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High efficiency transfection of glioma cell lines and primary cells for overexpression and RNAi experiments

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Abstract

In order to investigate the impact of signalling proteins on the phenotype and malignant behavior of glioblastoma cells, we optimized the transfection procedure of human glioblastoma cell lines U251, U373, GaMG and of primary cells obtained from a patient's tumor using nucleofection technology in conjunction with plasmid pmaxGFP. We describe the optimization procedure, show that a high percentage of the cells can be transfected and that nucleofection does not cause phenotypic alterations of the cells. Therefore, we conclude that nucleofection is a highly efficient tool to deliver plasmids for transient protein overexpression and siRNA for specific protein knock-down to different glioblastoma cell lines or primary cells.

Keywords: Glioblastoma multiforme; Glioma cell-line; Primary cells; Transfection; Electroporation; Nucleofection

1. Introduction

Astrocytomas are tumors derived from glia cells. They rarely metastasize, but are characterized by an aggressive local growth pattern and a marked degree of invasiveness which ultimately limits the prognosis of the patient.

Glioma cell invasion requires an intricate series of both, host and tumor related steps, involving tumor matrix disintegration and tumor cell migration. These events are highly regulated by different signalling cascades. Many additional proteins, like, e.g. matrix metalloproteases, are also required for cell invasion (Chandresakar et al., 2000; Lakka et al., 2000; Nabeshima et al., 2002; Rao, 2003; VanMeter et al., 2001; Yong et al., 1998). However, little is known about the influence of these pathways and factors on the degree of glioma malignancy. Potential therapeutic regimes profit from a more explicit understanding of the underlying molecular and cellbiological mechanisms involved (VanMeter et al., 2001). Therefore, we investigated the impact of certain signalling proteins on glioma-cell characteristics like, e.g. proliferation rate, invasiveness, adhesion capability, apoptosis, and MMP expression and activity in primary cultures of human astrocytomas using overexpression and siRNA techniques.

For these experiments, however, a high degree of transfection efficiency is required. Basically, two different transfection principles exist: viral and non-viral transfection. The first comprise systems with retroviruses, adenoviruses, herpes simplex viruses and lenti-viruses (Pan et al., 2003). They offer the advantage of very high transfection efficiencies but handling and production of suitable viruses is time consuming and strong laboratory security measures have to be met. Viral genes are introduced into the cell's genome and may cause unwanted side effects like, e.g. pathogenicity or they may influence the expression pattern with concomitant problems when analysing protein functions (Byrnes et al., 2004; Lenz et al., 2003; Maasho et al., 2004). Some non-viral transfection methods have been developed to circumvent limitations of viral systems. They include the calcium phosphate transfection method, the DEAE-Dextran approach, ultrasound, lipofection and electroporation (Hamm et al., 2002; Kim et al., 1996; Lorenz et al., 2004; Muramatsu et al., 1997; Pepe et al., 2004). Varying and low efficiency rates limit the calcium phosphate method (Yamamoto et al., 1999) and cytotoxicity is a major problem of DEAE-Dextran transfection (Luo et al., 2002). Transfection by ultrasound works very precisely in vivo, since ultrasound can be exactly focused and is easy to han-

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dle. However, the low transfection efficiency is a major drawback (Pepe et al., 2004). The most broadly used transfection method is lipofection. Negatively charged DNA binds to cationic lipids and DNA–lipid-complexes are formed. By choosing an adequate ratio of the mixture, the complex remains positively charged and therefore is able to bind to the negatively charged surface of the cell. By endocytosis the DNA is transferred into the cell (da Cruz et al., 2004). However, cytotoxicity is a problem and transfection efficiencies of 40% are reached only very seldom. Electroporation may be an alternative to the so far mentioned approaches, but the transfection efficiencies achieved are in the same range as gained by lipofection.

