into the FerH- and Pol2-driven LVs to obtain FerH-Luc/GFP and Pol2-Luc/GFP labeling vectors. To test their functions, HeLa cells were infected with increasing amounts of FerH-Luc/GFP and Pol2-Luc/GFP. For cells in each infection condition, the activities of both reporter domains were determined by luciferase assay and FACS analysis, which gave the bioluminescence (BL) intensity and mean fluorescence intensity (MFI), respectively. The regression analysis of these two measurements was performed to examine their correlation. As shown in Fig. 4, for both LV reporters the BL intensity was well correlated with MFI, indicating both luciferase and GFP domains were functional. Similar correlations between GFP and luciferase activities have also been observed in FerH- or Pol2-Luc/GFP-labeled p19NR-Mel, HGF-Mel and B16 melanoma cell lines (data not shown).

Function and consistency of FerH- and Pol2-Luc/GFP labeling in syngeneic mouse models

In this study, we aimed to develop cell-labeling LVs for use in immunocompetent mouse models of melanoma. For this purpose, we used mouse B16BL6, a metastatic subline of the B16 cell line which is syngeneic to the C57BL/6 strain of mice. Melanoma tissue grown from B16BL6 cells was infected with FerH-Luc/GFP or Pol2-Luc/GFP, and then subcutaneously transplanted into albino C57BL/6 mice. In all tumors labeled with either LVs, the BL intensity increased roughly in parallel with tumor growth, although the rate of increase varied among individual tumors (Fig. 5A). Cell suspensions were prepared from resected tumors, and the GFP-positive cells from the labeled tumors were analyzed by FACS, as shown in Fig. 5B. These results demonstrated that the Luc/GFP labeling allows

the monitoring of B16BL6 tumor growth by BL imaging and the sorting of GFP-positive cells from tissues by FACS.

By the end of the third week, the subcutaneous tumors were resected, and the pieces transplanted into eight mice as the next passage. Following the initial transplantation, two more such passages were performed. Fig. 5C shows the labeled B16BL6 tumors and their BL signal in the mice from three consecutive passages. In the first passage, most of the FerH-Luc/GFP-labeled tumors had stronger BL signal than the Pol2-Luc/GFP-labeled tumors, although the variation between tumors was obvious. This was expected since FerH is a much stronger promoter than Pol2 (Fig. 1B). The BL activities of Pol2-Luc/GFP-labeled tumors appeared to be maintained in similar levels over the three passages. In FerH-Luc/GFP-labeled tumors, however, overall BL activities decreased as a function of passage number (Fig. 5C). Since the overall BL activity is related to the size of the labeled tumors, the reporter activities among tumors can be compared only after normalizing for size. Therefore, in each passage, we calculated the ratio of BL to size (BL/size) of individual tumors at Day 14 post-inoculation, a time point of growth during which no necrosis was observed in any of the tumors (Fig. 5D). For Pol2-Luc/GFP-labeled tumors, the BL/size ratios were not significantly different between the three passages, suggesting stable expression of reporter over time (Fig. 5D, left panel). For FerH-Luc/GFP-labeled tumors, most of their BL/size ratios in the first passages were several folds higher than those of Pol2-Luc/GFP-labeled tumors, as expected based on the higher activity of the FerH promoter. However, their BL/size ratios decreased and varied widely over subsequent passages. By the third passage, the median value had become significantly lower than that of the first passage ($p < 0.005$), with values dipping below those observed in the Pol2-Luc/GFP-labeled tumors (Fig. 5D, right panel). Taken together, these results indicate that the Pol2-Luc/GFP activities are sustainable and consistent in the immunocompetent syngeneic mouse model of B16BL6 melanoma. In contrast, FerH-Luc/GFP labeling was not sustainable over multiple passages in this model.

