Detecting cancers through tumor-activatable minicircles that lead to a detectable blood biomarker


Earlier detection of cancers can dramatically improve the efficacy of available treatment strategies. However, despite decades of effort on blood-based biomarker cancer detection, many promising endogenous biomarkers have failed clinically because of intractable problems such as highly variable background expression from nonmalignant tissues and tumor heterogeneity. In this work we present a tumor-detection strategy based on systemic administration of tumor-activatable minicircles that use the pan-tumor-specific Survivin promoter to drive expression of a secretable reporter that is detectable in the blood nearly exclusively in tumor-bearing subjects. After systemic administration we demonstrate a robust ability to differentiate mice bearing human melanoma metastases from tumor-free subjects for up to 2 wk simply by measuring blood reporter levels. Cumulative change in reporter levels also identified tumor-bearing subjects, and a receiver operator characteristic analysis of data from a limited number of these mice yielded an area of 0.918 ± 0.084. Lung tumor burden additionally correlated (r² = 0.714; P < 0.05) with cumulative reporter levels, indicating that determination of disease extent was possible. Continued development of our system could improve tumor detectability dramatically because of the temporally controlled, high reporter expression in tumors and nearly zero background from healthy tissues. Our strategy’s highly modular nature also allows it to be iteratively optimized over time to improve the test’s sensitivity and specificity. We envision this system could be used first in patients at high risk for tumor recurrence, followed by screening high-risk populations before tumor diagnosis, and, if proven safe and effective, eventually may have potential as a powerful cancer-screening tool for the general population.

Cancer is an enormous global health problem. The American Cancer Society estimates that in 2008 alone there were an estimated 1.27 million new diagnoses of cancer and 7.6 million deaths caused by cancer (1). The time at which a cancer is detected, both at initial cancer diagnosis and during tumor recurrence, is one of the most important factors affecting patient outcome, because if cancer is detected early, current treatments are likely to be more effective (2). Unfortunately, the majority of cancers are detected relatively late, leading to high mortality rates. These rates are expected to double by 2030 unless more effective detection strategies and treatments are developed. To stem the tremendous loss of life caused by this terrible disease, a broadly applicable tool capable of detecting cancers in their earliest stages is urgently needed.

One strategy for improving detection of cancers includes the development of blood-based assays that detect endogenous cancer biomarkers (protein, microRNA, circulating tumor cells, and others) that are shed or released into the bloodstream. This is highly attractive because it facilitates affordable cancer-screening programs but often suffers from sensitivity and specificity issues resulting from low blood biomarker concentrations (3), rapid in vivo and ex vivo biomarker degradation (4), tumor heterogeneity, and highly variable background expression in nonmalignant tissues (5). Using current clinical biomarker assays, we have computationally estimated that a tumor can grow for 10–12 y and reach a spherical diameter >2.5 cm before endogenous blood biomarkers reach sufficient levels to indicate disease (6). Of the thousands of potential blood biomarkers reported, only a small percentage (<1%) are used in the clinic (7), and the implementation of new blood biomarkers in the clinical setting is decreasing because of their lack of validated specificity and diagnostic value (4, 7). Although enormous effort has been devoted to developing tools for detecting endogenous cancer blood biomarkers, there have been very few successes.

To overcome the limitations of endogenous biomarker detection, we envisioned an alternative strategy based on the identification of tumor-bearing individuals using blood-based detection of exogenously delivered, genetically encoded reporters that produce tumor-driven biomarkers. The main potential advantage of this strategy is the ability to tailor biomarker expression exclusively in cells of a particular phenotype (i.e., tumor cells), thereby reducing the number of false positives caused by protein production in nonmalignant tissues. Based on this premise, we hypothesized that systemic administration of a tumor-activatable vector encoding a secretable reporter gene could be used to identify tumor-bearing subjects provided that transgene expression was transcriptionally targeted to cancer cells using a tumor-specific promoter (a promoter of a protein that is only present in tumors) (Fig. 1). For this strategy to be translated into the clinic more readily, the safety, specificity, sensitivity, and broad applicability are of utmost importance, and each component of our system was chosen carefully to maximize translational potential. Specifically, the authors declare no conflict of interest.

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in this first-generation system we developed nonviral tumor-activatable minicircles (MCs) encoding the reporter gene human secreted embryonic alkaline phosphatase (SEAP) and attained tumor specificity through the use of the tumor-specific Survivin promoter (pSurv).

