Improvement of the monitoring and biosafety of encapsulated cells using the pSFGNESTGL triple reporter system

Raúl Catena[1][*], Edorta Santos[2][*], Gorka Orive[2], Rosa María Hernández[2], José Luis Pedraz[2], Alfonso Calvo[1]

[1]Laboratory of Novel Therapeutic Targets, Centre for Applied Biomedical Research (CIMA), University of Navarra. Avenida Pío XII 57, 31007 Pamplona, Spain.

[2]Laboratory of Pharmacy and Pharmaceutical Technology, University of the Basque Country (UPV/EHU), Paseo de la Universidad 7, Vitoria-Gasteiz, 01006, Spain.

[*]These two authors contributed equally to the study.

Corresponding Author: Dr. Alfonso Calvo
                        Laboratory 1.05
                        Division of Oncology
                        CIMA-University of Navarra
                        Pio XII, 55
                        31008-PAMPLONA
                        SPAIN
                        E-mail: acalvo@unav.es
                        Tl: +34 948 194700 Ext:1011
                        Fax: +34 948 194714

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ABSTRACT

Cell microencapsulation may represent a breakthrough to overcome problems associated with cell therapy. Advances in material biocompatibility and production protocols have put this field close to its clinical application. However, issues such as the possibility of tracking cell-containing microcapsules, monitoring cell viability, and discontinuation of the therapeutic activity when necessary, still remain unsolved. We demonstrate here simultaneous monitoring and pharmacological control of myoblasts-containing alginate microcapsules, injected in immunocompetent mice after transduction with the pSFG$_{NES}$TGL triple reporter retroviral vector, which contains green fluorescence protein (GFP), Firefly Luciferase and Herpes Simplex virus type 1 thymidine-kinase (HSV1-TK). Naked (as controls) or microencapsulated cells were subcutaneously injected in C57BL/6J mice and followed up by luminometry. Signal for naked cells disappeared 2 weeks after cell injection, whereas signal for microencapsulated cells remained strong for 8 months, thus demonstrating the presence of living cells. Treatment of mice with the thymidine-kinase substrate ganciclovir caused death of microencapsulated myoblasts, as seen by a drastic decay in the light emission and histological analysis. Hence, we conclude that incorporation of the SFG$_{NES}$TGL vector into microencapsulated cells represents an accurate tool for controlling cell location and viability in a non-invasive way. Moreover, cell death can be induced by administration of ganciclovir, in case therapy needs to be interrupted. This system may represent a step forward in the control and biosafety of cell- and gene-therapy-based microencapsulation protocols.

INTRODUCTION

In recent years, advances in the field of cell microencapsulation have turned this technology into much more than a mere promise, leading to the design of several strategies for a wide number of disorders and diseases [1-3], as well as novel research approaches for tissue engineering [4]. A broad range of cell lines have been successfully enclosed within semipermeable and biocompatible immobilization devices that control the bidirectional diffusion of molecules. Genetically-modified encapsulated cells are used to secrete hormones, neurotransmitters, and growth or inhibition factors for long periods of time without the need of immunosuppressants [5, 6].

In spite of such advances, many issues still remain unsolved. Two of the most critical challenges are spreading of particles out of the implanted area and the difficulty to retrieve the whole implant, in case side effects occur or once the therapy reaches its goal [7]. Furthermore, cell viability of enclosed cells during the implantation period has not been previously monitored in detail, because of the absence of appropriate biological tools. In addition, the lack of a tight control over these cells once they are implanted (for instance, by inducing cell apoptosis when desired), represents a challenge to lead this technology closer to its clinical use. Thus, the continuous monitoring of encapsulated cells and the improvement in biosafety by means of external drug control are still pending issues in the field.