We next tested these two labeling vectors in a non-melanoma mouse tumor model built into a different genetic background. Rhabdomyosarcoma tissue derived from the HGF-transgenic, Ink4a/Arf-deficient mouse (HGF-RMS) (Sharp *et al.*, 2002) was infected with FerH- or Pol2-Luc/GFP, and then transplanted into immunocompetent syngeneic FVB/N mice. From day 6 to 13, the size of the tumors labeled with either LV increased in a similar manner (Fig. 6A, upper panels). During the same period, the overall BL activities associated with the Pol2-Luc/GFP-labeled tumors increased with tumor growth (Fig. 6A, lower left panel). However, BL activity in most (four out of six) FerH-Luc/GFP-labeled tumors decreased rapidly over time (Fig. 6A, lower right panel). Increases in BL signal intensity were modest in the other two FerH-Luc/GFP-labeled tumors. This trend was also revealed in the BL images of the mice (Fig. 6B). Again, we calculated BL/size ratios to analyze the time-course changes in reporter activities (Fig. 6C). For Pol2-Luc/GFP-labeled tumors, BL/size ratios at day 6 and day 13 were not significantly different (Fig. 6C, left panel). During the same period, however, the BL/size ratios of the FerH-Luc/GFP-labeled tumors were reduced significantly ($p < 0.05$; right panel of Fig. 6C), dropping more than 100-fold. When fragments from tumors with the highest reporter activities were transplanted into FVB/N mice for the second passage, they showed trends similar to the first passage: BL signals in Pol2-Luc/GFP-labeled tumors were maintained during tumor growth, while those in most FerH-Luc/GFP-labeled tumors diminished rapidly (data not shown). Clearly, for HGF-RMS tumors, Pol2-Luc/GFP is a much more consistent reporter than FerH-Luc/GFP in the syngeneic FVB/N mouse model.

To summarize, we demonstrate the potential of FerH- and Pol2-Luc/GFP as cell-labeling vectors for syngeneic mouse models of melanoma. In the case of the B16BL6 melanoma,

both FerH- and Pol2-Luc/GFP labeling were maintained during tumor growth in C57BL/6 mice, and both allowed tumor monitoring *in vivo* and FACS analysis. However, Pol2-Luc/GFP labeling was more sustainable and consistent than FerH-Luc/GFP labeling during serial tumor passage in mice. We also show that reporter consistency depended on the type of tumor cells and/or mouse strain. In the two immunocompetent, syngeneic mouse tumor models, Pol2-Luc/GFP labeling provided weaker but more consistent reporter activity.

DISCUSSION

The analysis of the *in vivo* behavior of human tumors, including melanoma and other cancer types, as xenografts in immunocompromised mice has been an important contributor to our understanding of mechanisms associated with tumor genesis and progression. However, the lack of a functional immune system and the aberrant tumor-host interactions engendered by the need to grow human cells in immunocompromised host mice has severely limited the utility and the relevance of these studies (Talmadge *et al.*, 2007). Transplantation of mouse melanoma into syngeneic mice harboring an intact immune system can overcome many of these deficiencies (Zaidi *et al.*, 2008). However, murine tumor cells attempting to grow in an immunocompetent mouse encounter significant environmental hurdles, many of which can affect the expression of foreign genes, and/or selection of the cells that express them. In fact, the lack of sustainability and consistency in expression of transgenes has been reported as a limiting factor for gene delivery into immunocompetent mouse (Hafenrichter *et al.*, 1994, Follenzi *et al.*, 2004, Kimura *et al.*, 2007). To overcome the difficulties, we here characterize two bifunctional LVs, FerH-Luc/GFP and Pol2-Luc/GFP, for use in mouse melanoma labeling, and demonstrated their potential use in establishing traceable melanoma models using immunocompetent mice.

The lentivirus is a type of retrovirus whose preintegration complex can actively penetrate the intact nuclear membrane of its target cell, thereby infecting both dividing and nondividing cells, including terminally differentiated cells and tissue stem cells (Haas *et al.*, 2000, Hanawa *et al.*, 2002). Upon entering the nucleus of the target cell, reverse transcriptase synthesizes the first strand of DNA from the RNA template, and the host DNA polymerase synthesizes the second strand to produce double-strand DNA, which is then inserted into the host genome by viral integrase. Contemporary self-inactivating LVs exclude genes for viral genome replication and virion production, not only providing safety for use, but also stabilizing the integrated gene. These features enable stable gene delivery without the need for drug selection (Federico, 2003). We have tested several HIV-based LVs and found that pSico has higher efficiency in transducing mouse cell lines (data not shown). All the LVs used in this study were based on pSico (Fig. 1). They were further tested in three types of mouse melanoma cells with distinct origins: B16 is a well-established cell line derived from a spontaneous mouse melanoma, HGF-Mel cell line is derived from UV-induced melanoma of HGF-transgenic mice, and p19NR-Mel cells are p19Arf-deficient, N-Ras-transformed primary mouse melanocytes. All three were efficiently transduced by the six developed LVs, demonstrating the feasibility of pSico-based LVs in labeling mouse melanoma cells.