Although much safer than viral vectors, traditional nonviral vectors (i.e., plasmids) have two drawbacks: low gene transfer rates and transient expression profiles. MCs are essentially plasmids that lack the prokaryotic backbone required only for expansion in bacteria. First described in 1997 by Darquet et al. (8), MCs have demonstrated improved expression profiles compared with their plasmid counterparts because of their smaller size and reduced transcriptional silencing (8–11). MCs also conform to regulatory “plasmids free of antibiotic resistance genes” (pFAR) principles (12). Although producing MCs traditionally was very labor intensive and time consuming, recent advances in MC production schemes have made it possible to produce large quantities in short periods of time with relative ease and reduced costs (13). Hence, MCs have become one of the most promising nonviral vector platforms in terms of translational potential, potency, and safety.

SEAP is the most commonly used secretable reporter protein and has many ideal characteristics. It is an artificial, C-terminally truncated, secretable form of human placental alkaline phosphatase (PLAP) that is expressed only during embryogenesis; thus it is a unique reporter not normally found in the blood and should have nearly zero background levels (14). Compared with PLAP, SEAP is unusually heat stable; thus heating samples to 65 °C allows SEAP to be assayed specifically (15). Commercial SEAP detection assays are extremely sensitive over at least a 4-log order concentration range, with detection limits in the picogram per milliliter range. SEAP also is an ideal protein-based reporter for translation into the clinic because (i) it has shown good potential for longitudinal monitoring of nonviral gene transfer in mice and large animals (16); (ii) its human origin implies it should have low or no immunogenic potential, as indicated with murine SEAP in immunocompetent mice (17); and (iii) it already has been used successfully in the clinic (18).

Last, our system uses the tumor-specific pSurv to drive the expression of SEAP. Baculoviral IAP repeat-containing protein 5 (BIRC5), also called Survivin, is a member of the apoptosis inhibitor family and helps control mitotic progression and prevent cell death. It is expressed in some fetal tissues (kidney, liver, lung, and brain) and in many cancers, such as melanoma, liver, lung, breast, colon, and ovarian, but is not expressed at detectable levels in healthy adult tissues (19–21). pSurv is well suited for transcriptional targeting of tumors as demonstrated in models of lung, melanoma, colon, breast, ovarian, and liver cancer (21–25). Thus, our pSurv-driven tumor-activatable MCs should have broad applicability for effective cancer screening across numerous tumor types and patient populations.

Here we have developed diagnostic tumor-activatable MCs and tested the ability to distinguish tumor-bearing from healthy subjects after their systemic administration by measuring blood levels of a genetically encoded cancer biomarker. Our results suggest that use of tumor-activatable MCs may be a promising platform technology for safe and efficacious detection of cancers in the future.

Results

Tumor-Activatable Minicircles Are Superior to Plasmids Across Multiple Melanoma Cell Lines. We first compared the transcriptional activity of two promising tumor-specific promoters, pSurv and the progression elevated gene-3 promoter (pPEG) (26), to assess which promoter would give us the lowest background in healthy tissues. To do so, we constructed plasmids expressing firefly luciferase (FLuc) driven by either pSurv or pPEG and delivered these plasmids systemically into healthy female Nu/Nu mice. After 2 d, pSurv-driven plasmids showed significantly lower background FLuc expression than pPEG-driven constructs, particularly in the heart and lung (Fig. S1). We also compared promoter activity in both primary human fibroblasts and two human tumor cell lines. Again, pSurv had lower background activity in human fibroblasts and equivalent or higher expression in tumor cell lines (Fig. S2). Thus, for all future experiments we decided to use pSurv in our tumor-activatable constructs.

We developed both tumor-activatable parental plasmids (PP; ≈7.9 kb) and MCs (≈4.1 kb) with pSurv driving SEAP expression (Fig. 2 A and B). To compare the SEAP concentration attainable with these two constructs, two human melanoma cell lines, MeWo (Fig. 2C) and SK-MEL-28 (Fig. S3), were transfected with an equal mass of PP and MC and an equal volume of a linear polyethyleneimine (PEI) transfection agent. After transfection, SEAP concentration was measured in the culture medium each day for up to 8 d. By day 3 in MeWo cells, MCs had significantly higher SEAP concentration in the medium than did PPs, and these differences were maintained until the last day (day 8) of the experiment (Fig. 2C). Similar results were obtained for SK-MEL-28 cells (Fig. S3). Therefore, MCs driven by the tumor-activatable pSurv have improved transgene expression profiles in melanoma cancer cells compared with their PP counterparts. To ensure that MCs provide an advantage over PPs in vivo, we also compared the transgene expression levels achieved by PPs and MCs driven by a constitutive promoter after systemic administration in mice (n = 5 for PPs and n = 4 for MCs) and found that MC expression was significantly higher than PP expression (P < 0.05) in lung at multiple time points postdelivery (Fig. S4).

