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RESEARCH ARTICLE

Efficient cold transfection of pea ferredoxin-NADP(H) oxidoreductase into rat hepatocytes†

María Gabriela Mediavilla[1,4]
Adriana Krapp[1,3]
Néstor Carrillo[1,3]
Joaquín V. Rodríguez[2]
Claudio Tiribelli[4]
Edgardo E. Guibert[1]*

[1]*Biología Molecular, Departamento de Ciencias Biológicas, Universidad Nacional de Rosario, Suipacha 531, [S2002LRK] Rosario, Santa Fe, Argentina*

[2]*Farmacología, Departamento de Ciencias Fisiológicas, Universidad Nacional de Rosario, Suipacha 531, [S2002LRK] Rosario, Santa Fe, Argentina*

[3]*IBR (Instituto de Biología Molecular y Celular de Rosario), CONICET, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario. Suipacha 531, [S2002LRK] Rosario, Santa Fe, Argentina*

[4]*Centro Studi Fegato, AREA Science Park Basovizza and Department BBCM, University of Trieste, 34012 Trieste, Italy*

*Correspondence to:
Edgardo E. Guibert, Biología Molecular, Dpto. Cs. Biológicas, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Suipacha 531, [S2002LRK] Rosario, Santa Fe, Argentina.
E-mail: eguibert@fbioyf.unr.edu.ar

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Abstract

We describe the use of a non-viral, polyethylenimine-based vector to transfect rat hepatocytes preserved under hypothermic storage. DNA sequences encoding *Escherichia coli* β-galactosidase and pea ferredoxin-NADP(H) oxidoreductase (FNR), cloned into plasmids pCH110 and pKM4 respectively, were used. FNR was detected in the liver of animals transplanted with transfected cells; no reactivity was observed in endogenous parenchyma. The expression of the transgene was transient as it was detectable up to 96 h subsequently declining to undetectable levels. In contrast to non-transfected cells, the engraftment of FNR-positive cells was not associated with inflammatory reaction. The percentage of FNR-positive implanted hepatocytes was at least five times higher than the original transfection efficiency measured *in vitro*, while the percentage of β-galactosidase-positive cells was similar for both methods. These data indicate that the transfection system is effective in the transfer of plasmid DNA into hepatocytes under cold preservation and suggest the advantage of pKM4-transfected hepatocytes on engraftment in the recipient parenchyma. Copyright © 2005 John Wiley & Sons, Ltd.

Keywords pea FNR; transfection; UW solution; hepatocyte transplantation; oxidative stress; PEI

Introduction

Hepatocyte transplantation is considered an alternative therapy to liver transplantation and is a required step in hepatic cellular and *ex vivo* gene therapies [1]. Healthy hepatocytes are delivered to the liver or spleen of recipients by infusion of cell suspensions and are expected to engraft and function in these organs. The functional contribution of the transplanted hepatocytes, which depends on the number of implanted cells, must be large enough to ameliorate or cure different acute and chronic hepatic diseases, either inherited or acquired [1,2]. However, several problems must still be solved to make this technique suitable for routine clinical application. A major issue is to increase the number of transplanted cells in the recipient liver to achieve a notable therapeutic effect [2,3]. Different strategies can be adopted to address this problem such as increasing the number of injected hepatocytes, making several separate infusions, or somehow augmenting the proportion of injected cells that finally engraft. The last option relies on the observation that about one-half of the transplanted hepatocytes is either lost or eliminated by the recipient organ and, consequently, would not contribute to functional replacement [4]. A number of approaches have been suggested to improve hepatocyte engraftment and/or avoid elimination by the host such as co-administration of a vasodilator [5] or genetic modification [6].

