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A 3D *in vitro* spheroid model as a way to study the mechanisms of electroporation

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ABSTRACT

Electropermeabilization is a physical method to deliver molecules into cells and tissues. Clinical applications have been successfully developed for antitumoral drug delivery and clinical trials for gene electrotransfer are currently underway. However, little is known about the mechanisms involved in this transfer. The main difficulties stem from the lack of single cell models which reliably replicate the complex *in vivo* environment. In order to increase our understanding of the DNA electrotransfer process, we exploited multicellular tumor spheroids as an *ex vivo* model of tumor. We used confocal microscopy to visualize the repartition of permeabilized cells in spheroids subjected to electric pulses. Our results reveal that even if cells can be efficiently permeabilized with electric fields, including those cells present inside the spheroids, gene expression is by contrast limited to the external layers of cells. Taken together, these results, in agreement with the ones obtained in tumors, indicate that the spheroid model is more relevant to an *in vivo* situation than cells cultured as monolayers. They validate the spheroid model as a way to study electro-mediated gene delivery processes.

1. Introduction

The delivery of therapeutic molecules into cells and tissues remains a major challenge in pharmacology. This is because the plasma membrane of the cell acts as a selective barrier that hinders the uptake of small as well as large hydrophilic molecules into cells. Therefore, efficient and safe delivery methods are essential to overcome this barrier. Delivery of DNA can be achieved by taking advantages of natural vectors such as viruses. However efficient, their use is limited due to their lack of safety (Hacein-Bey-Abina et al., 2002). For the past 20 years a huge variety of non-viral methods, including chemical and physical vectors, have been developed. In recent years, significant efforts have been made to use the potential of nanotechnology in drug delivery (Hamidi et al., 2008; Brigger et al., 2002). Amongst physical methods, electropermeabilization, also named electroporation, which was introduced in the 1970s, has been further developed in the 1980s for drug and gene delivery (Neumann et al., 1982; Kinosita and Tsong, 1979). The method, based on the transient permeabilization of the plasma membrane by applying electric field pulses, has been used with increasing popularity (Heller and Heller, 2006; Cemazar et al., 2006b; Gehl, 2003; Giraud et al., 1996). Applications such as electrochemotherapy, have been successfully developed for antitumoral drug delivery (Mir et al., 1998; Belehradek et al., 1993; Rols et al., 2002) as well as others for the transfer of genes (Titomirov et al., 1991; Heller et al., 1996; Rols et al., 1998; Aihara and Miyazaki, 1998; Cemazar et al., 2006b; Mir et al., 1999). At present, electrochemotherapy is accepted in a number of countries as a palliative treatment (Gehl, 2008). Clinical trials of gene electrotransfer are currently under investigation. A phase I dose escalation trial of plasmid interleukin electroporation has been carried out in patients with metastatic melanoma and has shown encouraging results (Daud et al., 2008). However, one has to notice that even if *in vitro* electrotransfer is usually efficient in almost all cell lines, *in vivo* gene delivery and expression faces some problems. For instance in tumors, efficiency remains low with only a few percent of transfected cells (Rols et al., 1998). Those cells are in addition only found at the periphery of the tumors (Mesojednik et al., 2007).

The mechanisms behind the improved gene delivery *in vivo* remain largely unknown. A better understanding of these mechanisms, and their efficiencies in overcoming obstacles for *in vivo* delivery, is critical to the improvement and advancement of this therapeutic strategy. While the mechanism of membrane permeabilization and pore formation is still a subject of continuous debate, it is accepted that the driving force behind electropermeabilization is the applied field-induced transmembrane voltage