Intratumoral Injection of Tumor-Activatable MCs Leads to Detectable Plasma SEAP Concentration. We next determined whether direct intratumoral (I.T.) administration of tumor-activatable MCs would lead to a detectable SEAP signal in the blood. Mice bearing s.c. MeWo xenografts (≈50–80 mm³) were I.T. administered 20 μg of MCs complexed with PEI (n = 4) or only with PBS as
For each mouse, plasma SEAP concentration was measured before (day 0) and on days 1, 3, 7, 11, and 14 after tail-vein administration of 40 μg of MC (tumor + MC). As control groups, healthy (tumor-free) mice also received either MC (control + MC; \( n = 6 \)) or 5% glucose (control – MC; \( n = 5 \)). As seen in Fig. 3A–C, plasma SEAP concentration was elevated after MC injection in individual tumor-bearing mice. On average, plasma SEAP concentration profiles between days 3 and 14 postadministration were significantly higher (\( P < 0.05 \)) in the tumor-bearing mice than in either control group (Fig. 3D). It should be noted that although some healthy mice receiving MC showed a slightly positive SEAP signal (most likely reflecting promoter leakiness, as also noted with pSurv in Fig. S1), overall no significant differences were noted between the two groups of control mice (Fig. 3D). Therefore, measurement of plasma SEAP levels after the systemic administration of tumor-activatable MCs could differentiate between tumor-bearing and healthy subjects, and a wide window of opportunity (>1 wk) was available to identify tumor-bearing subjects.

Because SEAP levels were elevated at multiple time points after MC administration, we evaluated the cumulative shedding of SEAP into plasma by calculating the plasma SEAP concentration area under the curve (AUC) for each mouse. Comparison of this single metric across all mice revealed no differences between the two control groups (control ± MC) but significantly (\( P < 0.05 \)) elevated values in tumor-bearing mice as compared with either control group (Fig. 4A). We then evaluated our assay’s ability to distinguish between tumor-bearing and healthy subjects by performing receiver operator-characteristic curve (ROC) analysis (Fig. 4B). We found a significant (\( P < 0.05 \)) area of 0.918 (±0.084 SE) and a 95% confidence interval of 0.754–1.083. Hence, with this first-generation vector used at the MC doses described, our assay was significantly reliable in identifying tumor-bearing subjects.
Finally, we noted some tumor-bearing subjects had AUC values that were only slightly above the mean of the control mice receiving MC (Fig. 4A). Moreover, as shown in Fig. 3A–C, the change in plasma SEAP concentration appeared to correspond qualitatively to the degree of tumor burden. Based on these two observations, we hypothesized that SEAP AUCs would correlate with lung tumor burden (as assessed by BLI within 3 d before MC administration). Because tumors were located primarily within the lungs, and the optical BLI signal is dependent on tissue depth, we restricted our evaluation to mice with only lung tumors (n = 6). One mouse with multiple metastatic foci outside the lung was excluded, although inclusion of this mouse showed an \( r^2 \) of 0.56 and a \( P \) value close to significance (\( P = 0.0541 \)). As expected, region-of-interest (ROI) analysis of the lung BLI signal before MC administration revealed a wide range of lung tumor sizes (Fig. 4C). We found that lung tumor burden was correlated significantly with SEAP AUC values (\( r^2 = 0.714; P < 0.05 \)) (Fig. 4C). Therefore, our tumor-activatable MC system not only shows a robust ability to identify tumor-bearing subjects but also can be used to evaluate disease extent, provided tumor burden is restricted to one organ.