In long-term culture (Fig. 2) and colony forming assay (Fig. 3) of transduced cells, the relative consistency of the six reporters exhibited the same trend in all the three tested cell lines (FerH- and Pol2-GFP > CAG- and GRP94-GFP > GRP78-GFP > CMV-GFP), indicating that the promoter type is the essential determinant of reporter consistency. Interestingly, this trend is in accordance with the categorization of the six promoters. The core elements of the FerH, Pol2 and CAG promoters are derived from the housekeeping genes, ferritin, RNA polymerase II and actin, respectively. GRP78 and GRP94 are stress-responsive molecular chaperones of the endoplasmic reticulum (ER), and their gene promoters are constitutively active, but can be further enhanced upon cellular stress such as

hypoxia or glucose starvation (Little *et al.*, 1994). The CMV promoter is derived from the immediate/early promoter/enhancer of cytomegalovirus. It is one of the most common promoters used to drive transgenes in eukaryotic cells. Though it is often regarded as a constitutively active promoter, studies have shown that the CMV promoter can be activated by a variety of mitotic signals and environmental stresses (Bruening *et al.*, 1998, Ramanathan *et al.*, 2005). These data imply that promoters derived from cellular housekeeping genes are more consistent than those derived from the stress-responsive genes; mammalian promoters are more sustainable than the viral promoter. In accordance with our results, it is well recognized that promoter silencing is the major cause of transgene expression diminution in transduced cells (Mutskov and Felsenfeld, 2004). Also, it has been shown that ubiquitous mammalian promoters are more consistent than viral promoters for long-term expression (Rettinger *et al.*, 1994). Taken together, we concluded that a ubiquitous promoter derived from mammalian housekeeping genes can drive more consistent expression of a reporter gene in LV-transduced cells. We also identify FerH and Pol2 as two promoters for sustainable melanoma cell labeling. Not surprisingly, the sustainability of reporter also depends on the cell type being transduced. For example, HGF-Mel cells maintained reporter activity more consistently than B16 and p19NR-Mel cells, regardless of their promoter types. Therefore, LVs driven by GRP78, GRP94, CMV, and CAG may be useful for transducing specific types of cells.

To generate a reporter with both luciferase and GFP functions, we linked these two genes to form a Luc/GFP fusion gene reporter. We have shown that this Luc/GFP reporter allows not only *in vivo* monitoring of labeled cells (Fig. 5 and 6), but also their analysis and isolation by FACS (Fig. 2 and 5B). The physical link between the luciferase and GFP genes ensures that the signals from these two reporters will be co-linear (Fig. 4). When the cells are sorted according to their GFP activities, all the isolated labeled cells harbor luciferase activity, whose level is proportional to the GFP-emitted fluorescence intensity of individual cells. Alternatively, when the tumors are detected in mice by BL imaging, they consist of a GFP-positive population whose mean fluorescence intensity is correlated with the BL intensity. Compared to this simple strategy, the linking of two reporter genes through an internal ribosome entry site (IRES) does not ensure the direct coupling of both activities, potentially increasing the variation in selecting cells. The Luc/GFP fusion reporter can also be used to overcome the failure of LV infection to reach 100% efficiency; FACS can be used to purify the infected, Luc/GFP-labeled cells. The luciferase activity contained within the purified cells assures their monitoring by BL imaging. Moreover, with proper selection of promoters for reporter genes, as we have shown in this study, the sorted cells can maintain sustainable and consistent dual reporter activities without the need for drug selection. This is important, especially for *in vivo* studies, since long-term drug selection procedures may alter the cell being studied. Moreover, drug selection marker genes have been reported to alter native cellular characteristics and increase the risk of immune response (Persons *et al.*, 1998). Taken together, our data demonstrate the advantages of the Luc/GFP fusion gene reporter for *in vivo* studies.