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(Teissie et al., 2005). The transmembrane voltage depends on the shape and orientation of the cells and the microenvironment surrounding the cells (Valic et al., 2003; Kotnik and Miklavcic, 2000). Moreover, electromediated drug delivery depends on the nature of the drugs themselves. Small molecules enter the cell across part of the membrane facing the two electrodes by a diffusion process (Rols and Teissie, 1990; Puc et al., 2003; Sun et al., 2006). In contrast, the introduction of DNA is much more complicated. The electric field induces a key reaction between the electropermeabilized membrane and the DNA which is electrophoretically accumulated at the interface. DNA does not enter into the cell during electric pulses but it is first "trapped into" the plasma membrane before entering the cell a long time after pulses application (Golzio et al., 2002). These dependences lead to spatially heterogeneous cellular uptake of solutes into tumors during electroporation, making it difficult to achieve a uniform cellular delivery of genes to tissues (Henshaw and Yuan, 2008). Studies performed on cells in culture, even if instructive, are of limited use when relating to cells within a tissue environment since in the former, delivered molecules have direct access to these cells. Indeed, in tissues, the diffusion of molecules is impaired with large molecules such as DNA having no direct or free access to the entire cell population. Thus, an abiding limitation of studies performed in animals is the lack of reductionist experimental approaches which at a single cell level reliably mirror the ongoing processes at an organ level.

The aim of the present study was to increase our understanding of the DNA electrotransfer process. Our adopted strategy used a 3D *in vitro* spheroid model as a way to study the mechanisms of electropermeabilization. To this end, we exploited multicellular tumor spheroids as an *ex vivo* model of tumor. Upon growth, spheroids display a gradient of proliferating cells (Sutherland, 1988). These proliferating cells are located in the outer cell-layers and the quiescent cells are located more centrally. When the central cells become deprived in oxygen and glucose, cell death and necrosis occur. This cell heterogeneity is similar to that found in avascular microregions of tumors. They can be used to evaluate tumor response to therapeutic agent and are useful to identify new markers of the response to endocrine treatment and to investigate the effects of drugs combination (Truchet et al., 2008). We used confocal microscopy to visualize the repartition of permeabilized cells in spheroids submitted to electric pulses. Our results reveal that despite small molecules, such as propidium iodide, being efficiently transferred into cells, including cells present in the center of the spheroids, transfer and expression was limited to the external layers of cells in agreement with the ones obtained in tumors.

2. Materials and methods

2.1. Cells

The HCT 116 cell line was derived from a human colorectal carcinoma and the LPB cell line was derived from methylcholanthrene-induced C57Bl/6 mouse sarcoma. They were selected for their ability to grow plated on Petri dishes and to form spheroids. HCT 116 cells were grown in DMEM media (Gibco) containing 4.5 g/l glucose, L-glutamine and pyruvate and supplemented with 10% (v/v) of heat inactivated fetal calf serum, 100 U/ml penicillin and 100 mg/ml streptomycin. LPB cells were maintained in Eagle's minimum essential medium (EMEM; Gibco-Invitrogen, Carlsbad, CA, USA) with 10% fetal calf serum (Gibco), penicillin (100 units/ml) (Gibco-Invitrogen), streptomycin (100 mg/ml, Gibco-Invitrogen) and L-glutamine (0.58 mg/ml; Eurobio, France) in a 5% CO_2 humidified incubator at 37 °C (Jouan, St. Herblain, France). LPB cells were transduced with viral vectors to stably express eGFP (Cemazar et al., 2006a). To that purpose, a retro viral vector, MFG-eGFP, encoding the eGFP under the control of 50 long terminal repeat (LTR) was used. 293T cells, generously provided by the Genethon (Evry, France), were transiently transfected using the calcium phosphate co-precipitation protocol with pMDG encoding VSV-G protein, pGagPol encoding gag and pol and MFG-eGFP. Virus containing supernatants was collected 36–72 h after transfection, filtered and concentrated to titers of 1–5109 colony forming units (c.f.u.)/ml. LPB cells were plated in 35 mm culture dish 24 h prior transduction. On day 0 cells were transduced with viral vectors at a MOI of 100:1. After transduction (48 h) cells were harvested for FACS analysis on a Becton–Dickinson FACScan to select LPB cells stably expressing eGFP.

2.2. Generation of spheroids

The hanging-drop method has been adapted to produce spheroids of similar diameter (Del Duca et al., 2004). Drops (20 μl) containing 500 cells were suspended on the lid of agar coated 24-well dishes containing 500 μl of culture media. After the 72 h required for cell aggregation, the spheroids were transferred to the bottom of the well containing 500 μl of culture medium. Multicellular spheroids were then allowed to grow for 10 days and the spheroids used in the experiments sized in average around 500 μm of diameter.