Discussion

A strategy using an exogenously delivered, genetically encoded cancer blood biomarker vector could overcome some of the inherent limitations of screening for cancers using endogenous cancer blood biomarkers such as high background expression in healthy tissues, tumor heterogeneity, and random fluctuations in biomarker expression over time. We report here a tumor-activatable MC system that can be administered systemically to identify tumor-bearing subjects using a simple and relatively inexpensive blood-based assay. In this first-generation system our assay showed reliable detection capabilities, and assessment of disease extent was possible. Thus, we demonstrate the feasibility of tumor-activatable MCs as a highly robust detection system for cancers.

The holy grail of cancer gene therapy is to express a therapeutic transgene specifically within a tumor so that healthy cells are not harmed. To reach this goal, several strategies have been explored including transcriptional targeting of tumors using tumor-specific promoters (24, 27, 28) and enhanced tumor tropism of both viral and nonviral vectors (29, 30). Our efforts are quite similar to these exciting advancements, but instead of a therapeutic transgene for tumor treatment we propose the expression of a secretable reporter gene for the purposes of cancer detection. With this application comes the additional challenge of overcoming heightened safety concerns, because as a potential diagnostic tool the vectors presumably would be used in some patients who have no evidence of a cancer. Therefore, all components of this type of system need to be safe, including the delivery vehicle, the transgene expressed, and the DNA vector itself. Although many delivery formulations are possible, we chose an in vivo transfection agent that has an excellent safety profile (i.e., no immunostimulation) (31) and is in phase I/II clinical trials (32–34). We chose the transgene SEAP because it is of low immunogenicity (17), and already has shown promise in the clinic (18). Finally, although nonviral vectors are much safer than viral vectors, there still is concern regarding immunostimulatory prokaryotic CpG motifs in the backbone of traditional plasmids. This concern is alleviated in MCs because these vectors lack a prokaryotic backbone. Although integration and possible insertional mutagenesis also are safety concerns with many gene vectors, particularly viruses, even with very effective delivery methods (e.g., electroporation), the in vivo integration rates of nonviral vectors (plasmids and MCs) are approximately three orders of magnitude below the rate of spontaneous gene-inactivating mutations (35–39). MCs also are more resistant than plasmids to shearing stress and linearization (40, 41), an important characteristic that has been correlated with integration rates (42). Therefore, although extensive safety testing must be done before eventual clinical translation, our tumor-activatable MC system should be considered relatively safe. Because safety issues arising from the transfection reagent used to deliver the gene are transfected, it should be noted that our system can be modified to use emerging delivery vehicles, such as more tumor-specific polymeric transfection agents that limit normal tissue transfection (29, 43), and/or delivery vehicles targeted to a protein expressed at high levels by cancer cells (44). These newer delivery agents also may yield benefits in the sensitivity and specificity of tumor detection.

A few groups previously have explored the use of tumor-activatable reporter gene-expressing vectors for cancer detection (26, 45–48). However, the vector systems chosen (adenoviruses, Herpes simplex viruses, and plasmids) all have safety issues that will hamper clinical translation significantly. Viruses are highly immunogenic, and preexisting viral immunity in humans is a widespread problem (48–50). Plasmids also are immunogenic because of unmethylated CpG sequences in the prokaryotic backbone (necessary only for plasmid production) (51) and also carry the risk that encoded antibiotic-resistance genes will be disseminated to endogenous flora (12). Thus, tumor-activatable MCs have many advantages over these other vectors and should have greater translational potential arising primarily from easier good manufacturing practices (GMP) (compared with viruses) and a better safety profile. In addition to genetically encoded reporter genes, another approach for generating synthetic cancer biomarkers was described recently (52). This exciting strategy involved the development of mass-encoded biomarkers coupled to nanoparticles called “nanoworms.” Once injected systematically,
these peptide-based biomarkers are released into the blood following specific cleavage by peptidases at sites of disease (including cancer) and can be detected in the urine. In terms of translatability, our system may be accepted more readily, because nonviral cancer gene therapy has been studied for decades and routinely is being tested and optimized in clinical trials, whereas nanoparticle-based cancer therapy or cancer detection still is arguable in its infancy. Furthermore, the ability of nanoparticles to reach all tumor sites is not yet fully understood.