Many types of melanocytes and melanoma cells are pigmented, including B16 and B16BL6 cells. Cell pigmentation, by its function in nature, serves to absorb light. Its impact on optical imaging depends on the absorption spectrum of melanin (300 to 700 nm, with a peak at 330 nm), the emission spectrum of the light source, and their relative levels. GFP has a relatively narrow band of light emission (500–550nm, with a peak at 520nm); therefore, the fluorescence absorption by melanin can be substantial if a cell is heavily pigmented (Fig. 3C). However, due to variation of pigmentation among cells, the impact can vary greatly. Compared to GFP-emitted fluorescence, BL generated from the firefly luciferase reaction has a relatively broad spectrum (500–700 nm) and a peak at a longer wavelength (562 nm), so the effect of melanin on BL is fairly minimal, even for quantitation purposes. In this

study, the fluorescence from the Luc/GFP-labeled B16 and B16BL6 cells still allowed successful analysis and isolation by FACS (Fig. 2B and 5B), and no significant effect of pigmentation on BL imaging was observed in our animal models.

Ideally, the overall reporter activity should be proportional to the number of tumor cells, and the ratio of the reporter activity to the tumor size should remain constant in animal models. In this study, we use the BL/size ratio to measure the reporter activity in each tumor. The change in the median BL/size value between the transplant passages reflects the sustainability of the reporter, and the variation among the BL/size ratio of the individual tumors in the same passage is an index of the reporter consistency. The sustainability and consistency of the Pol2-Luc/GFP-labeled tumors were demonstrated by the similar median and variation of the BL/size ratios in the three passages. In contrast, for the FerH-Luc/GFP-labeled tumors, the median BL/size ratio decreased through all three passages, indicating that the sustainability of this reporter was limited in this model. Moreover, the variation of BL/size ratio was actually increased from the first to the second passage (Fig. 5D, right panel), implying that alterations in reporter activity were occurring during tumor growth. In our HGF-RMS models, Pol2-Luc/GFP labeling was also more sustainable and consistent than the FerH-Luc/GFP labeling, suggesting that the Pol2-driving reporter is superior in immunocompetent, syngeneic mouse tumor models. As mentioned above, overexpression of reporter genes may cause cellular toxicity or adverse immune response in animals. We postulate that lower reporter expression levels driven by a weaker promoter such as the Pol2 promoter may be advantageous because they reduce such risks, resulting in higher *in vivo* consistency and sustainability. In fact, we have observed that FerH-driven reporter activity was better maintained in immunocompromised mice compared to syngeneic mice (data not shown). Our results suggest that host immune response plays a role in cellular labeling sustainability and consistency.

In conclusion, we characterized pSico-based LVs harboring FerH- and Pol2-driven reporter genes and demonstrate sustainable and consistent melanoma cell labeling. We also show that a Luc/GFP fusion gene reporter permits real-time tracking of tumors in mice and isolation of tumor cells by FACS. Importantly, we demonstrated the feasibility of establishing traceable melanoma models with syngeneic, immunocompetent mice using cells labeled with the Pol2-Luc/GFP and/or FerH-Luc/GFP LVs.

METHODS

Construction of lentiviral vectors

The murine Pol2 promoter DNA was a generous gift from Dr. Steve Hughes, National Cancer Institute. The FerH, FerL, Grp78, Grp94 and CAG promoter/enhancers were derived from pVivo series vectors (Invivogen, San Diego, CA). The pSico LV vector was a kind gift from Dr. Tyler Jacks, Whitehead Institute, MIT. The U6-lox-CMV-GFP region of the pSico vector was removed by restriction digestion with XbaI and XhoI, and a Gateway Multisite vector conversion cassette (Invitrogen, Carlsbad, CA) was inserted to generate a Gateway Destination vector. Promoters were cloned using the manufacturer's protocols into Gateway Entry clones in the attL4-attR1 format for Multisite Gateway, while reporters were cloned into Gateway Entry clones in the standard attL1-attL2 format. The Luc2-eGFP reporter was generated using overlap PCR to fuse the firefly luciferase gene from pGL4.13 (Promega, Madison, WI) and the eGFP gene. All LV cloning was done in the E. coli MDS42 host (ScarabGenomics, Madison, WI) to minimize unwanted LTR recombination. Final expression clone DNA was prepared using GenElute XP maxiprep kits (Sigma, St. Louis, MO). The detailed information of promoter sequence and construction is provided upon request to D. Esposito (esposidod@mail.nih.gov).