2.3. Plasmid extraction

A 4.7 kb plasmid (pEGFP-C1) containing the gene coding for the Green Fluorescent Protein under control of the CMV promoter was obtained from Clonetech (Palo Alto, CA). It was purified from *Escherichia coli* transfected cells by using Maxiprep DNA purification system according to Qiagen instructions (Courtaboeuf, France).

2.4. Electropulsation

Electropulsation was achieved by using a CNRS cell electropulsator (Jouan, St Herblain, France) which delivered square-wave electric pulses. An oscilloscope (Enertec, St. Etienne, France) monitored pulse shape. Stainless steel flat parallel electrodes (1 cm length, 1 cm width) were used.

2.5. Electropermeabilization

Spheroids were resuspended in 200 μl of pulsing buffer (10 mM K_2HPO_4/KH_2PO_4 buffer, 1 mM $MgCl_2$, 250 mM sucrose, pH 7.4). Permeabilization was performed on Petri dish by application of long electric pulses required to transfer genes and to load macromolecules into cells. Penetration of propidium iodide (100 μM, in pulsing buffer) was used to monitor permeabilization (Golzio et al., 2002). Ten pulses lasting 5 ms at a frequency of 1 Hz were applied at a given electric field intensity at room temperature, conditions known to induce efficient and transient cell permeabilization (Rols and Teissie, 1998).

2.6. Electrotransfection

Spheroids were resuspended in plasmid DNA containing pulsing buffer. For each condition, 10 μg of pEGFP-C1 plasmid was used. As with the electropermeabilization protocol, 10 pulses lasting 5 ms were applied at various pulses amplitude. Spheroids were incubated for 5 min at room temperature and cultured in agar coated wells of 24-wells plate with 500 μl of culture medium at 37 °C in a 5% CO_2 incubator for 24 h.

2.7. Confocal microscopy

Spheroids were observed with a Zeiss LSM 510 confocal microscope. For the GFP fluorescence, samples were excited with an Argon laser set at a 488 nm wavelength and emission was collected through a band pass filter from 500 nm to 530 nm. PI fluorescence was excited with a Helium–Neon laser set at 543 nm wavelength and emitted light was collected through a 560–615 nm band pass filter. Several sections of the spheroid were imaged until the loss of fluorescent signal. Images represent the compilation of all the optical slices in a 3D projection.

3. Results

In an attempt to gain insight into the process of drug delivery mediated by applied electric pulses, we used multicellular spheroids as a way to explore the mechanisms of electrotransfer at the single cell level on an *ex vivo* tumor model. According to our previous works, electric pulses were applied in the millisecond time range. Such conditions are known to allow transfer of macromolecules (Rols and Teissie, 1998). Propidium iodide was used as a marker of membrane permeabilization. A plasmid coding for the GFP was used to detect DNA delivery and gene expression with time. In the present study, we used confocal microscopy as a convenient tool to image the spatial distribution of those molecules which have been taken up by live cells, i.e. without any fixation or slicing of the sample.

3.1. Electropermeabilization of multicellular spheroids

Electropermeabilization in the presence of propidium iodide of spheroids from LPB cells, constitutively expressing GFP, allowed for the study of both the entry of external molecules (propidium iodide) and the release of internal molecules (GFP). Spheroids were subjected to 10 electric pulses lasting 5 ms at the following intensities: control (0 kV/cm), 0.1 kV/cm, 0.3 kV/cm and 0.5 kV/cm. As shown in Fig. 1, control spheroids showed heterogeneous expression of GFP in the cells. No detection of propidium iodide was present indicating the absence of dead cells. Application of electric pulses led to a decrease of the green intensity associated to the GFP with a concomitant increase of the red fluorescence associated with propidium iodide uptake. At 0.1 kV/cm, only a few cells were permeabilized. At 0.3 kV/cm, more than 50% of the cells had taken up propidium iodide. At 0.5 kV/cm, practically the entire cell population was permeabilized to propidium iodide. The fluorescent pictures are a 3D compilation of confocal slices and allowed for the visualization of cells up to a depth of 140 μm. Permeabilized cells appeared to be homogeneously distributed in the core of the spheroids, at least in the regions where their detection has been performed.

