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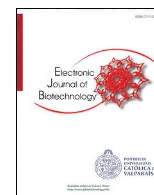
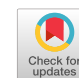


Short communication

Melanoma transplants in “green” mice: Fluorescent cells in tumors are not equivalent to host-derived cells



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A B S T R A C T

Background: To examine the usefulness of green fluorescent protein (GFP) mice for studying the interactions between normal cells and tumor cells in a host, we used a melanoma model in such “green” mice [C57BL/6-Tg (CAG-EGFP)1Osb mice]. Mice were given a subcutaneous injection of B16-F10 cells, and the resultant primary tumors were removed. Then cells from individual tumors were cultured.
Results: The proportion of EFGP+ cells was determined by fluorescence-activated cell sorting (FACS) and was 6.8% ± 3.2% (mean ± s.d.) on day 1 of culture, 0.6% ± 0.3% on day 2, and 0.02% ± 0.01% at day 7. In all cases, isolated cells grew at a constant rate, but fluorescence decreased over time and became undetectable on day 14. Cells were tested using PCR for the presence of an *EGFP*-specific sequence, and results were negative in all cases, thus indicating that the cells did not harbor the host's reporter gene. Cells were also tested for the presence of *EGFP* mRNA, which was consistently detected for 22 days after the start of culture. The tumorogenicity of the cultured cells was confirmed in GFP mice injected with cells from a selection of cultures.
Conclusions: In a melanoma model in GFP mice, the detection of “green” cells in tumors was not equivalent to the detection of host-derived cells. Such “masking” was caused by a transient, but lasting, transfer of *EGFP* mRNA from the host's normal cells to tumor cells. Thus, an analysis of tumors *postmortem* by techniques that yield only a single snapshot can lead to incorrect interpretations and erroneous conclusions.
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1. Introduction

Interactions between normal cells and tumor cells in a host play a significant role in the development of tumors [1]. Available evidence suggests that the oncogenic transformation of host cells might occur through fusion [2] or by the uptake of microvesicles [3] or other particles [4,5].

Clarification of phenomena related to the recruitment and transformation of host cells might lead to new therapeutic approaches based on the inhibition of such phenomena. Cancer models in animals are particularly useful tools regarding this because they can be designed to enable cancer cells to be distinguished from host cells. For example, it is easy to distinguish human cells from mouse cells in xenograft cancer models. It is also possible to detect tumor cells that have been tagged with a fluorescent marker in syngeneic animals. The use of fluorescent proteins has been utilized in studies of interactions between cancer cells [6,7] and between a tumor and its microenvironment because, theoretically, this method enables host cells to be distinguished from tumor cells at single-cell resolution [8]. Moreover, the use of immunocompetent, as distinct from immunodeficient, animals facilitates analyses under conditions that more closely resemble the clinical setting.

“Green” mice (referred to, in this study, as GFP mice) are transgenic animals that express cDNA for the so-called enhanced green fluorescent protein (EGFP) under the control of a chicken β-actin promoter and a cytomegalovirus enhancer [9]. The fluorescence of cells from transgenic “green” mice has been utilized in a variety of cell transplantation experiments such as cell tracking [10,11], analysis of tumorigenesis, and studies of gene therapy [8,12], both in immunocompetent and in immunodeficient animals [12,13]. It has been reported that the expression of EGFP is heterogeneous, differing among tissues, within organs and tissues, and even among stages of development [14,15]. This

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heterogeneity cannot be ignored if one hopes to avoid misinterpretation of results.

The use of GFP mice to study recruitment of cells in the development of cancers appears to be an ideal strategy because the animals have an intact immune system, the host's cells are easily recognizable, and several lines of cancer cells have been generated in animals with a similar genetic background (e.g., B16-F10 melanoma cells).

The goal of the present study was to examine the utility of GFP mice for the study of the recruitment of host cells into tumors. We used a melanoma model in GFP mice, and we monitored the expression of the gene for EGFP to identify host-derived cells, both by cytometric detection of EGFP itself and by PCR-based techniques (Fig. 1).

2. Materials and methods

2.1. Cell culture

We cultured B16-F10 mouse melanoma cells (ATCC® CRL-6475™) in monolayer in Dulbecco's modified Eagle medium (DMEM) (Gibco™, Life Technologies Ltd., Paisley, Scotland), supplemented with 10% fetal bovine serum (Gibco™) and 1% penicillin/streptomycin mixture (Gibco™). Cells were passaged after dispersion in 0.125% trypsin in EDTA (Gibco™).