Production of lentivirus

VSV-G pseudotyped LVs were produced using the ViraPower™ Lentiviral Expression System (Invitrogen, Carlsbad, CA). For LV production, 293T cells were plated in a T-75 flask and transfected three days later. Each LV expression construct was co-transfected with the ViraPower™ Packaging Mix that includes three packaging plasmids. Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA) was used to make DNA-Lipofectamine complexes in Opti-MEM I medium (no serum). The complexes were gently added to the cells and the flasks were incubated overnight at 37°C, 5% CO₂ in a humidified incubator. The next day the transfection medium was removed, the cells were re-fed with Dulbecco's Modified Eagle's Medium (DMEM) containing 10% FBS and 1% Penicillin-Streptomycin and returned to the incubator. The cell culture supernatant containing the LV particles was harvested 48 hrs post-transfection and centrifuged at 3000 rpm for 20 minutes at 4°C. To concentrate virus particles, the LV stock was filtered through a Millex-HV 0.45 µm filter and 1 ml aliquots were prepared and stored at -80°C. For the animal studies, LVs were concentrated using an Amicon 15 filter (Millipore) and centrifuging at 2000 rpm for 15–25 min. Further information of LV production is provided upon request to B. Ortiz-Conde (conde@ncifcrf.gov).

Cell culture, transduction, and sorting

HeLa cells and B16 (ATCC CRL-6322) melanoma cells were grown at 37°C in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% FBS, 4 mM L-glutamine, 4.5 g/L glucose, 1 mM sodium pyruvate, and 1.5 g/L sodium bicarbonate. p19NR-Mel cells were generated by stably transducing the activated N-Ras gene (a gift from Dr. Paul Kavari, Stanford University Medical School, CA) into p19^{Arf}-null mouse melanocytes (a gift from Drs. Dot Bennett and Elena Sviderskaya, St. George's University of London, UK) as previously described (Ha *et al.*, 2007). They were grown at 37°C in RPMI medium 1640 with 10% FBS, 200 nM 12-O-tetradecanoyl phorbol 13-acetate, and 200 pM cholera toxin. The HGF-Mel cell line was derived from UV-induced melanoma in HGF-transgenic, Ink4a/Arf-deficient mouse line described previously (Recio *et al.*, 2002). The melanoma cells were maintained in DMEM containing 10% FBS, 2 mM L-glutamine, 4.5 g/L glucose, 5 µg/ml insulin, and 5 ng/ml EGF.

To transduce HeLa cells, LV supernatant was added into a culture plate containing 20–40% confluent cells. The plate was incubated at 37°C and the medium changed the next day. Two days after infection, the reporter expression was examined by using a fluorescence microscope or by luciferase assay (Promega). Mouse melanoma cells were transduced by spinoculation with LVs as described previously (O'Doherty *et al.*, 2000). Briefly, cells were grown in a 6-well plate to reach 20–40% confluency. The media harvested from LV-producing 293T cells was added into cultured cells in a specific titer. The plates were centrifuged at 1200xg for 1 hour. Immediately after centrifugation, each well received 2 ml of the specific cell medium, and the plates were returned to the incubator. The medium was changed the next day. Two days after spinoculation, the GFP expression was examined under a fluorescence microscope. The GFP-positive cells were sorted using non-transduced cells as a negative control in the FACS Core Facility (Laboratory of Cancer Biology and Genetics, National Cancer Institute, Bethesda, MD), and returned to culture immediately.