Imaging technologies have made possible an accurate non-invasive follow-up of engrafted tissues [8, 9]. Non-invasive imaging techniques using various reporter genes are complementary to *ex vivo* molecular-biological assays and include additional spatial and temporal dimensions. Indeed, invasive methods require the use of a large number of animals along the different time points of the experiments. With the aim of introducing multimodality imaging in the field of cell microencapsulation, we have used a reporter

vector, pSFG$_{NES}$TGL that codes for HSV1-TK, GFP and luciferase genes. This triple reporter gene was recently developed by V. Ponomarev *et al.* [10]. The TGL vector as well as other similar triple reporter fusion constructs [11, 12] have been used in several *in vivo* applications including monitoring of the immune response in various cancer treatments [13-15] and to study the development of neural progenitor cells [16]. The presence of GFP in the vector facilitates the recognition and purification of the cells that have been successfully transduced by means of fluorescence activated cell sorting (FACS). The presence of luciferase allows for the bioluminescent image analysis of the transduced cells, with the goal of monitoring cell location and viability *in vivo*. HSV1-TK was initially introduced in the vector for nuclear imaging (by addition of specific radioactive nucleotides that can be traced by PET) [17]. But another critical advantage is its use as a suicide gene, so that apoptosis can be induced at a desired time point, after administration of ganciclovir (GCV) [18].

In the present study, C$_2$C$_{12}$ myoblasts were transduced with a viral vector which codes for the triple reporter gene TGL, and microencapsulated cells were monitored both *in vitro* and *in vivo* in immunocompetent C57BL/6 mice. To our knowledge this is the first report showing that viability of microencapsulated cells can be closely monitored *in vivo* and selectively eliminated at a specific moment. This combined strategy is a step forward for the future design and control of novel gene and cell therapy approaches using microencapsulation techniques.

MATERIALS AND METHODS

Cell culture

C_2C_{12} myoblasts derived from the skeletal muscle of a C3H mouse and the BD-Retropack 293 (Clontech, Mountain View, CA) packaging cells were maintained in complete medium, consisting of: RPMI-1640 growth medium (Invitrogen, Carlsbad, CA) with Glutamax®, supplemented with 10% FBS, 100U/mL penicillin and 100 μg/mL streptomycin (both antibiotics from Invitrogen). Cells were grown at 37ºC in a 5% CO_2 atmosphere. After transduction of cells with the pSFG$_{NES}$TGL-containing virus, cells were treated with different concentrations of Ganciclovir (Invivogen) and the cell density was measured through crystal violet staining and subsequent image analysis with Scion ImageTM (NIH, Bethesda, MD).

Generation of triple reporter C_2C_{12} myoblasts (C_2C_{12}-TGL)

Purified plasmidic DNA for the pSFG$_{NES}$TGL retrovector (kindly provided by Dr. Ponomarev, Memorial Sloan Kettering Cancer Center, NY) was introduced into mammalian packaging BD-Retropack 293 cells through cationic lipid-based transfection with the reagent Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendations, in order to generate infective but replication-defective virions carrying the $_{NES}$TGL fusion construct. To transduce target C_2C_{12} cells with the retroviral vector, virion-containing supernatants from the transfected packaging cells obtained 48h and 96h after transfection were passed through 45 μm pore filters (Sartorius, Goettingen, Germany) and added to the cell culture of target cells at the moment of exponential growth phase. To enhance transduction efficiency, polybrene

(hexadimethrine bromide, Sigma, St. Louis, MO) was added at a concentration of 6 μg/mL to the culture medium of target cells. 96 h after retroviral transduction, vector-containing GFP+ myoblasts were analyzed by FACS using a FACSAria Device (BD, Franklin Lakes, NJ).

Encapsulation

C_2C_{12} myoblasts genetically engineered with the triple reporter gene TGL (C_2C_{12}-TGL, 2B7 clone, generated in our laboratory) were encapsulated into alginate-poly-*L*-lysine-alginate microcapsules using an electrostatic droplet generator following a brief modification of Lim & Sun's procedure [19]. Ultra pure low-viscosity high glucuronic acid alginate (UPLVG) was purchased from FMC Biopolymer, Norway. Poly-*L*-lysine (PLL hidrobromide M_w 15 000–30 000 Da) was obtained from Sigma. Briefly, cells were harvested from monolayer cultures using trypsin-EDTA (Invitrogen) and resuspended in 2% sodium alginate at a density of $5x10^6$ cells/mL. The resulted suspension was extruded in a sterile syringe through a 0.25 mm needle at a flow rate of 5.9 ml/h using a peristaltic pump. The drops were collected in a 55 mM $CaCl_2$ solution and maintained in a shaker for 10 min upon complete gelation. Subsequently, the beads were suspended in 0.05% PLL solution for 5 min and coated again with another layer of 0.1% alginate for 5 min. Finally, microcapsules were cultured in complete medium.