Gene transfer techniques applied to mammalian cells generally make use of viral-derived vectors because of their high efficiency [7,8]. However, the use of non-viral vectors seems to be preferable in clinical applications due to their

higher safety related to transient transfection. Unfortunately, they usually exhibit a lower efficiency as compared to viral vectors [6,9]. Previous work from the group of Shaked [7,10–12] using adenoviral vectors reported successful gene transfer into either hepatocytes or whole liver during hypothermic preservation in University of Wisconsin (UW) solution. Due to the caveats in the use of viral vectors in human applications, we became interested in exploring the possibility of transfecting hepatocytes during cold preservation in UW solution by a non-viral vector.

Ferredoxin-NADP(H) oxidoreductases (FNRs) are ubiquitous flavoenzymes present in animals, plants and prokaryotes, where they catalyze the reversible electron exchange between obligatory one- and two-electron carriers such as NADP(H) and ferredoxins, respectively [13]. Recently, FNRs have been shown to confer protection against oxidative stress in proteobacteria and cyanobacteria [14–17], a property that can be of great help during reperfusion of cold-stored hepatocytes. After preservation in ice-cold UW solution, reperfusion exposes cells to oxidative damage due to the presence of oxygen and higher temperature during the procedure [18,19]. Kupffer cells have also been implicated in the oxidative damage due to the local release of reactive oxygen species (ROS) induced by a transitory ischemia, resulting in an early clearance of transplanted hepatocytes [20].

We therefore investigated whether the introduction into hepatocytes of a DNA plasmid encoding the pea FNR gene [21] using a polyethylenimine (PEI)-based system [9] might result in a transient transfection and its effect in improving cell engraftment by reducing/preventing the oxidative damage following reperfusion.

Materials and methods

Reagents

Polyethylenimine (# P-3143) and collagenase type IV (# C-5138) were from Sigma-Aldrich Co. (St. Louis, MO, USA); MEM (# 1013126), M199 (# 1023126) and prednisolone 21-hemisuccinate (# 156347) were from ICN (Costa Mesa, CA, USA); jetPEI (# GDSP10110) was from Qbiogene (Illkirch, France); 5-(and -6)-carboxyfluorescein diacetate succinimidyl ester (CFSE) (# C-1157) was from Molecular Probes (Eugene, OR, USA); and 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) (# MB 1001) was from Melford (Ipswich, UK).

Hepatocyte isolation

Adult male Wistar rats weighing 250–300 g were used in all experiments. Rats were fed *ad libitum* and received human care in compliance with international regulations. The National Council Committee of Argentina approved the protocols. Rats were anesthetized with sodium thiopental (70 mg/kg body weight, intaperitonally), and the hepatocytes isolated by the collagenase perfusion procedure described by Seglen [3] and modified in our laboratory [19,22]. Briefly, livers were perfused *in situ* for 5 min via portal vein with 100 mL of a modified Hanks' balanced salt solution (HBSS) supplemented with 5 mM Tris and 0.5 mM EGTA, pH 7.40, at 37 °C. The perfusate was oxygenated by passing through oxygen-permeable tubing inside an appropriate glass container filled with 95% O_2 and 5% CO_2, at a constant pressure of 80 mm Hg. Air bubbles were avoided by connecting a disposable nylon filter in line between the oxygenator and the inflow. After the initial flush-out, the perfusion was followed in a recirculating system with 150 mL of EGTA-free HBSS supplemented with 1.2 mM $CaCl_2$, 0.025% collagenase type IV and 1% bovine serum albumin (BSA). The cell suspension was centrifuged (50 *g* for 3 min), and the pellet was resuspended twice in HBSS, 1% BSA, 1.2 mM $CaCl_2$ solution.

The viability of the cells was tested by the exclusion of 0.4% Trypan Blue dye (TBE) in phosphate-buffered saline (PBS). A minimum of 6×10^8 cells were usually obtained per liver. Preparations with a TBE greater than 85% were considered suitable for the experiments.

Hypothermic preservation

Hepatocytes were suspended at a density of 3×10^6 cell/mL in cold modified UW solution saturated with N_2. Cells suspended in modified UW solution were stored in an ice bath (0.0 ± 0.2 °C) for 24 or 48 h. The composition of the modified UW solution is 100 mM lactobionic acid, 25 mM KH_2PO_4, 5 mM $MgSO_4$, 30 mM raffinose, 2.5 mM adenosine, 1 mM allopurinol, 15 mM glycine, 5% PEG 8000, 0.25 mg/mL streptomycin, 10 IU/mL penicillin G, and 3 mM GSH, pH 7.40 [19,22].