2.2. Animals

C57BL/6-Tg(CAG-EGFP)1Osb mice [9] (referred to, in this study, as GFP mice) were kindly donated by Mr. Jesús Martínez-Palacio (CIEMAT, Madrid, Spain) and bred at the Experimental Research Unit of the University General Hospital of Albacete (Spain). C57BL/6J mice (referred to, in this study, as C57 mice; Charles River Laboratories, Barcelona, Spain) were used as wild-type animals. For breeding, we used mixed pairs (female C57 mice × male GFP mice), and for experiments, we used heterozygous GFP mice. All animals were housed and handled according to the protocol approved by the Ethics Committee for Animal Research of Castilla-La Mancha University (Spain), under supervision by the staff of the animal facility of the University General Hospital of Albacete. The studies were conducted in accordance with European and Spanish laws (Directive 2010/63/UE and Real Decreto 53/2013, respectively).

As recommended by the Federation of European Laboratory Animal Science Associations (FELASA), mice in the animal facility were tested periodically to ensure that the colony remained free of pathogens.

We used both male and female GFP mice and C57 mice, from 7 to 38 weeks of age (mean ± s.d., 15 ± 10 weeks; median, 10 weeks). From birth to the end of the experiments, all mice had unlimited access to water and standard rat chow (Teklad Lab Animal Diets; Harlan Laboratories, Barcelona, Spain).

2.3. Implantation of cells and design of experiments

The design of the study is schematically shown in Fig. 1.

Tumors were generated in the back of 14 GFP mice by unilateral subcutaneous injection of B16-F10 cells. Cells in culture were trypsinized, washed, and resuspended in phosphate-buffered saline (PBS). Then 0.2 ml of the suspension, containing 1×10^6 cells, was injected in each mouse. Animals were examined daily, and the growth of subcutaneous tumors was monitored and recorded weekly. We measured the greatest diameter of each tumor with electronic calipers. Animals were sacrificed between 19 and 23 days after injection of cells.

Using a similar procedure, nine C57 mice were injected with cells from cultures obtained from tumors generated in GFP mice.

2.4. Collection and culture of primary tumors

Mice were killed by CO_2 inhalation. Lungs and lymph nodes were inspected visually for the presence or absence of metastases, which was recorded. Primary tumors were removed, and tumor cells from