3.2. Electrotransfer of gene to multicellular spheroids

Spheroids from HCT116 cells were transfected by electropermeabilization in the presence of a plasmid coding for the green fluorescent protein (GFP). Electro-gene transfer was performed by

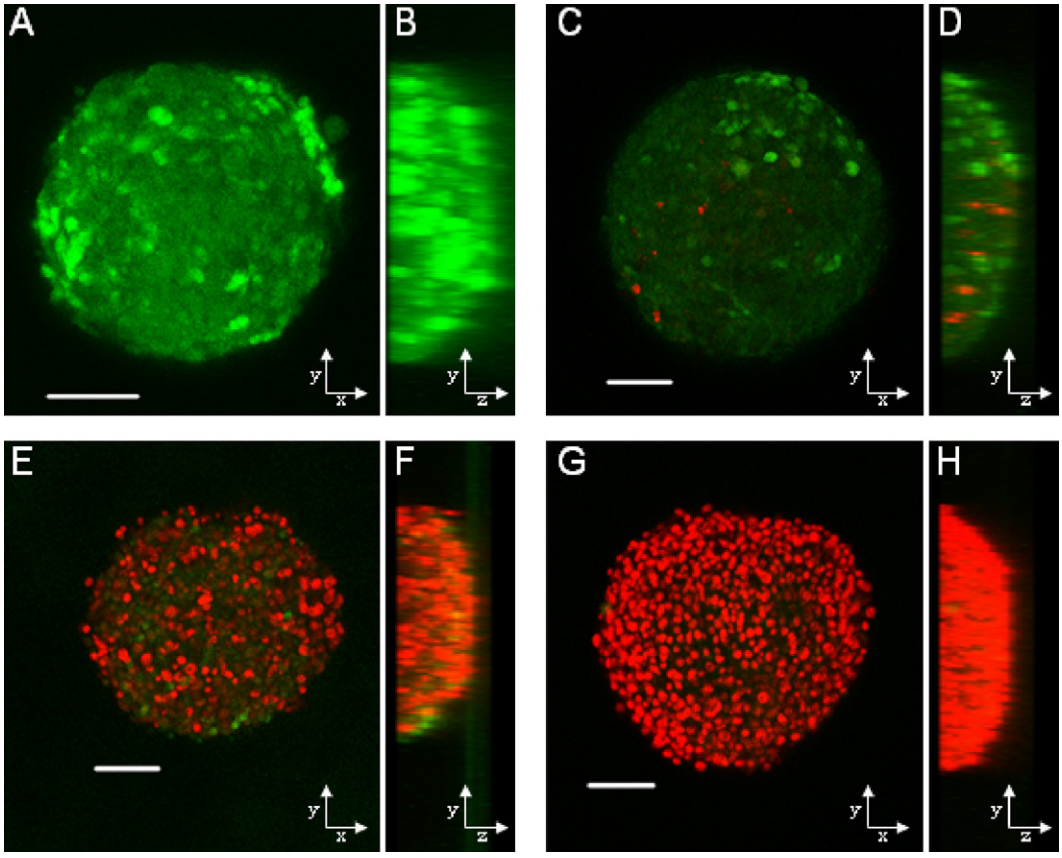

Fig. 1. Electropermeabilization of LPB spheroids with propidium iodide. Spheroids from LPB cells constitutively expressing the GFP (green fluorescence) were electropermeabilized (red fluorescence). Electropermeabilization was performed as described in Section 2 and three different intensities were tested: control (A and B); 0.1 kV/cm (C and D); 0.3 kV/cm (E and F) and 0.5 kV/cm (G and H). The fluorescent images are a 3D compilation of 15 optical slices of up to 140 μm depth in total. A, C, E and G show the image in the *X*–*Y*-axis of the 3D compilation with B, D, F and H showing the image in the *Z*-axis. The scale bars represent 200 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