Our tumor-activatable MC system has many distinct advantages over the detection of endogenously expressed cancer blood biomarkers. First is the opportunity to optimize our system iteratively to improve the stringency of tumor-activatable gene expression, to generate more potent vectors, and to enhance vector delivery to the tumor. Although ways to augment endogenous tumor biomarker secretion rates exogenously are being developed (53), sensitivity using endogenous biomarkers will be inherently limited by the amount of biomarker produced by the tumor (6). In contrast, we can tune our MC system’s sensitivity. We currently are testing what effects dose may have on tumor-detection sensitivity by exploring different MC and/or transfection agent doses and dosing regimens (single versus multiple doses). Our system also can provide improved specificity through two mechanisms: (i) the uniqueness of the biomarker in the blood, because no SEAP is detectable before MC administration, and (ii) the ability to drive expression strictly within the tumor, thereby alleviating signal in healthy tumor-free subjects. In our current system we did observe slight SEAP signal from tumor-free mice receiving MC; we believe this signal arises from leakiness of pSurv, and therefore there is room for improvement. Although we have seen success with pSurv, we are not limited to this promoter and can explore alternative tumor-activatable promoters such as the Id1 or hTERT promoters (46, 54) or others as they emerge.

One of the limitations of this study is the lack of an absolute measure of the lowest tumor burden detectable with our system. This stems from our chosen animal model, because, rather than a single measureable tumor forming in the lung, we found numerous and extremely infiltrative tumors throughout the lung. Current studies are testing our strategy using models with more defined tumor boundaries so that tumor volume can be measured and detection sensitivity can be assessed. We recognize the inability to correct for mouse tumor burden for first-generation system described here is possible melanotic tumors were seen throughout in the lungs 2 wk following MC administration (Fig. S7), but, with future improvements in both the vector itself and the delivery of the vector to tumors, it should be possible to detect smaller and smaller tumors over time. An important point for clinical translation is that the blood reporter produced by our MCs will be diluted by the much larger (~3,500-fold) blood volume in humans (5 L) as compared with mice (2 mL). Once future studies define the minimal detectable tumor volume in mice, it will be important to consider that the minimal detectable volume in humans will be relatively larger because of the differences in total blood volume. Assuming that dosing is linearly scalable, tumor transfection efficiencies are equivalent, and biomarker secretion/degradation rates are similar between the two species, we note that the tumors detectable in mouse and man will not be similar in terms of absolute tumor volume but likely will be comparable in terms of percentage of total body weight (i.e., similar values for tumor weight per kilogram of body weight). However, it also is quite possible that scaling across species will be nonlinear. For example, we have developed a mathematical model for relating tumor volume and endogenous blood biomarkers (6), and a biomarker detectable in the blood of a mouse bearing a 5-mm3 tumor can still be detectable in the blood of a human even if their tumors are not 3,500-fold larger. Another potential issue to consider for translation is whether the production of enough MC for a human dose is feasible. Although the technology we used to generate our MCs yields amounts similar to those obtained with conventional plasmid preparations, and the MC production appears to be scalable (13), the ability to produce the amounts needed for human doses under GMP conditions remains to be fully validated.

One of the advantages of endogenous blood biomarkers is that they can be used to determine what type of cancer a person may harbor (e.g., a high prostate-specific antigen level may indicate prostate cancer). Our system was developed to be useful for screening for all cancers, not a particular tumor type. However, it is possible to explore alternative promoters useful for screening patients at high risk for a particular cancer, such as variants of the prostate-specific antigen enhancer/promoter for prostate cancer (28, 55, 56) or the mucin-1 promoter for breast cancer (57). Finally, another limitation of exogenous blood biomarkers (i.e., reporters) is the inability to localize the site(s) in the body where the biomarker originated. By replacing or coexpressing SEAP with an imaging reporter gene (e.g., herpes simplex virus thymidine kinase 1 for PET), our system also may allow the tumor location to be visualized. Bhang et al. (26) recently described the ability to image tumors using both BLI and single photon emission computed tomography after systemic administration of tumor-activatable plasmids expressing the appropriate imaging reporter gene. This strategy also was pursued with the SEAP-expressing viral vectors described to date, because these vectors coexpressed fluorescent proteins for cancer visualization using fluorescence stereomicroscopy (45–47). Rather than one vector system expressing two reporters, it may be possible to deliver two different vectors designed for specific applications—one for tumor localization and one for tumor identification. Finally, the ability of our systems to incorporate reporters that can be used in the urine or other easily accessible bodily fluids.