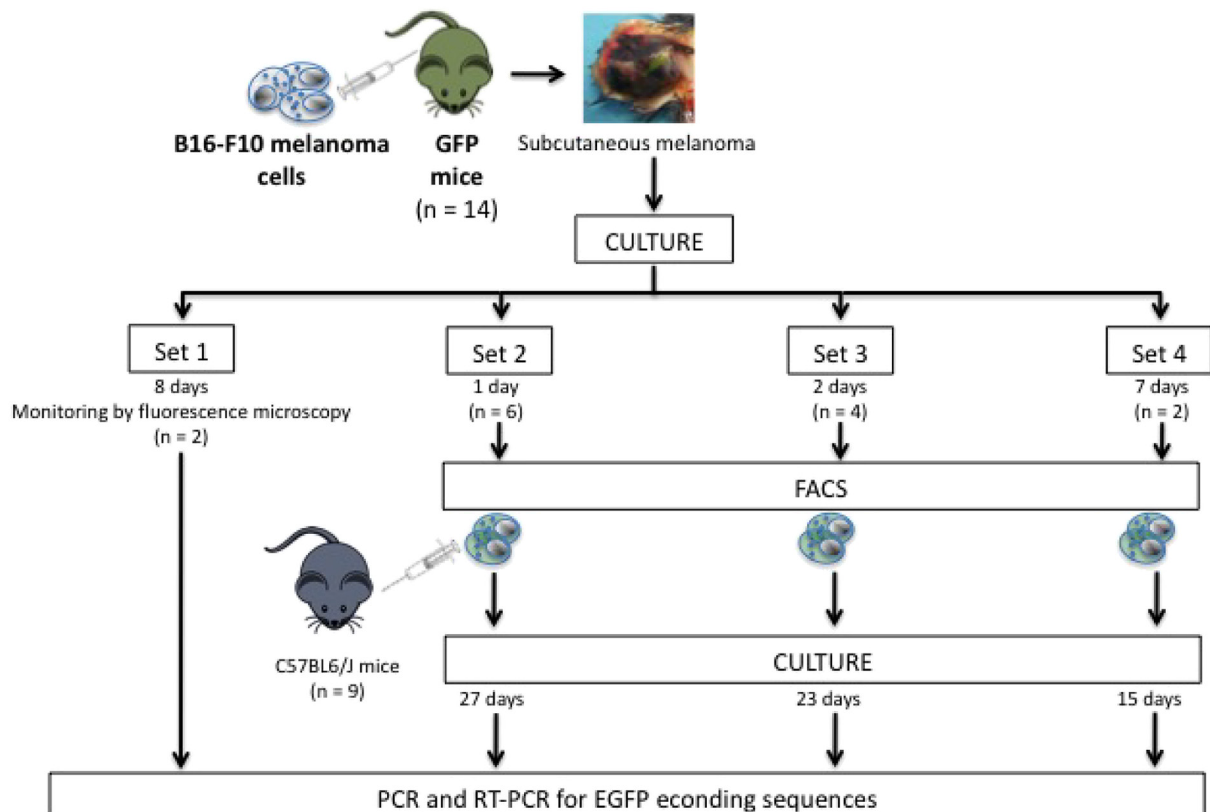


Fig. 1. Schematic representation of the study design.

individual tumors were cultured as follows. Tumor samples were dissociated with the GentleMACS™ Dissociator (Miltenyi Biotec GmbH, Gladbach, Germany) according to the protocol suggested by the manufacturer. In brief, up to two pieces of tumor 5-mm large each were under mechanical dissociation, which was followed by enzymatic digestion with collagenase I and dispase. Then DNase I was added and mechanical dissociation was repeated. The resultant suspension was passed through a 70-μm-pore filter and then centrifuged at 300x g for 10 min. The resultant cell pellet was suspended in the culture medium, and cells were allowed to proliferate in flasks at 37°C in an atmosphere of 5% CO_2 in air. The composition of the culture medium was the same as that of B16-F10 cells.

Each individual culture was generated from one single tumor, which was derived from one animal. In other words, each culture corresponded to an individual animal. To monitor the changes in proportion of "green" cells with time, we established four sets of cultures from these primary cultures, as follows (Fig. 1):

- Set 1 (n = 2): Cultures of tumor cells were maintained for a maximum of 8 days. Cells were monitored daily by fluorescence microscopy and, periodically, cells were counted; the proportion of green-fluorescing cells was calculated. For counting, we dispersed cells in 0.125% trypsin in EDTA and used a Neubauer chamber to identify fluorescent and nonfluorescent cells.
- Set 2 (n = 6): On day 1, green-fluorescing cells were quantified and isolated by fluorescence-activated cell sorting (FACS). Then all isolated cells were cultured for a maximum of 27 days. On day 14 (when we achieved enough cells), cells from three such cultures were injected into nine C57 mice (three sets with three mice in each set), as described above for GFP mice. The resultant primary tumors in C57 mice were harvested, and the tumor cells were cultured.
- Set 3 (n = 4): On day 2, green-fluorescing cells were quantified and isolated by FACS. Then they were cultured for a maximum of 23 days.
- Set 4 (n = 2): On day 7, green-fluorescing cells were quantified and isolated by FACS. Then they were cultured for a maximum of 15 days.

Thus, each set of cultures was designed with a specific goal:

- Set 1 was a control set, in which cells were not subjected to FACS.
- In Sets 2, 3, and 4, cells were subjected to FACS at different times after the start of culture for accurate counting of each population of cells. In addition, green-fluorescing cells (theoretically, host cells) were isolated to follow their growth without interaction with nonfluorescent cells (tumor cells).

2.5. Fluorescence-activated cell sorting (FACS)

For selection of $EGFP^+$ cells, we used a BD InFlux® cell sorter (BD Biosciences, San José, CA, USA) that was equipped with a sterile hood. Cells were sorted at a rate 10,000 cells/s with a 100-μm nozzle tip. Double sorting was performed for each sample, and this resulted in cell cultures of 100% purity, with the exception of one culture, which was 96.3% pure (mean purity: 99.6%; Fig. 2).