Transfection

Plasmids pCH110 (Pharmacia # 27-4508-01) and pKM4 were used (Figure 1). pCH110 drives the expression of β-galactosidase under the control of the SV40 promoter. pKM4 was constructed from pcDNA3 (Invitrogen # V79020) (Figure 1) by insertion of the mitochondrial import sequence from the mouse ferredoxin reductase gene fused in phase with the mature portion of the pea *fnr* gene, encoding FNR, between the *Hind* III and *Eco* RI sites of the multiple cloning site of the plasmid.

Transfection complexes were formed by mixing 0.5–2 μg of plasmid DNA per 10^6 cells and the appropriate amount of PEI, dissolved in saline. The PEI-plasmid proportion was 9 amine groups of PEI per phosphate group of DNA (N/P = 9) for Sigma PEI as determined by Boussif *et al*. [9], while a N/P = 5 was used for jetPEI as recommended by the supplier. The transfection vector was added to the hepatocyte suspension immediately after resuspension in UW solution, mixed by gentle inversion and left undisturbed

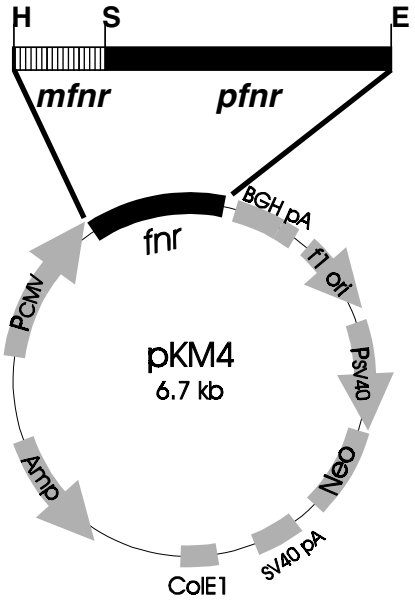

Figure 1. Schematic structure of pKM4. *fnr*: fusion gene composed of the mitochondrial localization sequence of mouse FNR (*mfnr*) and the mature portion sequence of pea FNR (*pfnr*). H, S, E: *Hind* III, *Sac* I and *Eco* RI restriction enzyme recognition sequences, respectively. PCMV: Cytomegalovirus promoter sequence. BGH pA: polyadenylation signal from bovine growth hormone gene. f1 ori: f1 single-strand DNA origin. PSV40: SV40 promoter. Neo: neomycin phosphotransferase gene. SV40 pA: polyadenylation signal. ColE1: ColE1 origin of replication. AMP: β-lactamase gene

at 0 °C for 24 or 48 h, respectively. Hepatocytes were then either transplanted into the spleens of recipient rats [23] or settled in culture [24].

Hepatocytes used as control were transfected with pCH110 which drives the expression of β-galactosidase following the same procedures used for pKM4 described above.

Hepatocyte transplantation

pKM4- and pCH110-transfected hepatocytes were transplanted into Wistar male rats under light ether anesthesia. Before transplantation, hepatocytes were labeled with 10 μM CFSE for 15 min at 37 °C under agitation [25]. Transplantation was performed through an intrasplenic injection of about 2×10^7 hepatocytes at high cellular concentration (5×10^7 cell/mL) [5,23], representing 1–2% of total rat liver hepatocyte mass [3]. Animals were sacrificed at different time points after transplantation and livers were harvested for histological examination and transgene expression analysis. For each time point, the livers of three animals were analyzed. For each organ, four different tissue fragments ($1 \times 1 \times 1$ cm^3) were collected from the left liver lobe. Liver necropsies obtained from animals transplanted with pKM4-transfected hepatocytes were fixed in 4% formalin, dehydrated and paraffin-embedded. Tissue fragments from animals transplanted with pCH110-transfected hepatocytes were embedded freshly in OCT and cryosectioned. Tissue sections of 0.5 μ thickness were used for quantitative analysis. After detection of the two transgenic products, tissue sections were examined to count cells expressing transgene products and/or CSFE-labeled. The assessment was performed by two distinct expert investigators (MGM and EEG).