A further alternative is a new technology to introduce DNA directly into the nucleus of cells without the necessity of dividing cells (Christine and Siebenkotten, 2000; Rothmann-Cosic et al., 2002). The DNA is bound to proteins containing a nuclear localisation signal (Christine and Siebenkotten, 2000). This complex then is transferred into the cell by electroporation. The DNA–protein complex is actively transported by the cell into its nucleus and allows gene expression of exogenous DNA shortly after transfection. Using nucleofection, primary neurons of the mouse have been transfected up to 60%, embryonic stem cells more than 60% and human leukemia cells up to 75% (Dityateva et al., 2003; Lakshmipathy et al., 2004; Schakowski et al., 2004). This new technology achieves very high transfection efficiencies without major preparation and security measures. In addition, expression of introduced recombinant proteins is already detectable a couple of hours after transfection. Nucleofection, however, has to be optimized for every type of cell. Each cell-line and primary cell culture has its own characteristics, including a unique composition of the cell membrane. Therefore, the parameters of the electric field for electroporation and the suitable transfection solution have to be determined for every cell type in an optimization process.

Primary cells from human glioma sections grow very slowly, are difficult to acquire and too valuable to be used for optimization of transfection efficiencies. In addition, each primary cell culture is derived from a tumor of a different patient. It is therefore likely that there are differences in the cells which might influence transfection efficiency. To overcome these problems, we decided to optimize the transfection efficiency for an established glioma cell line, U251, and to check afterwards, whether the closely related, but distinctive glioma cell lines U373 and GaMG can be transfected to a satisfying degree using the same protocol. In a next step we then transfected primary cells without further optimization, determined transfection efficiencies and tested possible alterations in the cell's phenotype and behavior using diverse cell biological assays.

2. Materials and methods

2.1. Cell lines and cell culture

Primary cells from a human glioblastoma multiforme (WHO IV) were prepared as described elsewhere (Wagner et al., 1999). Informed consent of the patient was obtained for acquisition of tumor material as approved by the local ethics committee. Human glioma cell lines U251 and U373 were purchased from ATCC (American Type Culture Collection, Rockville, MD) and GaMG was established from a patient with glioblastoma multiforme (Gade Institut of the University Bergen, Norway) (Akslen et al., 1988).

Cell lines and primary cells were grown under the same conditions in DMEM (CytoGen, Sinn, Germany) with 10% heat inactivated fetal calf serum (FCS), four times the prescribed concentration of nonessential amino acids, 3 mM L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin (all from Invitrogen, Carlsbad, USA), as monolayers in 75 cm^2 flasks (Corning, New York, USA). Cells were maintained at 37 °C, 5.0% CO_2 and 100% humidity.

2.2. Spheroid formation

One gram Noble Agar (Becton Dickinson Biosciences, New York, USA) was dissolved in 20 ml ultra pure water (Biochrom, Berlin, Germany) by shortly boiling the mixture in the microwave. Eighty milliliters of culture medium as described above were added and 2 ml were applied to each well of a six-well plate (Corning, New York, USA) for base-coating. After polymerization, plates were stored at 4 °C. Multicellular spheroids from cell lines and primary cells were generated by seeding 1×10^6 cells per well filled with 5 ml of medium, each. After 48 h, spheroids with a diameter of 100–300 μm were selected under microscopic control (Wilovert, Hund, Wetzlar, Germany) with a Pasteur-pipet for further experiments.

2.3. Lipofection of pmaxGFP

Cells were seeded into six-well plates in a density that they reached 1×10^6 cells the next day. Lipofection was performed according to the manufacturer's manual with 2 μg pmaxGFP DNA (Amaxa, Cologne, Germany) and 7.5 μl Lipofectamin Reagent (Invitrogen, Carlsbad, USA) per well. Since the manual describes transfection of cells cultured in 24-well plates, the volume of each solution used was multiplied by a factor of three to adjust it to the larger surface of the six-well plates. Twenty-four hours after lipofection, transfection efficiencies were evaluated as described below.

2.4. Nucleofection of plasmids and siRNA

Cells were passaged 2 days prior to nucleofection and grown to 80% confluency. Subsequently, cells were trypsinised (0.25% Trypsin–EDTA; Invitrogen), washed with PBS and for each transfection 1×10^6 cells were resuspended in 100 μl nucleofector solution. Three such solutions are offered. They differ in their buffer capacity (pH 7–8, 25 °C), which covers a range between 20 and 80 mmol/l × pH, and their ionic strength, which varies between 200 and 500 mmol/l (Rothmann-Cosic et al., 2002). In addition the solutions contain a concentration of potassium ions between 2 and 6 mmol/l K^+, a concentration of sodium ions between 100 and 150 mmol/l Na^+, and additional components like, e.g. sodium chloride, sodium succinate, manitol, glucose,