Animals and Histology

Six adult female C57/BL mice per group were used as allogeneic recipients. The mice were anesthetized by intraperitoneal administration of ketamine (150 mg/kg) and xylazine (10 mg/kg) mixture, and a total volume of 200 μL of cell-loaded

microcapsules suspended in additional 200 µL of PBS were implanted subcutaneously using a 20-gauge catheter (Nipro; Nissho Corp, Belgium). The equivalent number of non-encapsulated cells was administered as control. A group of mice were injected daily for a week with 150 mg/kg ganciclovir in saline solution, intraperitoneally. Control mice were injected with saline. At the end of the experiments, mice were sacrificed and samples were collected and fixed in formalin for further analysis. Fixed samples were paraffin-embedded and 5 µm thick sections were obtained to perform Masson's thrichromic staining. Image analysis of different randomly taken images was performed to quantify the number of cells within the microcapsules.

Detection of Luciferase activity

C_2C_{12}-TGL-containing microcapsules or naked cells were detected either *in vitro* or *in vivo* through luciferase-mediated light emission. Mice or culture plates were placed in a Xenogen IVIS 100 series luminometer (Caliper Life Sciences, Hopkinton, MA) living image system after injection or addition of D-luciferin (Promega, Madison, WI) to the cell culture medium, respectively. Mice were injected under anaesthesia intraperitoneally with 3 mg of D-Luciferin in 100 µL saline, 5 min before light caption. Mice were shaved before light caption to avoid hair-mediated light-emission decay [20]. Images were analyzed to quantify the luminescent signal with the Igor-Pro 2.20 software (Caliper Life Sciences).

RESULTS

Generation of triple reporter mouse C$_2$C$_{12}$ myoblasts and *in vitro* activity