Hepatocyte culture

To estimate transfection efficiency, hepatocytes were placed in monolayer cultures. Culture medium consisted of MEM/M199 (3 : 1) plus 1 g/L BSA, 2.2 g/L $NaHCO_3$, 5 mg/L insulin, 133 IU/L penicillin, 0.1 mg/L streptomycin, 133 IU/L kanamycin and 10% fetal bovine serum (FBS) [24]. In the first 4 h after seeding, the medium also included 50 μM prednisolone 21-hemisuccinate. Hepatocytes were seeded at a density of 10^5 cells/cm^2 in 12-well plastic dishes and incubated at 37 °C under 5% CO_2 atmosphere. The medium was changed every 24 h and the presence of the transgenic protein checked at 24, 48 or 72 h of culture, respectively. Transfection efficiency was expressed as the percentage of cells expressing the transgene over the total cell number.

Detection of transgenic protein

β-Galactosidase activity was revealed by X-gal substrate (1 mg/mL X-gal, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM $MgCl_2$ in PBS). FNR was inmunodetected using a rabbit polyclonal antibody and the VECTASTAIN ABC-AP system (Vector # AK-5001) for development with alkaline phosphatase substrate (0.02% naphthol AS, 0.05% Fast Red, 1 mM levamisole in 0.1 M Tris, pH 8.20).

Statistical analysis

Results are expressed as mean ± standard deviation (SD). Groups of data were compared by analysis of variance (ANOVA) and Student's t-test analysis as convenient. The test used and the number (n) of experiments are reported in the Results section.

Results

Hepatocyte transplantation

Animals recovered in less than 15 min after the surgery, and did not present any post-operative complications until the time of sacrifice. The sequence of delivery, integration and distribution of transfected hepatocytes into the liver parenchyma of transplanted rats was followed by CFSE staining and compared to that previously reported by us after transplanting non-transfected freshly isolated or preserved liver cells [23]. In line with previous observations [26], immediately after intrasplenic