2.6. Analysis by PCR

Each culture was examined at least once by PCR for the presence of an EGFP-encoding sequence.

DNA was extracted from cells by using a commercial kit (QIAamp DNA Mini Kit; QIAGEN, Hilden, Germany) according to the instructions from the manufacturer. To verify the effectiveness of the extraction, the DNA was quantified by spectrophotometry using a NanoDrop ND-1000 system (NanoDrop Technologies, Inc., Wilmington, DE, USA). In addition, to ensure the presence of amplifiable DNA, we performed a first PCR for the detection of a sequence of a constitutive gene (*k-ras* gene).

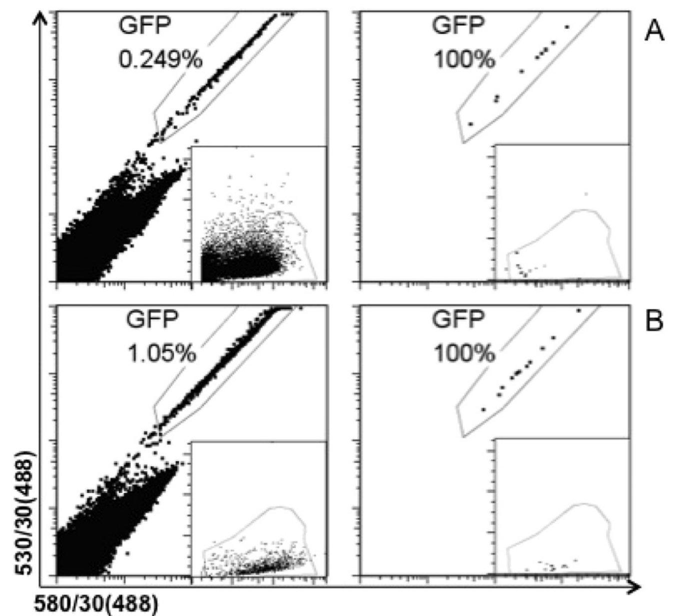


Fig. 2. Sorted $EGFP^+$ cells. $EGFP^+$ cells were selected on the basis of fluorescence in the [580/30 (488)] channel (see text for details). Dot plots from two representative cultures of tumor cells (A and B) are shown. Each sample was subjected to double sorting (left, right), thereby resulting in a mean purity of 99.6% for isolated cells.

Then, for detection of the EGFP-encoding sequence, we used the following primers to amplify a specific 173-bp fragment: forward, 5′-aagttcatctgcaccaccg; and reverse, 5′-tccttgaagaagatggtgcg. The reaction mixture, with a total volume of 12 μl, contained a minimum of 25 ng of template DNA in a volume of 2 μl. It also contained 1 μM of each primer and 2 mM $MgCl_2$.

Each PCR reaction was performed in a thermal cycler (iCycler; Bio-Rad, Hercules, CA, USA), with initial denaturation by incubation at 94°C for 1.5 min. Then amplification was allowed to proceed for 35 cycles of incubation for 30 s at 94°C, for 1 min at 60°C, and for 1 min at 72°C, with final extension for 2 min at 72°C.

In these amplification experiments, we included DNA from tail biopsies obtained from GFP mice as the positive control and water as the negative control.

We evaluated the sensitivity of the technique with serial dilutions of DNA from biopsies of GFP mice in samples of DNA from B16-F10 cells. We were able to detect EGFP-expressing cells at a maximum dilution of 1/500.

2.7. Analysis by RT-PCR

We selected five cultures to be tested at least twice over time for the detection of *EGFP* mRNA sequences. Specifically, we chose two cultures in Set 1, one in Set 2, and two in Set 4, each of which was tested before and after FACS.

RNA was extracted from cells by using a commercial kit (RNeasy Mini kit; QIAGEN), according to the instructions from the manufacturer. Then samples were subjected to DNase digestion followed by retrotranscription using a commercial kit specific for these two steps (QuantiTect Reverse Transcription Kit; QIAGEN).

To ensure the absence of DNA in the samples of RNA before reverse transcription, we performed a PCR for the detection of the EGFP-encoding sequence as described above. After reverse transcription, samples of cDNA were analyzed by the same technique.