The mouse myoblast cell line C2C12 was retrovirally transduced in order to generate clones stably expressing the SFGNESTGL triple reporter retroviral vector (Fig. 1A). C2C12-TGL containing cells were sorted by FACS for the enrichment of the GFP+ cell population, to obtain more than 90% of GFP+ cells (Fig. 1B). Microencapsulated cells in vitro (Fig. 1C) also expressed strongly GFP, even after 1 week in culture (Fig. 1D). In addition, strong light emission was observed in C2C12-TGL cultured cells (Fig. 1E). The functionality of the triple reporter chimerical protein was further demonstrated by sensitivity of the cells to GCV treatment. When C2C12-TGL cells were treated with different doses of the drug, cell death was observed (Fig. 1G) and light emission dropped as well (Fig. 1E–F, H). When a dose of 2.3 μM GCV was added to the medium photon flux decayed to almost undetectable levels by day 6 (Fig. 1E–F). C2C12 cells carrying the SFGNESTGL vector showed an almost 1000-fold increased sensitivity to the drug compared to control cells (Fig. 1G). In keeping with this result, light emission curves paralleled with those of cell proliferation when cells were treated with GCV (Fig. 1G, H). Untransduced cells were GFP and luciferase negative (not shown).
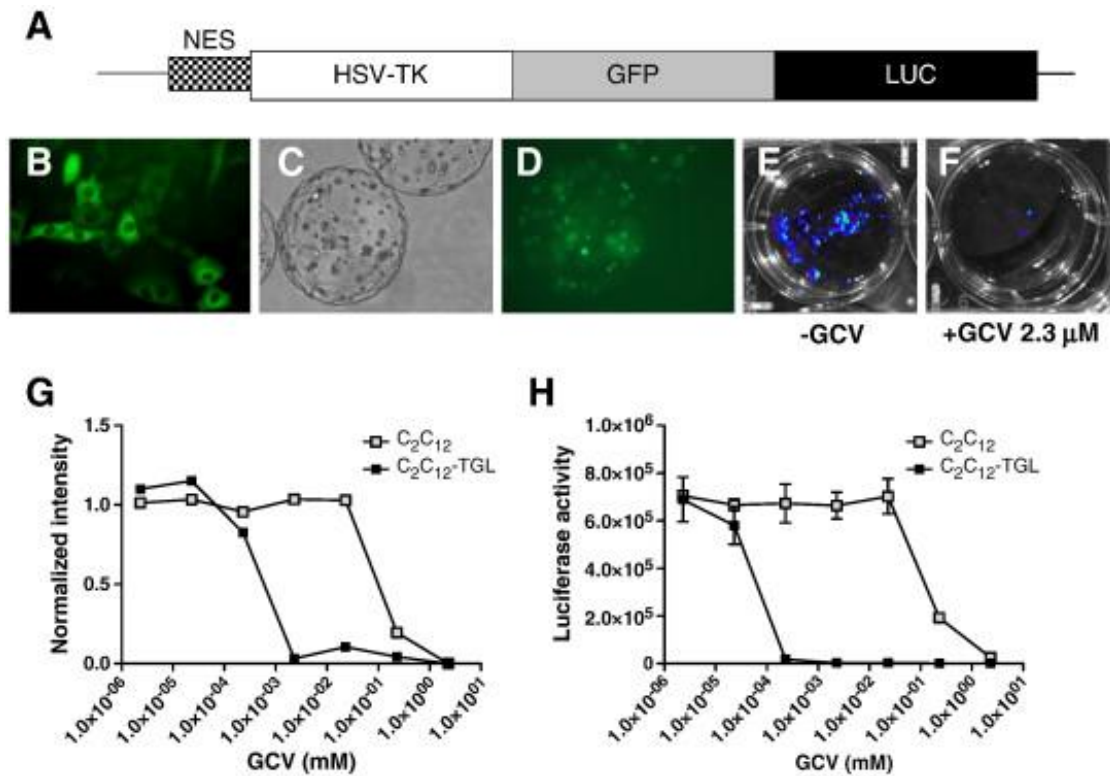
Figure 1.

Microencapsulated but not naked cells remain viable *in vivo* and express the triple reporter chimerical protein for a long period of time

Mice were injected subcutaneously with 200 μL of cell-loaded microcapsules. The total number of microcapsules was counted in several doses of 200 µl. The result for each of them was approximately 13.500 microcapsules (data not shown). Considering that initial cell load is about 5 million cells/mL, the number of cells in 200µl is around 1 million. This translates into an average of 74-75 cells/capsule for this determinate particle size (300 µm diameter).