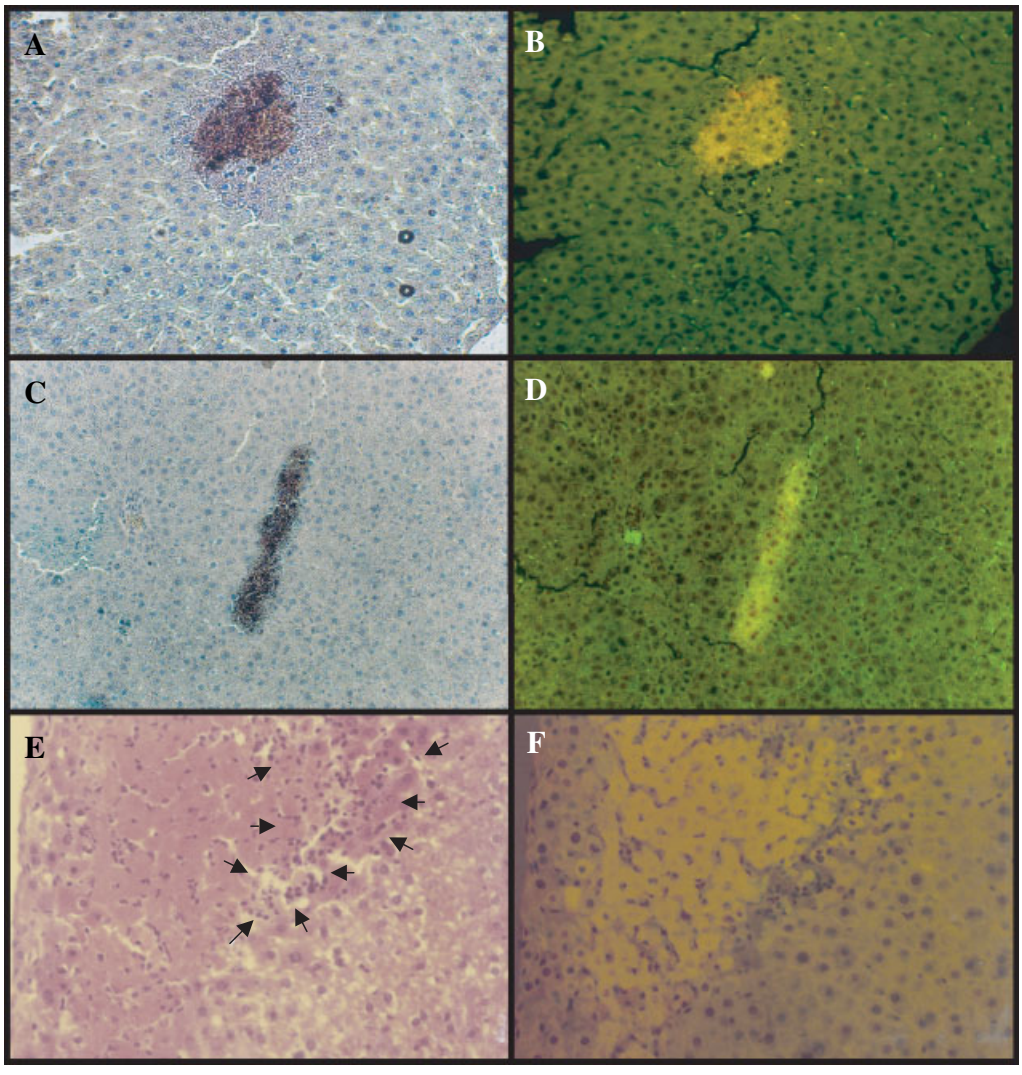

Figure 2. Liver sections of rats transplanted with transfected (A–D) and non-transfected (E, F) hepatocytes. (A) Immunodetection of FNR on tissue sections obtained 24 h after pKM4-transfected hepatocyte transplantation. A cluster of positive cells can be recognized among surrounding negative liver cells. Magnification 33×. (B) Detection of CSFE-labeled hepatocytes under fluorescence microscopy in the same field shown in (A). Magnification 33×. (C) Immunodetection of FNR on tissue sections obtained 48 h after pKM4-transfected hepatocyte transplantation. Positive cells are organized in a trabecular-shaped cluster and can be easily distinguished from the surrounding negative parenchyma. Magnification 33×. (D) Detection of CSFE-labeled hepatocytes under fluorescence microscopy in the same field shown in (C). Magnification 33×. (E) Hematoxylin-eosin staining of tissue section obtained 24 h after non-transfected hepatocyte transplantation. Note the inflammatory infiltrates (arrows). Magnification 33×. (F) Detection of CSFE-labeled hepatocytes under fluorescence microscopy in the same field shown in (E). Note that inflammatory infiltrates seen in (E) are surrounding CSFE-labeled hepatocytes. Magnification 33×

administration, hepatocytes were detected in portal vein branches and, 3 h later, a few cells were observed entering sinusoids. Twenty-four hours after injection, CFSE-labeled cells (266 ± 33 cells/cm^2) were localized in the liver parenchyma with a minor perturbation of the normal morphological architecture of the tissue (Figure 2A). After 48, 72 and 96 h, cells were integrated and not distinguishable from endogenous parenchymal cells under light microscopy. The density of stained cells remained constant 48 and 72 h after transplantation (200 ± 57 and 249 ± 73 cells/cm^2, respectively) and was also maintained 7 days after transplantation (213 ± 28 cells/cm^2). In summary, the spatial and temporal distribution was comparable between transfected and non-transfected hepatocytes.