3. Results

Melanoma tumors were detected in 36% of the injected mice (5/14) one week after inoculation of cells. During the second week, all animals

had palpable tumors. At euthanasia, the greatest diameter of the tumors was 1.7 ± 0.6 cm (n = 14). Macrometastases were not detected by visual inspection in any of the mice. There was no statistically significant correlation between tumor growth and gender or age of the host.

In all tumors, fluorescence was visually detected both before extraction and after dissociation (Fig. 1).

In all subsequent cultures examined by PCR, we first verified the presence of amplifiable DNA, through amplification of murine KRAS-encoding sequences.

In Set 1, the proportion of green-fluorescing cells, as determined visually under a fluorescence microscope, decreased from 2.5% at the first observation to 0.5% after 8 days in culture (Fig. 3). Cells were examined by PCR and no EGFP-encoding sequences were detected in cultured cells. Two cultures in this set were also examined by RT-PCR, and *EGFP* mRNA was detected on days 2, 4, 5, and 8 after the seeding of the primary tumors (Fig. 4).

In Set 2, the proportion of green-fluorescing cells, as determined by FACS on day 1, ranged from 2.8% to 10.2% (mean, 6.8% ± 3.2%). These cells were isolated and cultured for a maximum of 27 days (Fig. 1). Fluorescence, as determined visually, decreased with time and became undetectable on day 14 after cell sorting. However, the cells proliferated at a normal rate and results of PCR were, again, negative for EGFP-specific sequences in all cases. One culture in this set was examined for the presence of *EGFP* mRNA on days 15 and 22 after selection by FACS; the first analysis yielded a positive result and the second did not. Flow cytometric analysis, 4 weeks after selection and the start of culture, indicated that there were no EGFP + cells in the culture.

In Set 3, the proportion of green-fluorescing cells, as determined by FACS on day 2, ranged from 0.2% to 1.0% (mean, 0.6% ± 0.3%). As in Set 2, the fluorescence of $EGFP^+$ sorted cells, as determined visually, decreased with time, but the cells proliferated normally. The results of PCR were negative.

In Set 4, the proportion of green-fluorescing cells, as determined by FACS on day 7, ranged from 0.01% to 0.02% (mean, 0.02 ± 0.01%). Although only a small number of $EGFP^+$ cells were isolated, the cells proliferated. However, no fluorescence was observed. The results of PCR were also negative. Two cultures in this set were examined for the presence of *EGFP* mRNA on days 4 and 7 after tumor seeding (before selection by FACS) and on day 22 (15 days after selection by FACS). All samples yielded positive results (Fig. 4).

Nine mice were injected with cells from three cultures in Set 2. No palpable tumors were detectable in any of the animals one week after inoculation. During the second week, one animal developed a palpable tumor (14%). During the third week, three animals had no evidence of tumors, whereas four developed large tumors and had to be euthanized in accordance with ethical endpoint criteria. After five weeks, all animals have had tumors macroscopically identical to those produced by B16-F10 cells. However, the course of tumor development was more heterogeneous than that in GFP mice that had been inoculated with B16-F10 cells.

Cells from the tumors were cultured and two resultant cultures were selected for analysis by FACS on day 1 of culture. No green-fluorescing cells were detected.

4. Discussion

In the present study, we examined the recruitment of host cells by tumors in a mouse melanoma model. In designing our study, we considered many reports of the limitations and artifacts associated with models based on the EGFP marker. It has been suggested that