The same number of naked cells was used as control. Photon flux measurements for microencapsulated cells were very low at the initiation of the experiment but increased during the first two weeks after implantation (Figs 2A and 2B). Eight months after implantation of microencapsulated cells, mice still displayed strong signal (Fig 2A). A group of mice received daily injections of GCV for a week and an almost complete signal clearance was observed (Figs 2A and 2B). On the contrary, injection of naked C_2C_{12}-TGL cells produced a high light signal in the first days of assay, but this signal was progressively lost (Fig 2C). For light detection, shaving of mice was critical, since black hair was able to absorb up to 99% of emitted light from the subcutaneous compartment. This result is in agreement to that of previously reported [20]. Injection of C_2C_{12}-TGL containing microcapsules in athymic Nu/Nu mice demonstrated that skin itself is not as important as the presence of black hair in order to detect luciferase activity (results not shown).
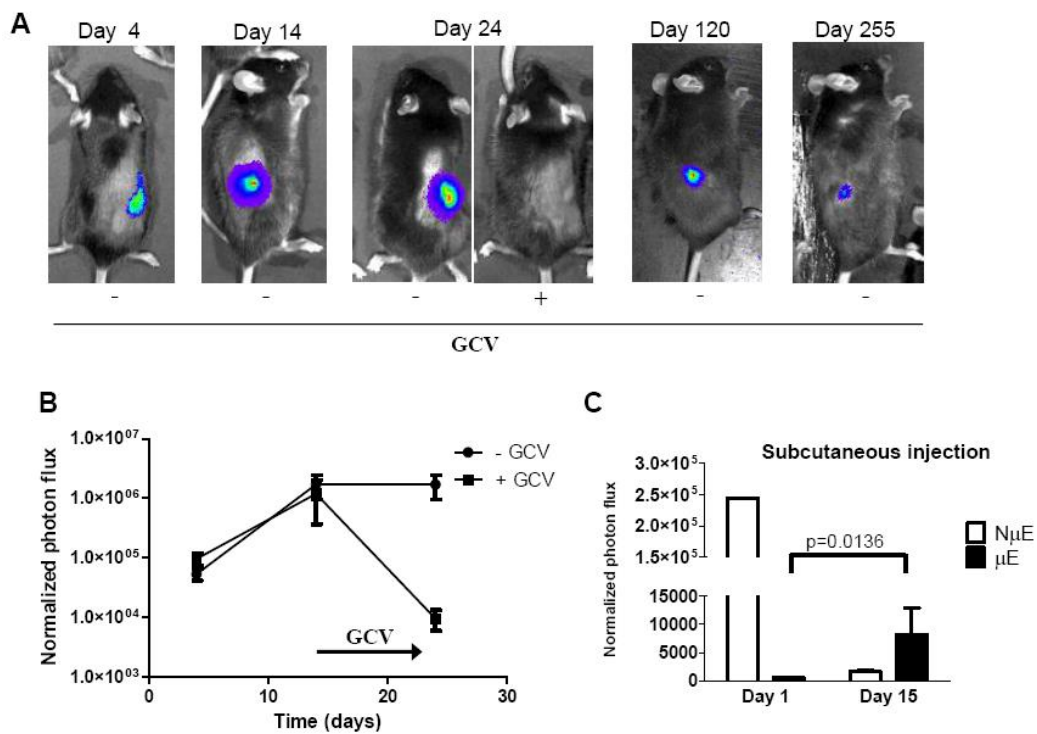

Figure 2.

