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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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ABSTRACT

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

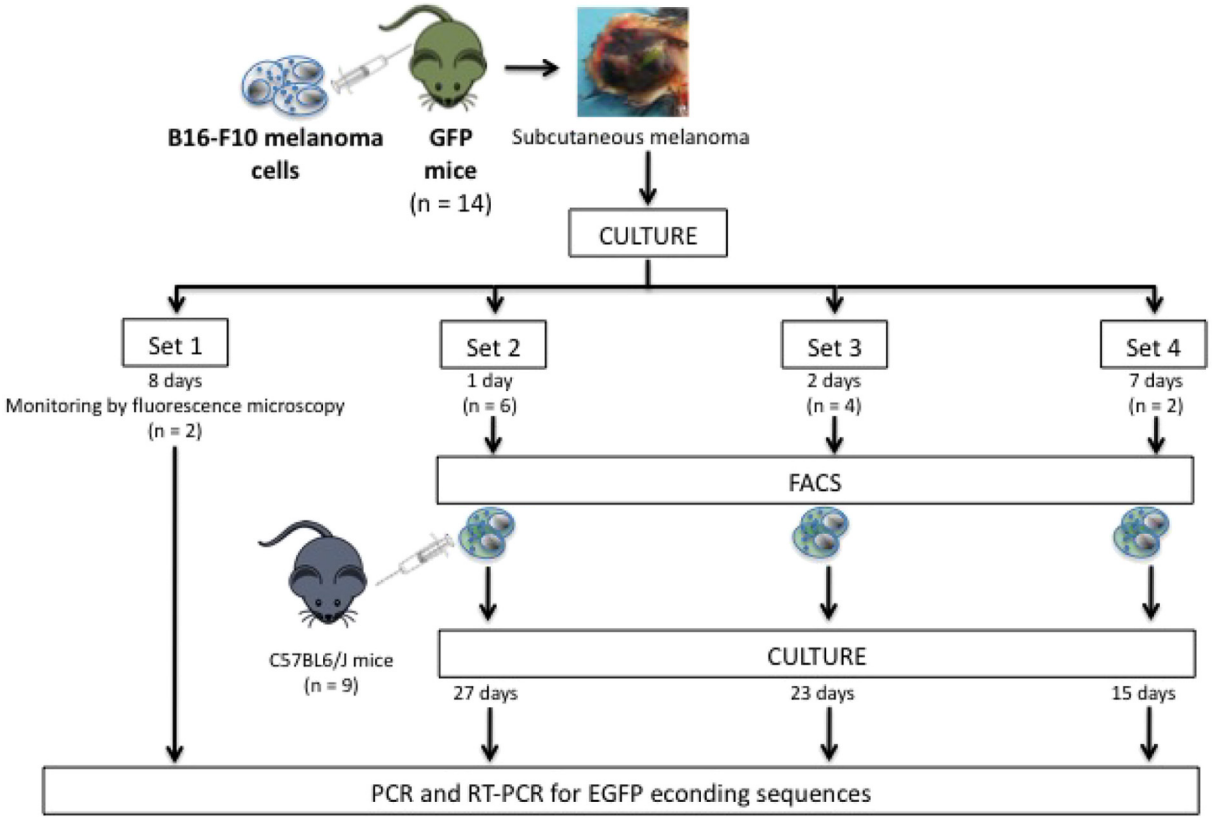

Fig. 1. Schematic representation of the study design.