Colony-forming assay

1.5 ml of 0.5% agar containing 1x cell culture medium described above was added into each well of the 6-well plate to form the base agar. Five-thousand GFP-labeled cells were mixed with melted 0.35% agarose containing 1x cell culture medium in 40°C, and then overlaid on the base agar. After the agarose solidified, 2 ml of cell culture medium were added to each well. The plates were incubated at 37°C in a humidified incubator for three to four weeks,

and the medium was changed every three days. The grown colonies were examined and imaged using a fluorescence microscope equipped with a CCD camera.

Mouse models of labeled tumors

B16BL6 cells have been described previously (Weber *et al.*, 1987) and were grown in the same medium as B16 cells. HGF-RMS tumors were generated from HGF-transgenic, Ink4a/Arf-deficient mouse line described previously (Sharp *et al.*, 2002). Both were kept in the cell repository bank of NCI-Frederick, MD. To grow tumors, B16BL6 cells were inoculated into C57BL/6 mice subcutaneously, and HGF-RMS fragments were transplanted into FVB/N mice subcutaneously. When the size of tumors reached 500 mg, they were resected and treated with 5 ml of 1.6% collagenase in RPMI 1640 medium for 90 min. The released tumor cells were washed, placed with RPMI 1640 medium containing 10% FBS. Then 5×10^6 tumor cells were mixed with 0.5 ml of high-titer LV supernatant ($> 2 \times 10^7$ /ml) in a 14-ml round-bottom tube (Falcon 352059), whose inner wall was coated with 1% gelatin. The tube was centrifuged at $1200 \times g$ for 1 hour in room temperature. After the centrifugation, the supernatant was removed. The cells were mixed with 500 μ l of RPMI 1640 medium and inoculated into five syngeneic mice immediately. Tumor size was measured with a caliper every three days, and the BL of the tumors was imaged using a Xenogen IVIS system (Caliper Life Sciences, Alameda, CA) every four to five days. All mouse studies were performed in accordance with Animal Study Protocols approved by the Animal Care and Use Committee (ACUC), National Cancer Institute, National Institutes of Health.

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Fig. 1. LVs developed in this study

(A) The structures of the six pSico-based LVs adopted and developed in this study. The name of each vector is listed to the left of each structural scheme. All vectors contain self-inactivating (SIN) LTRs, the LV packaging site (Psi), the Rev-responsive element (RRE), a central polypurine tract (cPPT), and the woodchuck hepatitis virus posttranscriptional regulatory element (WPRE). FerH, the hybrid SV40 enhancer (SV40e)-human ferritin promoter. Pol2, the RNA polymerase II gene promoter. GRP78, the hybrid SV40 enhancer (SV40e)-glucose regulation protein 78 gene promoter. GRP94, the hybrid SV40 enhancer (SV40e)-glucose regulation protein 94 gene promoter. CAG, the combination of CMV enhancer (CMVe)/chicken β -actin promoter (Act)/globin first intron (G-In). (B) The reporter promoter activities of the six LVs in HeLa and melanoma cells. The four cell lines were transduced with the LVs to reach a GFP-positive ratio of between 1% to 10%, and median fluorescence intensity was measured as the index of the promoter activity.

Fig. 2. Sustainability and consistency of LV-mediated labeling

p19NR-Mel, HGF-Mel, and B16 cells were transduced with GFP reporter gene by the LVs described in Fig. 1. The GFP-positive cells were sorted and maintained in culture continuously for 32 to 35 days. During this period, cells were split and analyzed by FACS every three days. (A) Time-course change in GFP-positive percentages for p19NR-Mel cells (left panel), HGF-Mel cells (middle panel), and B16 cells (right panel). The key is placed next to the right panel; FerH stands for FerH-GFP labeling, and the same for other reporters thereafter in this figure. (B) The FACS histograms of GFP intensity distribution at three time points for p19NR-Mel cells (upper panels), HGF-Mel cells (middle panels), and B16 cells (lower panels). The key for the time points of FACS analysis is at the bottom; the last time points (bright green line) for p19NR-Mel, HGF-Mel and B16 cells were day 32, 33 and 34, respectively.