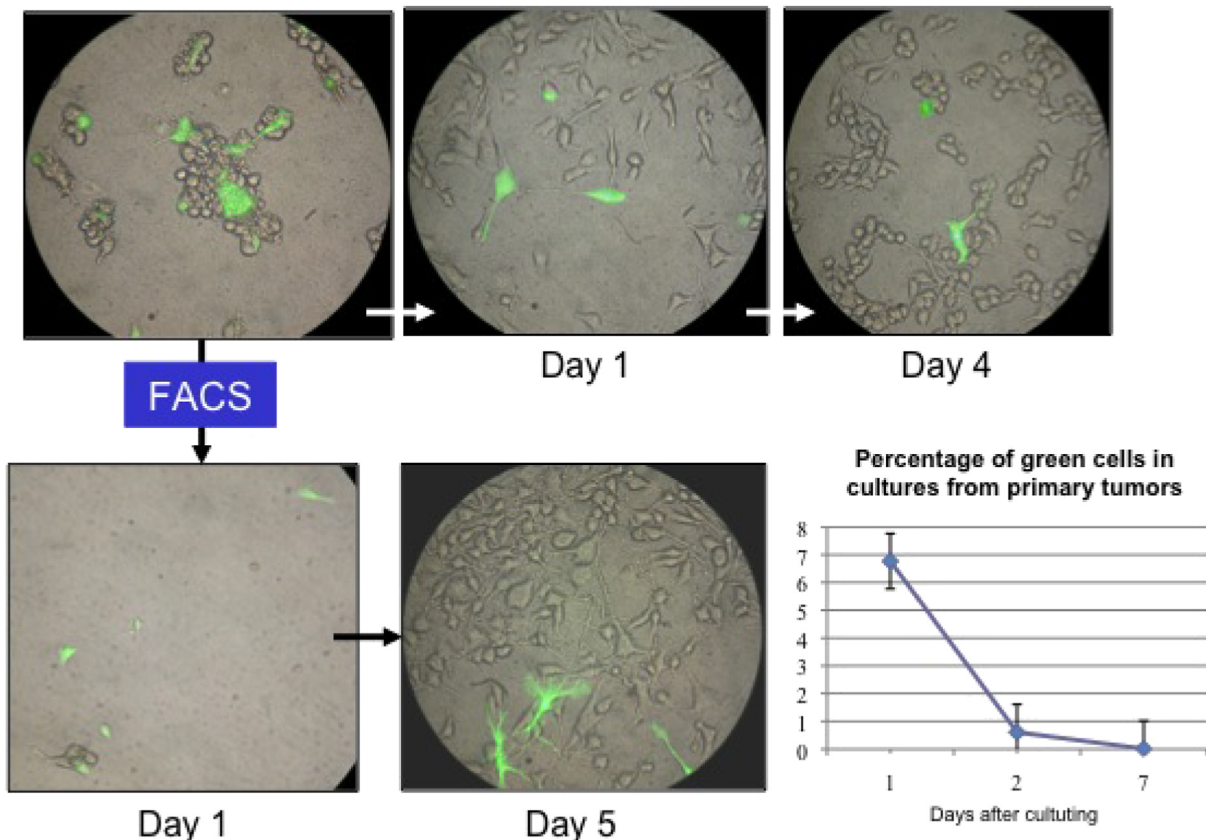
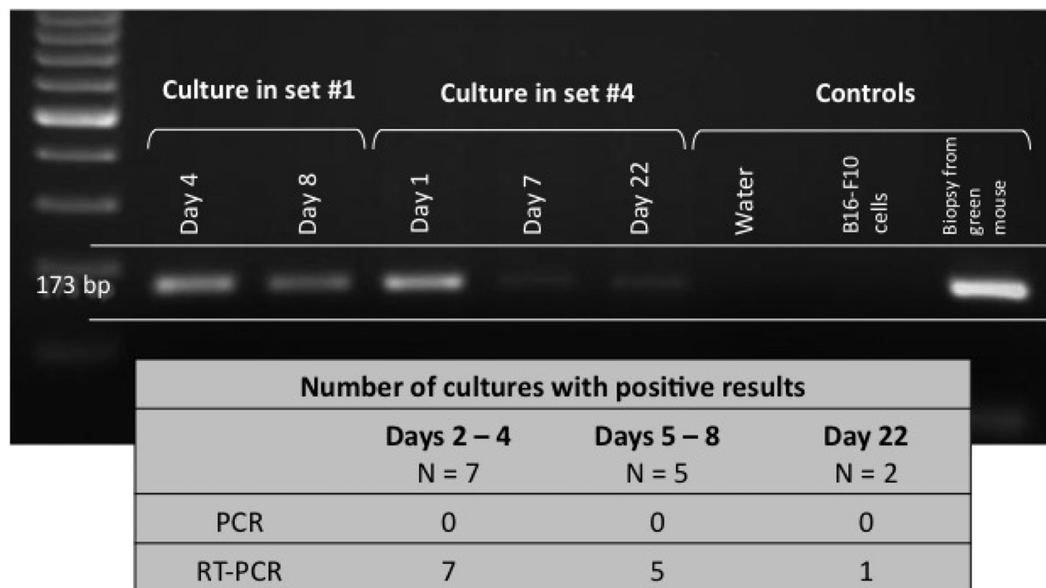


Fig. 3. The experimental procedure with microscopic images of primary tumors after culturing of cells. Each image was assembled from overlapping photographs that had been acquired by phase-contrast microscopy and fluorescence microscopy (blue light), respectively. Magnification: 20×. The graph shows the decrease in the mean percentage of green cells detected by FACS in cultures.