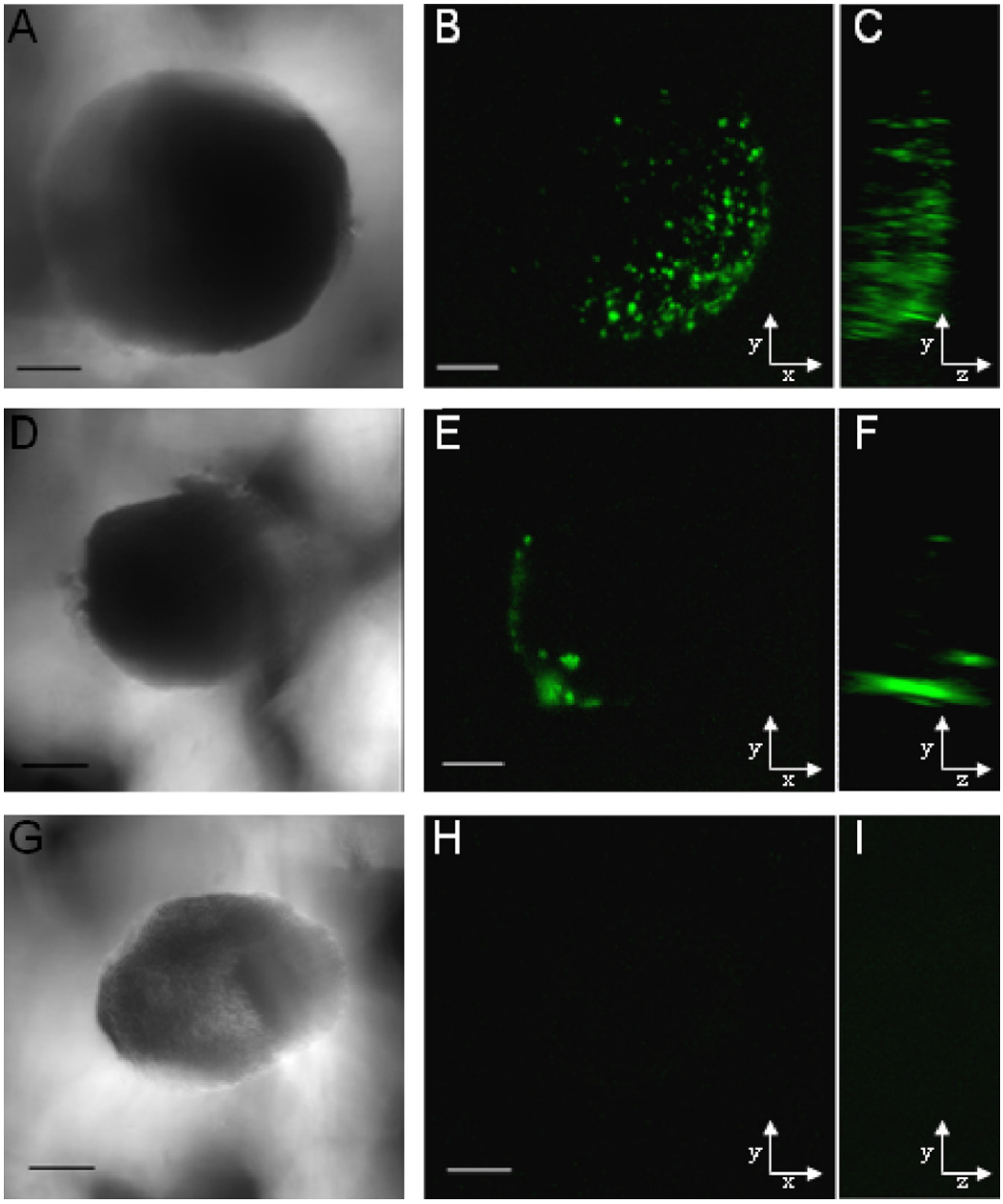

Fig. 2. Electro-gene transfer of multicellular spheroids. Spheroids from HCT116 cells were transfected by electropermeabilization with a plasmid coding for the green fluorescent protein (GFP). The efficiency of expression and its distribution were analyzed by confocal microscopy 24 h after transfection. Electro-gene transfer was performed as described in Section 2 and three intensities were tested for the applied electric field: 0.3 kV/cm (A–C); 0.5 kV/cm (D–F) and 0.8 kV/cm (G–I). A, D and G show transmitted light images of the spheroid with B, C, E, F, H and I being the respective fluorescent images. The fluorescent images are a 3D compilation of confocal slices of up to 460 μm, 550 μm and 380 μm in depth for the first, second and third spheroid respectively. B, E, and H are in the *X*–*Y*-axis with C, F, and I showing images in the corresponding *Z*-axis. The scale bars represent 100 μm.