Histology of C$_2$C$_{12}$-TGL containing microcapsules

Microscopic analysis of microcapsules revealed a morphology that was consistent with that described before (Fig. 3A) [21]. Myoblasts were visible within the microcapsules (Fig. 3A). Subcutaneous implantation of the microcapsules in mice generated fibrotic masses (Fig. 3A–B). In GCV-treated mice, microcapsules appeared empty of myoblasts, but cell debris could be observed (Fig. 3B). Quantification of the number of cells within the microcapsules showed a dramatic decrease after GCV treatment (Fig. 3C), which is in keeping with the reduction in light emission found in these mice. Blood vessels could be clearly seen in the stromal compartment of the plugs, in many instances in close proximity to the capsules (Fig. 4A–B). C2C12-TGL cells within the microcapsules exhibited strong expression of GFP in animals that were untreated with GCV, even 8 months after implantation, thus confirming the viability of these cells in vivo (Fig. 4C). No GFP positive cells were observed in mice that were administered with GCV (not shown).
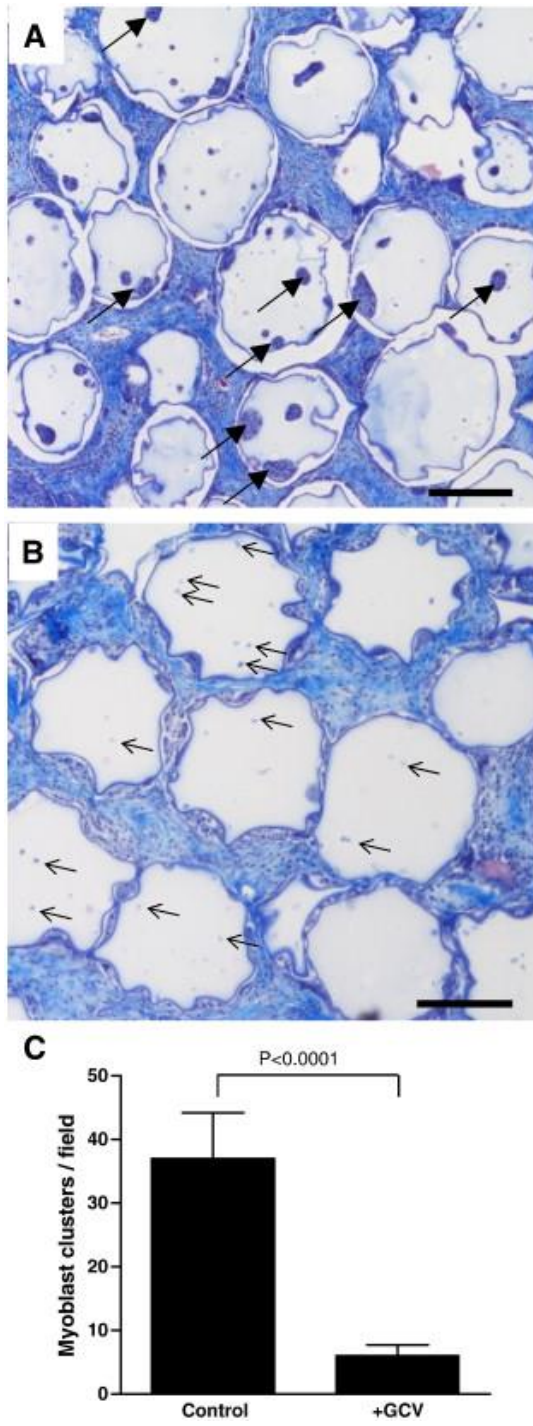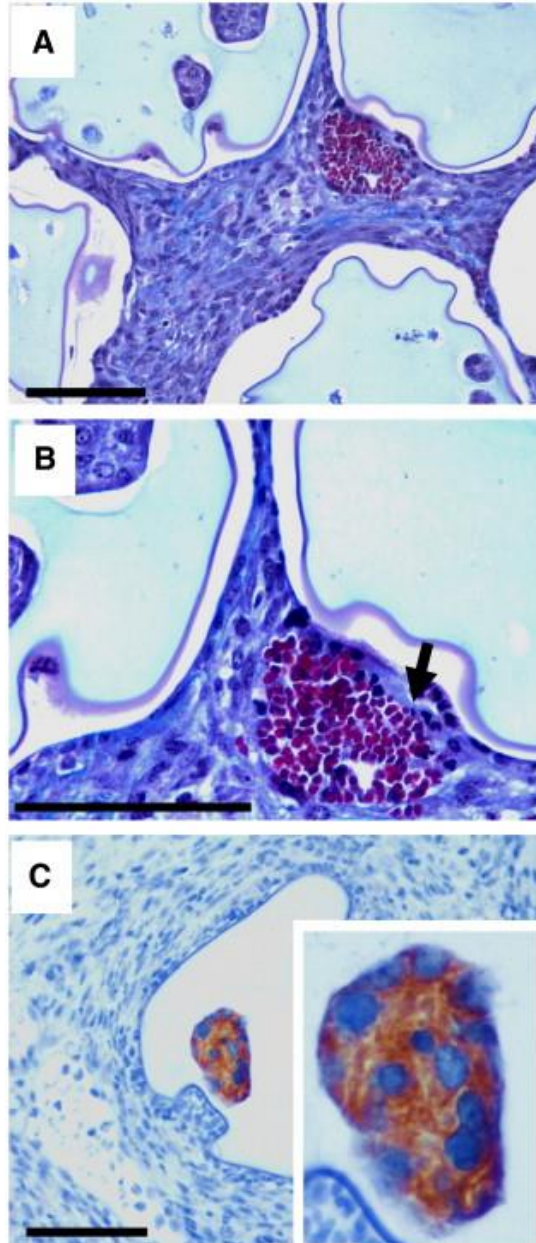
Figure 3.

Figure 4.

DISCUSSION

Important advances in the field of cell microencapsulation, including biocompatibility [22-24], mechanical stability [25-27], functionalization of matrices for cell-biomaterial interaction [28-30] and in standardization of production protocols [31] have been made. However, additional efforts are needed to overcome remaining challenges, such as the possibility of continuously localizing the implanted microparticles and even inhibiting externally the function of the immobilized cells. In fact, to our knowledge, a method to control tightly the location of encapsulated cells and their functionality has not yet been developed. A first approach to address these issues was reported by B. Gimi *et al.* using self-assembled three dimensional radio-frequency shielded containers but this system did not fulfil the real necessities of the technology, since it did not provide any information about functionality of enclosed cells [32].