Localization of transgenic proteins

Immunolocalization of FNR showed positive hepatocytes 24, 48 (Figures 2A and 2C), 72 and 96 h (data not shown) after transplantation in liver sections obtained from rats transplanted with hepatocytes transfected with pKM4. No positive hepatocytes were observed at 3 h and 7 days after transplantation, as well as in rats transplanted with non-transfected hepatocytes or not transplanted (data not shown). Positive cells were stained brown and appeared co-labeled with CSFE (Figures 2A–2D). Four tissue sections were analyzed for each liver and three livers were analyzed for each time point. FNR-positive hepatocytes averaged 20–50 cells/cm^2 of tissue section and were usually arranged in clusters containing

4–15 cells. They represented 77.0 ± 15.1% of total CSFE-stained hepatocytes at 24 h post-transplantation, 63.8 ± 11.3% at 48 h, and 66.7 ± 9.4% at 72 h, without significant difference (ANOVA) at the three different time intervals. As shown in Figures 2A and 2C, no inflammatory infiltrates were observed surrounding the FNR-positive cells, different from what is usually found when non-transfected hepatocytes are injected (Figures 2E and 2F) [23].

In contrast, rats transplanted with hepatocytes transfected with pCH110 displayed less than one β-galactosidase-positive blue cell per tissue section (data not shown). They represented 0.003 ± 0.001, 0.007 ± 0.001 and 0.006 ± 0.001% of total CFSE-stained cells at 24, 48 and 72 h post-transplantation (not significant, ANOVA). As in the case of FNR, no β-galactosidase-positive cells were observed in sections of rats either transplanted with non-transfected hepatocytes or not transplanted.

Transfection efficiency

Due to the rather evident difference in the quantity of FNR- and β-galactosidase-positive cells per liver section, we decided to determine the transfection efficiency of the two vectors. To this end, hepatocytes transfected in ice-cold UW solution were cultured for 24–72 h and transgene expression was detected thereafter (Figures 3A and 3B).

As reported in Table 1, the efficiency, expressed as percentage of positive cells, was comparable for the different amounts of DNA plasmid used and for the different times of culture. No difference was also observed between the two sources of PEI. In contrast, the efficiency was significantly higher for pKM4 than pCH110, as also shown in Figure 4, where the percentage of positive cells was much greater for cells transfected with the FNR gene than with the β-galactosidase gene (ANOVA, $p < 0.0001$). Noteworthy, transfection efficiencies were similar when determined at 24, 48 or 72 h of culture and no difference was observed between the genes by varying the amount of plasmid used for transfection (Table 1, Figure 4).

To assess whether the different transfection efficiency was related to intrinsic characteristics of each plasmid, the two vectors were used to transfect freshly isolated hepatocytes in culture. Freshly isolated liver cells were seeded in culture plates and, 24 h later, they were transfected with the PEI/DNA complexes. No significant differences were found in the efficiency obtained with pKM4 or pCH110 with Sigma PEI (0.090 ± 0.150% vs. 0.155 ± 0.230%, respectively) or jetPEI (4.75 ± 0.80% vs. 10.80 ± 5.80%, respectively) (Figures 3C and 3D).