applying 10 electric pulses lasting 5 ms at the following intensities: 0.3 kV/cm, 0.5 kV/cm and 0.8 kV/cm. The expression efficiency and the distribution of the GFP protein were analyzed by confocal microscopy 24 h after electric pulses application. Fluorescent pictures are a 3D compilation of confocal slices up to 460 μm, 550 μm and 380 μm in depth for the three electric pulse conditions, respectively. As shown in Fig. 2, gene expression could be detected. The highest efficiency was obtained at 0.3 kV/cm electric pulses intensity. Under this condition, few cells were transfected. However, in contrast to propidium iodide uptake experiments, increasing the pulse intensity did not result in any increase in transfection efficiency. The percentage of green cells dramatically decreased at 0.5 kV/cm and no cells expressing the GFP protein could be detected at 0.8 kV/cm. This observation correlated well with a decrease in cell viability as observed by an arrest of the spheroid growth following application of electric pulses above 0.4 kV/cm (data not shown). A striking observation is that transfected cells were not homogeneously distributed into the spheroids. Indeed, they appeared to be only localized in the outer layers of the spheroids. Also of note was that expression was only detected in one cap of the spheroids. This result was consonant with those obtained in cells *in vitro* where only one pole of the cells, the one facing the negative electrode was competent for DNA delivery (Fig. 3A).

4. Discussion

In this study, we used multicellular tumor spheroids of LPB and HCT116 cancer cells as a tissue model to assess the distribution

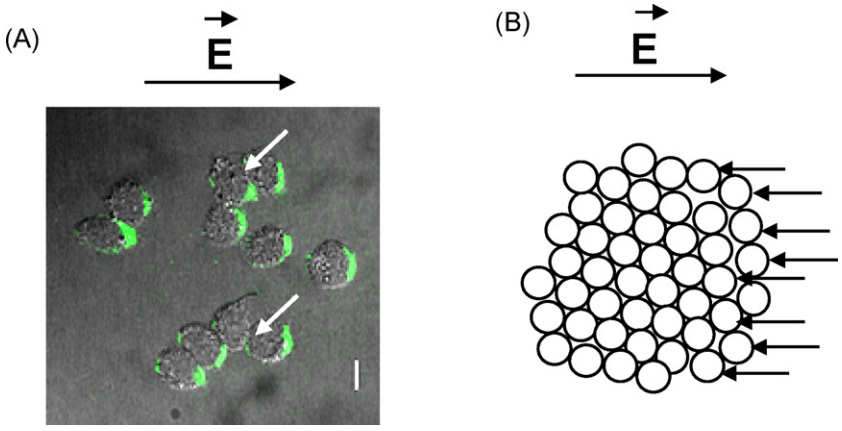

Fig. 3. DNA transfer in cells and spheroids. (A) Image of cells subjected to electric field in the presence of fluorescent plasmid DNA taken 1 min following pulses application as in Faurie et al. (2004). White arrows indicate areas of contact between cells where DNA cannot interact with. (B) Schematic representation of cells in a spheroid and their potential interaction with plasmid DNA (black arrows indicate the direction of DNA diffusion movement in an electric field).