Number of cultures with positive results			
	Days 2 – 4 N = 7	Days 5 – 8 N = 5	Day 22 N = 2
PCR	0	0	0
RT-PCR	7	5	1

Fig. 4. Photograph of an agarose gel showing the results of analysis by RT-PCR for the detection of EGFP-encoding cDNA sequences. Lane 1: Molecular mass markers. Lanes 2–6: Amplification of cDNA obtained by reverse transcription of RNA extracted from primary cultures of B16-F10 tumors generated in "green" mice. Lanes 7–9: Controls for PCR, namely, water (no template); DNA from B16-F10 cells (negative control for the detection of the EGFP-encoding sequence); DNA from ear tissue of a "green" mouse (positive control for the amplification by PCR). bp: base pairs. The additional Table shows the number of cultures that gave positive results in the PCR-based analysis for detection of DNA and RNA sequences associated with the gene for EGFP.

the use of protein products of transgenes as markers for following the fate of grafts is not a robust strategy because it is virtually impossible to achieve ubiquitous expression of a transgene [16]. Moreover, autofluorescence can occur as an artifact in mammalian skeletal muscle fibers [17] and the brain [18]. In addition, potential false-negative data, related to repression of the expression of the gene for EGFP, can result from an epigenetic silencing phenotype [19]. Autofluorescence and loss of expression appear to be the main causes of ambiguity associated with the use of EGFP as a marker. Use of GFP mice injected with nonfluorescent cells might allow avoidance of such problems, but additional limitations, based on differential expression in the tissues and cells of GFP mice, have been reported [14,15]. The design of our present study enabled us to circumvent the likely artifacts related to expression of EGFP. However, we observed a new and confounding phenomenon, namely, the detection of green-fluorescing cells in tumors was not equivalent to the detection of host-derived cells.

In our study, we injected nonfluorescent melanoma cells into host mice, and thus, theoretically, the presence of green-fluorescing cells in tumors should have been equivalent to the detection of host-derived cells in immunocompetent animals. After subcutaneous injection of murine melanoma cells, tumors grew at a steady rate. After euthanasia, microscopic examination revealed the presence of green-fluorescing cells in tumors. Thus, apparently, tumor cells included host cells. However, when we cultured such tumors, we found that the fluorescence diminished progressively and vanished completely within a few days. This finding might be explained by a higher growth rate of nonfluorescent cancer cells, as compared to potentially EGFP-expressing tumor cells. To examine this hypothesis, we isolated green-fluorescing cells from primary cultures of tumor cells by cell sorting. After isolation, green-fluorescing cells were allowed to proliferate in culture. Again, fluorescence diminished progressively and then disappeared completely within a few days as had occurred with our first tumor cultures (Fig. 3).

It has been reported that the fluorescence of EGFP decreases with reduced oxygenation, which is a characteristic of both experimental and clinical tumors [20]. However, in our study, the reoxygenation of cells when tumors were excised and their component cells were cultured should have allowed the recovery of fluorescence within several hours, as reported previously [20]. Nonetheless, to circumvent problems related to factors that might affect the expression of EGFP, we examined the presence of the EGFP-encoding sequence by PCR in the cultures that we had established from animal tumors. The sequence was never detected, not even in green-fluorescing cells that had been isolated by cell sorting. This result proved that the expression of EGFP in cultures had not failed but, rather, that cells did not harbor the host's reporter gene.

In a study by Yang et al. [12] in 2003, tumor-stroma interactions were analyzed with a dual-color model, using GFP mice and B16-F10 cells marked with the red fluorescent protein (RFP). The authors assumed that green-fluorescing cells were host-derived cells, whereas red-fluorescing cells were tumor cells, and they reported the presence of host-derived EGFP-expressing fibroblasts and endothelial cells within RFP-expressing melanomas, as well as dendritic host cells in contact with melanoma cells [12]. However, the presence of cells with both markers was not reported. By contrast, in EGFP-expressing nude mice grafted with human cancer cells, the same authors found cells that expressed the two markers. They identified these cells from their morphological features as macrophages that engulfed cancer cells [12]. The fact that an immune reaction, mediated by macrophages, was reported in nude mice and not in GFP mice, together with the fact that the conclusion was based on visual analysis alone, suggests that other phenomena, which might explain such findings, cannot be ruled out.