We carried out an approach for using and testing the triple gene TGL with the aim of adapting non-invasive molecular imaging techniques to cell microencapsulation. Retrovirally transduced C_2C_{12} myoblasts were entrapped in alginate-poly-*L*-lysine-alginate microcapsules. In order to test the functionality of the triple gene, we compared the sensitivity for GCV of transduced and not transduced cells *in vitro*. Transduced cells were almost 1000-fold more sensitive to GCV than non-transduced cells. This compound acts as a prodrug that is converted into a toxic drug by viral thymidine kinase phosphorylation. Cells infected with the virus vector produce highly-toxic triphosphates that lead to cell death [33].

Enclosed cells expressed the triple gene in a stable manner and, after addition of the luciferase substrate, we could clearly observe the light emitted by these cells. This

method resulted so sensitive and precise that we could even detect isolated microcapsules. *In vitro,* treatment with GCV functioned as expected and after addition of the drug for one week, cells underwent apoptosis and the light emission was switched off. It is remarkable that microencapsulation did not prevent the incorporation of luciferin into the myoblasts or the effect of GCV over enclosed cells.

Microcapsules were also administered subcutaneously in twelve C57 mice. At day 4, we noticed a slight luminometric signal in mice that rose considerably by day 14 and remained constant in subsequent measurements. Oxidation of luciferin by luciferase is dependent on Mg^{2+}, ATP and oxygen [34]. It is possible that luciferin and other factors may not be fully accessible to the cells inside the capsules for the first days after implantation. There are two possible not mutually exclusive explanations for this fact. The first one is the time required to reach optimal diffusion equilibrium through the capsule matrix, allowing a better delivery of luciferin and oxygen to the cells. The second explanation is that vascularization of the tissue surrounding the microcapsules is needed to provide oxygen and luciferin to the cells. The process of functional vascularisation takes several days to occur [35]. In the first days post-implantation, vasculature may not be fully developed and, therefore, no signal would be obtained. At the end of the experiment, histological analysis showed a functional microvascular network irrigating the whole implant.

Eight months after implantation, the encapsulated cells remained viable and functional, and emitted an intense signal detected by the photon flux chamber. These results are in agreement with those obtained by our group in previous experiments, in which a long-term expression of erythropoietin from encapsulated myoblasts was achieved for 330 days [28]. Hence, the immunobarrier function of microcapsules is be reinforced by the present results.

From the second fortnight onwards, half of the mice were treated with GCV everyday, for 8 days. As expected, in the treated group the signal intensity declined basically to undetectable levels by day 24, whereas the non-treated group remained with the activity shown at day 14. This could be considered an important breakthrough for the microencapsulation technology, that would allow for the elimination of the cells inside (or spread outside) the implant. Thus, a strict control over the encapsulated cells can be achieved, once the treatment reaches its goal or in case deleterious side effects may occur. The fact that mice treated with the drug showed capsules empty of cells demonstrates the safety and cleanliness of this inactivation mechanism.

Non-encapsulated cells displayed higher signal intensity at the beginning of the experiment compared with encapsulated cells, but such signal decreased progressively by day 15, suggesting clearance of cells by the immune system. Conversely, the emission from immobilized cells experienced a considerable rise by the end of the first fortnight, thus demonstrating the immunoprotective role of microcapsules. The use of HSV1-TK-derived reporters in non-encapsulated cells presents the potential hurdle of their known immunogenicity [36]. Since encapsulated cells are unable to physically interact with immune cells, HSV-TK would not elicit any cellular immune response, albeit has been shown previously to be very immunogenic in naked cells. The fact that HSV-TK-carrying microencapsulated cells were not rejected during months in this study confirms that microencapsulation prevents from deletereous immune attack. Thus, the use of microencapsulation in combination with HSV1-TK (or other gene-mediated suicide strategy) is not only an advance but should be mandatory in order to control cell viability in vivo.