It is well recognized that earlier diagnosis of cancers is paramount for more timely administration of treatments to maximize their benefit to patients. Endogenous biomarkers are continuing to be discovered and validated, and it is hoped that the real potential of these will be realized soon. However, alternative and complementary exogenous biomarker-detection strategies such as blood-based or imaging-based technologies are emerging and should be sought aggressively. We demonstrate the potential of a potent and safe nonviral vector platform technology to identify tumor-bearing subjects using a simple blood-based assay. Continued iterative optimization and validation of this system across multiple tumor types could provide clinicians with a sensitive tool to observe for tumor recurrence, to allow tumor detection in high-risk patient populations, or eventually to be used as a powerful cancer-screening tool for the general population.

Materials and Methods

All procedures performed on animals were approved by Stanford University’s Institutional Animal Care and Use Committee and were within the guidelines of humane care of laboratory animals. Materials and methods used in plasmid and minicircle construction, cell culture, and in vitro transfection experiments, s.c. tumor and inflammation models, and local vector administration, plasma collection, and statistical analyses are detailed in SI Materials and Methods.

Experimental Melanoma Metastases Model, BLI, and Systemic Administration of Minicircles. To evaluate the ability to detect tumors after systemic administration of MCs, we used an experimental metastases model described previously (26). MeWo cells (5 × 106) stably expressing a BRET fusion protein (RLuc8-H-TurboFP-BRET6) (58) were injected (200 µL of PBS total volume) into irradiated (5 Gy) female nude mice (Nu/Nu; Charles River Laboratories) via the tail vein. At weekly intervals after cell injection, tumor development was monitored with BLI immediately after i.v. administration of the substrate coelenterazine (33 µg per mouse diluted in 150 µL of PBS) using an IVIS-200 imaging system (PerkinElmer). Using the software package Living Image 4.1, ROIs were drawn over the lungs in each image to quantitate tumor burden. BLI data are expressed as lung average radiance in photons−1 cm−2 steradian−1.
Tumor-bearing mice (n = 7) or irradiated control mice (n = 7) were administered 40 μg of MC complexed with a linear polyethylenimine transfection agent (JetPEI; Polyscience Transfection, Inc.) at a N/P ratio of 8 and were resuspended in 400 μL of 5% (wt/vol) glucose. Mice were injected via the tail vein with two 200-μL injections with a 5-min interval between the first and second injection. An additional control group of irradiated mice (n = 5) was administered 400 μL of 5% (wt/vol) glucose alone.

SEAP Assay. To measure SEAP concentration in both medium and plasma, we used the Clontech Great EscAPE SEAP Chemiluminescence Assay kit 2 according to the manufacturer’s instructions. Briefly, 25 μL of medium or plasma was added to 1x dilution buffer, and endogenous alkaline phosphatase was heat-inactivated at 65 °C for 30 min. Samples were put on ice for 3 min and then were allowed to recover to room temperature. SEAP substrate (100 μL) was added and was incubated for 30 min at room temperature, and luminescence (expressed in relative light units, RLU) was measured over 10 s using a Turner 2020 luminometer (Turner Designs).

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Supporting Information

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SI Materials and Methods

Plasmid and Minicircle Construction. All plasmids were constructed using standard PCR and cloning technology and were sequenced by Sequenext. To generate both PPs and MCs, we used the system described by Kay et al. (1) (System Biosciences). The 977-bp Survivin promoter was subcloned from pSurv-FL (2) into the MN-100 PP backbone (System Biosciences) containing an SV40 polyA and woodchuck hepatitis virus posttranscriptional element (WPRE) to generate PP-pSurv-WPRE. Next, the SEAP transgene from pSELECT-zeo-SEAP (Invivogen) was subcloned into PP-pSurv-WPRE to generate PP-pSurv-SEAP-WPRE (Fig. 2A, Upper). Both PP-pSurv-SEAP-WPRE (PP) and MC-pSurv-SEAP-WPRE (MC) (Fig. 2A, Lower) were amplified and purified according to the protocol outlined in Kay et al. (1) and the supplier’s instructions (System Biosciences). Briefly, ZYCY10P3SST E. coli were transformed with the PP, colonies were selected, and E. coli were grown overnight in TB broth. To generate MCs, site-specific recombination via phiC31 integrase was initiated by the addition of equal volumes of LB broth containing 0.001% L-arabinose and 16 mL NaOH, and cultures were grown for an additional 5.5 h at 30 °C. For the PPs, the cells were grown in the same medium without L-arabinose supplementation. Endotoxin-free mega kits (Qiagen) were used to purify both PPs and MCs.