of molecular uptake in a multicellular environment as a function of electric field strength. These experiments were undertaken to gain an understanding of, as well as to compare the differences and similarities of responses between cells in a multicellular environment and cells in culture when submitted to electric pulses. Molecular uptake was consistently dependent on the size of the molecules to be delivered. Propidium iodide (MW: 668 Da) uptake could be observed in spheroids for field strengths of 0.3 kV/cm and higher, with the percentage of permeabilized cells increasing with an increase in field intensity. The method was highly efficient since practically the entire cell population was permeabilized. No clear difference could be detected between cells at the periphery of the tumor model and the cells located deeper within the spheroid's core. Moreover, uptake of exogenous propidium iodide correlated well with a decay in GFP intensity that could be due to a release of internal GFP molecules (MW: 27 kDa). Such data are in good agreement with data obtained *in vitro* in cells cultured in monolayer or in suspension (Rols and Teissie, 1990). In contrast, differences could be observed in the case of the uptake of macromolecules as plasmid DNA (MW: 3 MDa). Gene expression was lower for cells within the spheroids than for cells in culture, with only a limited number of cells expressing the reporter gene. Moreover, transfected cells were heterogeneously distributed, being only located in the first outer proliferating cell layers at one particular pole of the spheroids. Such a well defined localization of transfected cells reaffirms what was observed with cells in culture (Fig. 3), where only the cap facing the cathode is susceptible to gene transfer as previously reported (Golzio et al., 2002). Moreover, as shown by the arrow in Fig. 3A, close contacts between cells may act as physical barriers that limit the diffusion of plasmid DNA and therefore its access to cells present in the core of the spheroid as drawn in Fig. 3B. Taken together, these results indicate that the spheroid model is more relevant to an *in vivo* situation than cells in culture validating the spheroid model as a useful tool to study electro-mediated gene delivery processes.

Studies using spheroids as models that mimic *in vivo* situations have been reported by others groups working on gene delivery using different vectors. Grill and collaborators studied the interactions between tumor and adenoviruses using multicellular spheroids grown from primary brain tumor material. Using reporter genes expressed by replication-defective adenoviruses, they showed that infection was restricted to the first layer of cells (Grill et al., 2002). Using a replication-competent adenovirus, they showed that transgene expression in the spheroid was considerably enhanced and that viral spreading deep into the 3D structure took place. They concluded that organotypic spheroids offer a versatile *in vitro* system for studying distribution, spread, and oncolysis by adenoviruses in a clinically relevant model. In a study combining non-viral methods for gene delivery, Mellor and collaborators showed that PEI-mediated transfection was limited to cells at the MCTS periphery. Using fluorescent PEI, it was found that complexes could only penetrate the outer 3–5 proliferating cell layers of the MCTS, sparing the deeper quiescent cells. This 'resistance' to transfection observed in quiescent cells was overcome through the use of electroporation. However, despite the improved efficacy of electroporation in quiescent tissue, the gene expression was still confined to the outer regions of MCTS. The results suggest that limited access to central regions of an MCTS remain a significant barrier to gene delivery (Mellor et al., 2006). Clearly a prominent feature of solid neoplasms, the avascular regions represent a major obstacle to achieving widespread gene delivery into tumors. Various strategies could potentially improve the tissue penetration of vectors such as PEI for example, by using a receptor-mediated approach to increase membrane-association and endocytic uptake of polyplexes. Alternatively, disruption of tight junctions between adjacent tumor cells is a further option. Transfection of MCTS, using either PEI or electroporation to deliver the pDNA, resulted in transfection of only a small proportion (<5%) of total cells (Mellor et al., 2006). The higher levels of reporter gene expression and increased efficacy in quiescent cells afforded by electroporation make it a promising strategy for tumor gene therapy. However, full exploitation of this approach will only be possible by improving access to the deeper, avascular areas of solid tumors. The extracellular reservoir of molecules, and thus the efficiency of tumor electrochemotherapy, has been improved by pre-exposure of tumor tissue to the drug. Important discrepancy between *in vitro* conventional monolayer cultures and *in vivo* tissues is the three-dimensional environments (e.g., cell–cell and cell–ECM, extracellular matrix, interactions) for the cells. Han et al. (2007) showed that a reporter gene was expressed even at the inner region of MCTS where necrosis was considered to be developed; small spheroids have a relatively weak structure due to their immature development of cell–cell and cell–ECM interactions. In contrast, large spheroids have a heterogeneous structure consisting of outer layers of viable cells and a solid core of necrotic or hypoxic cells, resembling a monolayer cell culture where the cells adhere to the hard substrate. Such morphological differences between small and large spheroids may account for their different sensitivities against polyplex-induced cytotoxicity. Canatella and collaborators reported that molecule uptake progressively decreased for cells located deeper within a spheroid's interior. Overall reduced levels of uptake were explained by locally reduced electric field strength within spheroids due to spatially heterogeneous electrical properties and a reduced extracellular reservoir of solute within spheroids