In 2015, Dai et al. [21] showed that stromal fibroblasts undergo malignant transformation in a model of glioma in immunodeficient GFP mice. However, they were unable to determine whether this transformation had occurred in the animals themselves or in cells in culture, and the mechanism remained to be determined. In addition, Hoffman and colleagues developed and validated several dual-color models for human tumor xenografts in immunodeficient mice, and they found that gene transfer between cancer cells was associated with acquisition of strong metastatic behavior [6,22]. Nonetheless, it might be best to use immunocompetent models for studies of such phenomena because immunity might play an essential role.

Fusion of cancer cells and host cells has been proposed as a prominent mechanism for the horizontal transmission of malignancy

[2,23]. Furthermore, it has been suggested that, when a small number of marked cells is involved in a fusion phenomenon, a cytoplasmic marker protein would probably diffuse and become quite diluted as to become undetectable [17]. However, our observations (i) that the EGFP-encoding sequence was never detected in our cultures and (ii) that no cells with two nuclei were detected by microscopy fail to support the possibility that our results can be explained by a fusion mechanism.

After the isolation and culture of green-fluorescing cells, we confirmed their tumorogenicity by subcutaneous injection in mice. In all injected animals, we found melanomas that were qualitatively similar to those produced by the original B16-F10 cells, although tumor progression was slower. In other words, the passage of cells, through tumorigenesis in animals, changed the growth rate of the tumors. Thus, we cannot conclude that the injected cells were clonally derived from the original B16-F10 cells. In previous studies, serial passage of tumor cells in nude mice created more aggressive variants of some lines of cancer cells [24]. However, in our present study in immunocompetent animals, the first passage through the host led to a less aggressive cancer, perhaps as a result of interactions between the host's immune system and the tumor.

Di Castro et al. [25] reported false evidence of myogenic differentiation of human hematopoietic stem cells. This false evidence was based on protein transfer that led to the spurious acquisition of EGFP fluorescence. They proposed three mechanisms to explain their results: (i) the persistence of viral particles; (ii) the formation of nanotubes; and (iii) the exchange of microvesicles [25]. In our present study, the first mechanism can be excluded because we did not use cells infected with viral particles. However, protein transfer by either of the other two mechanisms might provide a plausible explanation. The half-life of EGFP is approximately 26 h [26]. Thus, simple transfer of this protein to cancer cells does not explain why fluorescent cells were detected by FACS even seven days after tumor excision and culture of tumor cells. The transfer of genetic material from host cells to cancer cells might explain our observations. In the present study, we demonstrated the transfer of RNA from host cells to tumor cells. Such transfer was transient but lasting, even when tumors were excised from animals and cultured. The capacity of cells to take up exogenous RNA both actively and qualitatively has been described and, also, proposed as a natural process associated with cell-to-cell communication [27,28]. Our observations were limited to the EGFP reporter but this phenomenon might apply to other genes in the context of a mutual cellular adaptation process as suggested for mesenchymal stem cells and cancer cells [29].

In conclusion, in the present study of melanoma tumors in GFP mice, we demonstrated the transfer of *EGFP* RNA from the host's normal cells to tumor cells, and this phenomenon led to the detection of EGFP in tumor cells for several weeks after tumor excision and the culture of tumor cells. Thus, because detection of EGFP in this model was not equivalent to the detection of host-derived cells, an analysis of tumors *postmortem* by techniques that yield only a single snapshot (e.g., microscopy or even flow cytometry) can lead to incorrect interpretations and erroneous conclusions.

Ethical approval

All procedures in this study were performed in accordance with the ethical standards of the Ethics Committee for Animal Research of Castilla-La Mancha University (Spain). The studies were conducted in accordance with European and Spanish laws (Directive 2010/63/UE and Real Decreto 53/2013, respectively).

Conflict of interest

The authors declare that they have no conflict of interest.

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