Cell Culture and Transfection. The human melanoma cell lines MDA-MB-231 and MeWo (both from ATCC) and SK-MEL-28 (a kind gift from Paul Khavari, Department of Dermatology, Stanford University, Stanford, CA) were maintained in Eagle’s Minimum Essential Medium and DMEM (Gibco), respectively. The medium was supplemented with 10% (vol/vol) FBS and 1x Antibiotic-Antimycotic solution (Life Technologies), and cells were maintained in a 5% CO2 incubator at 37 °C. Cells were routinely tested for mycoplasma contamination using a commercial kit (MycoAlert Mycoplasma Detection Kit, Lonza).

Cell lines were plated (1.25 × 105 cells per well) in 24-well plates 1 d before transfection. Cells were transfected with an equal mass of PPs or MCs (1 μg) and 2 μL of a linear polyethylenimine transfection agent (jetPEI; Polyplus Transfection) according to the manufacturer’s instructions. Medium was collected daily and centrifuged at 311 × g for 3 min, and the supernatant was stored at −20 °C until SEAP concentration was measured. After medium collection, each well was washed with PBS, and fresh medium was added; therefore SEAP measurements reflect protein production over a 24-h period.

Statistical Analysis. All statistical analyses were performed using Prism 6.0 software (GraphPad Software). SEAP measurements from cell culture medium were compared using two-way ANOVA followed by Sidak’s multiple comparisons test. Longitudinal plasma SEAP measurements from mice were compared using one-way repeated-measures ANOVA followed by Tukey’s multiple comparisons test. SEAP AUC measurements across mice were compared using two-way ANOVA followed by Tukey’s multiple comparisons test. ROC analysis was performed between SEAP AUC data from tumor-bearing and healthy mice receiving MCs. Finally, Pearson correlation analysis of SEAP AUC and measurements of lung tumor burden was performed. For all tests a nominal P value less than 0.05 was considered to be significant.

Fig. S1. Comparison of promoter activities in vivo in healthy (tumor-free) mice. Mice were systemically administered plasmids [30 μg, PGL4.2 backbone complexed with PEI (N/P = 6)] expressing the BLI reporter gene Fluc driven by pCMV (n = 3), pSurv (n = 5), or pPEG (n = 3). Mock-injected mice received 5% (wt/vol) glucose (n = 3). Each mouse also was coinjected with a plasmid expressing the BLI reporter gene humanized Renilla luciferase (hRluc) driven by pCMV to assess transfection efficiency (3 μg; 10-fold less than Fluc plasmid mass). (A) Representative BLI images 48 h postinjection. The scale of the image of the pCMV mouse is two orders of magnitude higher than the scale of the images of all other mice. BLI signal, primarily in the lungs, was seen in all mice receiving Fluc plasmid. (B) ROI analysis over the entire mouse was performed on BLI images, revealing significantly higher (~100-fold) BLI signal in mice receiving pCMV-Fluc plasmids (red bar) than in all other mice. A significantly higher BLI signal also was observed in pPEG mice (yellow bar) compared with mock-injected mice (white bar). *P < 0.05. Although qualitatively higher BLI signal was notable in pSurv mice compared with mock-injected mice, quantitative measures revealed only a trend (P = 0.16) toward higher BLI signal. Thus, in this mouse strain, Fluc expression in normal tissues was lowest with the tumor-specific pSurv. (C) Forty-eight hours after plasmid injection, ex vivo analysis of Fluc activity across numerous tissues revealed significantly higher (*P < 0.05) expression with pCMV (red bars) than in all other groups. With pPEG, significantly higher (*P < 0.05) Fluc activity was found in the heart, lung, and spleen compared with mock-injected animals. With pSurv, significantly higher (*P < 0.05) Fluc activity was observed in the spleen, and a trend (P = 0.13) toward higher activity was seen in the lung. (D) The only tissue showing higher hRluc activity above background was the lung (values presented are normalized to average background values from mock-injected mice). Therefore Fluc values determined from both imaging (B) and ex vivo tissue analysis (C) are not normalized by hRluc values. Importantly no significant differences in hRluc values within the lungs were seen across the three promoter mouse groups; thus the differences in Fluc measurements across the three groups are highly unlikely to be related to differences in transfection efficiency but probably are related to differences in promoter activity. Data are expressed as mean ± SD.
Fig. S2. Comparison of tumor-specific promoter activities in primary human fibroblasts and human cancer cell lines. Primary human fibroblasts, human breast cancer (MDA-MB-231) cells, and human melanoma (MeWo) cells were transfected with pPEG- or pSurv-driven plasmids (1 μg) expressing FLuc and cotransfected with a promoterless plasmid expressing hRluc (50 ng) to normalize for transfection efficiency. No differences in Rluc transfection efficiency were noted in any of the three cell types. pPEG-driven plasmids led to significantly higher FLuc activity in fibroblasts than pSurv (*P < 0.05). pSurv-driven plasmids led to significantly higher FLuc activity in MeWo cells (***P < 0.001) and equivalent activity in MDA-MB-231 cells. Data are expressed as mean ± SD.