Fig. 3. Reporter activity diversification among cells during colony formation

The GFP-positive labeled mouse melanoma cells described in Fig. 2 were grown in soft agar, which allowed colonies to form from single cells within three to four weeks. The LV-mediated reporter used to label the cells is denoted by the name of the promoter above each panel. For example, FerH stand for FerH-GFP. In each panel, the bright-field images and the corresponding GFP images are at the bottom and top, respectively. The yellow and blue bars mark scale of 250 and 50 μ M, respectively. (A) Colonies derived from GFP-positive p19NR-Mel cells. The dashed lines mark the edge of the colonies in the GFP images. p19NR-Mel cells are unpigmented; therefore, for all bright field images, the dark area was caused by the shadowing as the bright light projected in the middle of the colonies. (B) Colonies derived from GFP-positive HGF-Mel cells. HGF-Mel cells are unpigmented, and the darker area in the center of each colony in the bright-field images is also the shadowing from the bright light. (C) Colonies derived from GFP-positive B16 cells. Some B16 cells became pigmented in each colony.

Fig. 4. Correlative activities of the bifunctional reporter in the LVs

FerH-Luc/GFP and Pol2-Luc/GFP LVs were constructed by replacing the original GFP reporter gene with Luc/GFP fusion gene. HeLa cells were infected with different amounts of LV supernatant as described in Methods. The GFP and luciferase expression in the transduced cells were measured by the mean fluorescence intensity (MFI) by FACS, and bioluminescence intensity (BL) by luciferase assay, respectively. (A) The regression analysis of MFI and BL in FerH-Luc/GFP-transduced HeLa cells. The correlation coefficient (R^2) is 0.995. (B) The regression analysis of MFI and BL in Pol2-Luc/GFP-transduced HeLa cells. The correlation coefficient (R^2) is 0.982.

Fig. 5. Sustainability and consistency of FerH- and Pol2-Luc/GFP labeling in the B16BL6 melanoma mouse model

Tumors derived from B16BL6 cells were labeled with FerH-Luc/GFP or Pol2-Luc/GFP and then transplanted subcutaneously into C57BL/6 mice, as described in Methods. After three weeks, all tumors were resected, and one tumor with average BL was selected for transplantation into mice as the next passage. The B16BL6 tumors went through three passages in this study. (A) Size and BL intensity of the tumors labeled with Pol2-Luc/GFP (left panels) and FerH-Luc/GFP (right panels) in the first passage. Changes in growth and BL intensity of the individual tumors are shown in the upper and lower panels, respectively. (B) The representative FACS GFP-scatter profiles of the labeled B16BL6 tumors of the first passage. Upper panel, unlabeled control B16BL6 cells. Middle panel, Pol2-Luc/GFP-labeled tumor #7. Lower panel, FerH-Luc/GFP-labeled tumor #1. The tumor numbers correspond to those shown in the panels of (A). (C) The BL imaging of the tumors labeled with Pol2-Luc/

GFP (left pictures) and FerH-Luc/GFP (right pictures), at day 14 after transplantation, in the three passages. (D) The BL/size ratio of individual tumors labeled with Pol2-Luc/GFP (left panel) and FerH-Luc/GFP (right panel), at day 14 after transplantation in each passage. The red bar indicates the median of the values, and the blue whiskers mark the middle two quartiles. The significance is analyzed by 1-way ANOVA, Kruskal-Wallis test.

Fig. 6. Sustainability and consistency of FerH- and Pol2-Luc/GFP labeling in a rhabdomyosarcoma mouse model

HGF-RMS rhabdomyosarcoma cells were labeled with FerH-Luc/GFP or Pol2-Luc/GFP and transplanted subcutaneously into FVB/N mice, as described in Methods, and grown for two weeks. (A) Size and BL intensity of the tumors labeled with Pol2-Luc/GFP (left panels) and FerH-Luc/GFP (right panels) during the same period. Changes in growth and BL intensity of the individual tumors are shown in the upper and lower panels, respectively. (B) The BL imaging of the tumors labeled with Pol2-Luc/GFP (left panels) and FerH-Luc/GFP (right panels) at day 6 and 13 after transplantation. (C) The BL/size ratio of individual tumors labeled with Pol2-Luc/GFP (left panel) and FerH-Luc/GFP (right panel), at day 6 and 13 after transplantation. The thick bar indicates the median of the values, and the whiskers mark the middle two quartiles. The significance is analyzed by Wilcoxon matched pairs test.