due to dense packing of cells. The dependence of uptake on radial position within spheroids was explained by an extracellular solute concentration gradient during transient diffusional lag times and the observation that cell size decreased for cells located deeper within spheroids, which in turn led to smaller cell volumes and smaller transmembrane voltages (Canatella et al., 2004). These observations suggest that tissue electroporation could be enhanced by using larger field strengths than those used for cell suspensions (Canatella et al., 2004). However, as shown in the present work, increasing electric field strength induced cellular damage resulting in a decrease in gene expression (Fig. 2). Another interest of the spheroid model is related to junctions that exist between cells as well as the extracellular matrix (ECM) that makes it closer to the tissues reality than cells with high density such as cell pellet obtained by centribugation (Pucihar et al., 2007). In such situation, the influence of cell density on cell membrane electropermeabilization has been investigated on dense cell suspensions which represent a simple model for studying electropermeabilization of tissues. Permeabilization decrease was proposed to be due to the changes in the local electric field, which led to a decrease in the amplitude of the induced transmembrane voltage. Other barriers can exist that hinder pDNA transfer and therefore expression (Bureau et al., 2004). Other strategies have been therefore investigated to modify tissue ECM for improving interstitial pDNA transport during electric field application. Muscle pretreated with hyaluronidase was shown to result in a fivefold increase in reporter gene expression (Molnar et al., 2004). In tumors, the distance of DNA electrophoresis was correlated with tissue collagen content. Pretreatment of tumors with collagenase has led to an increase in the efficiency of gene delivery with adenovirus vectors (Henshaw and Yuan, 2008). However, the ECM modification may facilitate tumor metastasis since matrix metalloproteinase expression has been associated with metastasis.

Such observations lead us to propose methodological solutions based on electric field parameters to improve DNA delivery. The intratumoral electric field, which determines the efficiency of electric field-mediated drug and gene delivery, is significantly lower from the applied field (Mossop et al., 2006). Based on analytical and numerical models of the local electric field distribution, the applied voltage can be chosen specifically to achieve adequate electric field inside the target tissue while minimizing damages of the surrounding tissue (Corovic et al., 2007). However, in the case of irregularly shaped tissue structures, such as bulky tumors, electric field homogeneity is almost impossible to be achieved with current electrode arrangements. The use of conductive gels, matched to the conductivity of the tissues, has been proposed to fill dead spaces between plate electrodes gripping the tissue so that the electric field distribution becomes less heterogeneous (Ivorra et al., 2008). However, electric field strength if too high can damage the cells. Another alternative we can propose is to change the way electric pulses are delivered. Usually, electric pulses are applied in one direction. We previously showed that changing the polarity and direction of electric pulses increased transfection of cells in cultures and also cells in tissues, where both increases in the area of muscle as the local gene expression were obtained (Faurie et al., 2003; Faurie et al., 2004). The fraction of transfected cells can indeed be increased by changing the electric field direction between electrical pulses without affecting the cell viability (Rebersek et al., 2007). When cells are in clusters, which is representative of tissues, only the first layers of cells facing negative electrode can be transfected (Fig. 3B). Applying electric field pulses under different directions can therefore help DNA to interact with permeabilized cells all around the tumors. The problem of the access of DNA to the internal layers of cells still remains. A possible solution is the application of very low intensity but long pulses to electrophoretically push the DNA towards the center of the tumors, before applying standard electric pulses.

As a general conclusion, all these data suggest that the spheroid can be considered as a good model to study the mechanisms of electro-mediated molecules delivery. Furthermore, this approach lends itself to the philosophy of the 3R's which promotes reliable alternatives to animal experiments in an attempt to reduce the numbers of animals used.

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