Fig. S3. Comparison of tumor-activatable PPs and MCs in cultured SK-MEL-28 melanoma cells. SK-MEL-28 human melanoma cells were transfected with equal masses of tumor-activatable MCs (n = 3) and PPs (n = 3) and equal volumes of the transfection agent PEI. Significantly higher SEAP activity was observed in the medium of cells transfected with MCs from day 2 to day 7 (**P < 0.01; ***P < 0.001). Data are expressed as mean ± SD.

Fig. S4. Comparison of transgene expression of MCs and PPs driven by a strong constitutive promoter in healthy (tumor-free) mice. (A) Mice received systemic administration of either MCs (n = 4) (Top Row) or PPs (n = 5) (Middle Row) expressing hRluc driven by the strong constitutive EF1 promoter after complexation with PEI (40 μg; N/P = 8). BLI imaging was performed on days 1, 2, 3, 5, and 7 using the substrate coelenterazine. Representative images show higher BLI signal in MC-administered mice at all time points examined. (Bottom Row) Signal from a mouse receiving a 5% (wt/vol) glucose injection is shown for comparison (signal in liver is from oxidized coelenterazine). (B) ROI analysis over the lung region showed significantly higher BLI signal in mice administered MCs than in mice administered PPs on days 1, 2, and 5 (*P < 0.05; **P < 0.01). Data are expressed as mean ± SD.
Fig. S5. I.T. administration of tumor-activatable MCs leads to detectable blood reporter activity. (A) Nude mice bearing s.c. human melanoma xenografts were I.T. administered tumor-activatable MCs expressing SEAP (MC I.T.; n = 4) or 5% (wt/vol) glucose (mock; n = 3). A group of control mice also received i.m. injections of MCs (MC I.M.; n = 3). Plasma SEAP measurements before and for up to 2 wk after MC administration revealed that only MC I.T. mice had elevated SEAP levels from days 3–14 (*P < 0.05; **P < 0.01; ***P < 0.001). (B) BALB/c mice (n = 3) were administered turpentine oil to induce a local inflammatory reaction or no oil (control mice, n = 3). Twenty-four hours after the initial injury MCs were administered at the site of oil injection, and SEAP was measured both before MC administration and for up to 1 wk after MC administration. No detectable increase in blood SEAP was found after MC administration. All data are expressed as mean ± SD.

Fig. S6. Standard curve analysis of plasma SEAP assay. (A) Triplicate samples were measured at 10-fold dilutions of SEAP in 25 μL of plasma. SEAP activity was linear over five orders of magnitude and showed a detection limit of ∼3 × 10⁻⁷ μg (0.3 pg) in 25 μL of plasma. (B) SEAP measurements over the entire linear range were reproducible with coefficient of variance (%CV) measures less than 4%.
Fig. S7. Tumor burden before and after MC administration. (Left) BLI images of two representative mice before and 2 wk after MC administration. (Right) Corresponding ex vivo images of lungs at the time mice were killed (2 wk after MC administration). Values below each BLI image represent the average radiance in ROIs drawn over the lungs. Note the differences in image scales for the two mice. Indicating continual tumor growth, both mice showed an $\sim 4.5$-fold increase in BLI signal over the 2-wk period following MC administration. When mice were killed, tumors within the lungs were melanotic, and multiple tumor foci (white arrows) were observed throughout the lungs in both mice. Based on BLI signal changes, total tumor burden at the time of MC administration (2 wk before mice were killed) would have been $\sim 4.5$-fold less than seen in the ex vivo images presented here.