The initial lower signal intensity shown by encapsulated cells compared to naked cells may be a consequence of the above mentioned time required to reach luciferin

diffusion equilibrium through the capsule matrix, and the absence of adequate blood supply due to lack of vascularization at the time of injection.

In conclusion, the present study shows that incorporation of the pSFG$_{NES}$TGL into cells results in an accurate tool for holding a tight control on microencapsulated cells. More importantly, this method would provide information about enclosed cells at desired time points in a non-invasive way, without the necessity to retrieve the implant, with the additional advantage of cell inactivation, if needed. This would help determine the precise location of therapeutic biosystems *in vivo* and will improve the biosafety of cell microencapsulation. Such advance may lead this technology closer to a future clinical use.

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Acknowledgements

FIGURE LEGENDS

Fig. 1. Scheme of the SFG$_{NES}$TGL retrovector, which contains a triple fusion protein consisting of Herpes Simplex Virus Thymidine-kinase (HSV1-TK), Green Fluorescent Protein (GFP), and Firefly Luciferase (LUC) genes fused to a nuclear export signal (NES) in a single transcript (**A**). C2C12-TGL cells generated after transduction of C2C12 mouse myoblasts with the SFG$_{NES}$TGL retrovirus show GFP expression (**B**). Microencapsulated cells cultured for 1 week in vitro show strong expression of GFP; **C**: Bright field microscopy image of microencapsulated cells in vitro. **D**: Fluorescence image of the same microencapsulated cells, demonstrating the GFP signal. **E**: C2C12-TGL cells emitted light after addition of the luciferase substrate luciferin. **F**: After addition of GCV, light emission drops dramatically. **G**: Comparison of sensitivity to GCV in parental C2C12 and C2C12-TGL cells (cell proliferation). C2C12 cells carrying the SFG$_{NES}$TGL vector are ∼ 1000-fold more sensitive to the drug than control cells. **H**: A similar experiment showing light emission after treatment with different doses of GCV. Results are consistent with those of cell proliferation and indicate that C2C12-TGL cells can be effectively killed by GCV.

Fig. 2 Behaviour of C$_2$C$_{12}$-TGL microencapsulated cells *in vivo*. Microencapsulated C$_2$C$_{12}$-TGL cells exhibited light emission after being injected subcutaneously in C57/BL6 mice (**A-C**). Mice treated with 150 mg/kg/day Ganciclovir (GCV) for a week showed almost no signal (**A-B**). Cells within the microcapsules showed luciferase activity 255 days after injection (**A**). Quantification of light emission demonstrates an increase in the normalized photon flux during the first two weeks after implantation, probably due to vascularization of microcapsule plugs (**B-C**). Non-microencapsulated

C$_2$C$_{12}$-TGL cells displayed a marked decrease in light emission between days 1 to 15 (**C**). Conversely, microencapsulated myoblasts increased the emission during that period of time (**C**). µE: Microencapsulated cells. NµE: Non-microencapsulated cells.

Fig. 3. Histology of C2C12-TGL microencapsulated cells subcutaneously implanted into mice. Untreated encapsulated cells displayed an intact morphology (**A**). Arrows show myoblasts within the microcapsules. GCV-treated mice exhibited a dramatic decrease in the number of myoblasts within the microcapsules. Instead, cell debris could be seen (small arrows) (**B**). Quantification of myoblast clusters by image analysis demonstrates a dramatic reduction upon administration of GCV (**C**). Bars: 200 µm.

Fig. 4. Histological and immunohistochemical analysis further demonstrates the neovascularization of the plugs and viability of the C2C12-TGL-microencapsulated cells implanted in vivo, at the end of the experiment (**A–C**). Masson's trichrome staining allows for the identification of erythrocytes within the vessels (**B**, arrow). Vessels are located within the fibrotic tissue and, many times, in the vicinity of microcapsules (**A–B**). Immunohistochemistry for GFP shows strong staining in C2C12-TGL cells, thus indicating cell viability (**C**). The inset shows a detail of C2C12-TGL cells positively immunostained for GFP within the microcapsules. Bars: 100 µm.