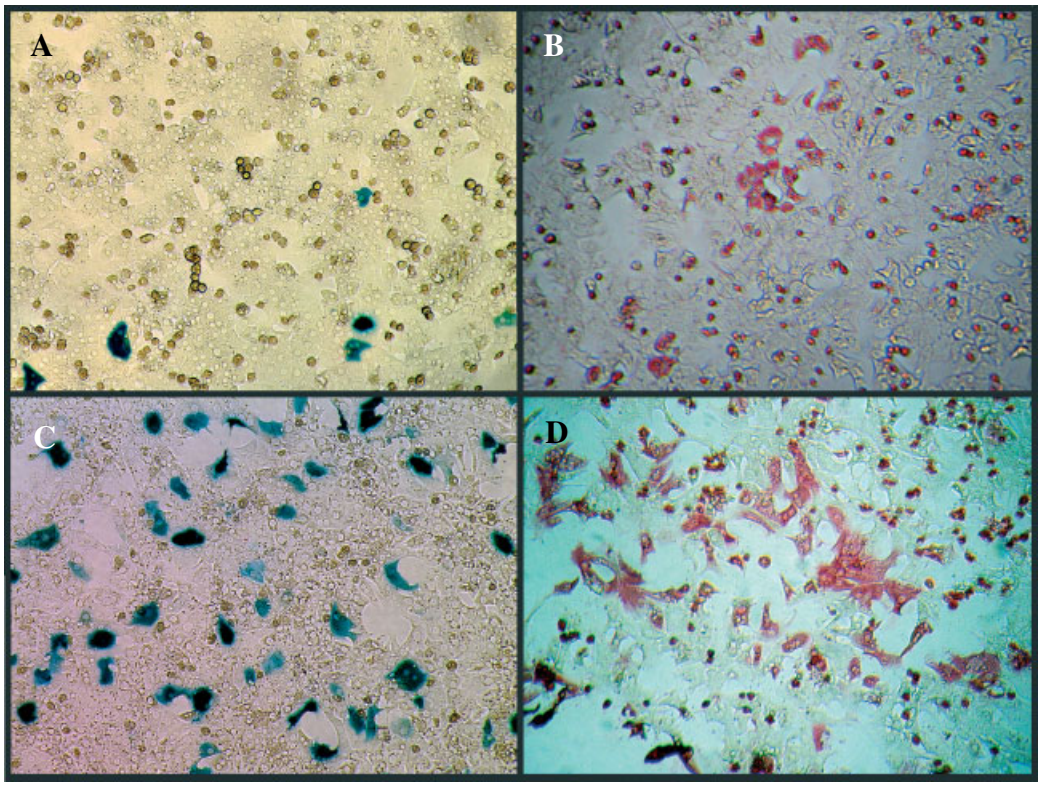

Figure 3. Detection of FNR and β-galactosidase in hepatocytes in culture. (A) X-gal staining of hepatocytes placed in culture for 48 h after transfection with pCH110 under hypothermic preservation. Blue cells indicate the expression of β-galactosidase. Magnification 100×. (B) FNR immunostaining of hepatocytes placed in culture for 48 h after transfection with pKM4 under hypothermic preservation. Red cells indicate the expression of FNR. Magnification 100×. (C) X-gal staining in hepatocytes transfected with pCH110 under culture conditions (culture medium, 37 °C, 5% CO_2) 48 h after transfection. Blue cells indicate the expression of β-galactosidase. Magnification 100×. (D) FNR immunostaining in hepatocytes transfected under culture conditions (culture medium, 37 °C, 5% CO_2) 48 h after transfection with pKM4. Red cells indicate the expression of FNR. Magnification 100×

Table 1. Efficiencies of transfection of hepatocytes during preservation in modified UW solution under hypothermic conditions, determined in primary cultures

Plasmid	µg/million cells	Incubation in UW (h)	PEI	Efficiency (%)	n[a]
pKM4[b]	1	24	Sigma	6.5 ± 3.2	3
	1	48	Sigma	9.7 ± 6.3	9
	1	24	jetPEI	4.3 ± 5.7	3
	1	48	jetPEI	14.7 ± 9.1	3
	0.5	48	Sigma	3.1 ± 0.5	3
	2	48	Sigma	11.2 ± 4.1	3
pCH110[c]	1	24	Sigma	$8.10^{-4} \pm 7.10^{-4}$	3
	1	48	Sigma	0.010 ± 0.003	9
	1	24	jetPEI	0.19 ± 0.05	3
	1	48	jetPEI	0.56 ± 0.49	3
	0.5	48	Sigma	0.008 ± 0.004	3
	2	48	Sigma	0.012 ± 0.07	3

[a]n is the number of transfection experiments performed on hepatocytes isolated from at least three different animals.
[b]Efficiency evaluated as the number of positively immunostained cells for FNR expression.
[c]Efficiency evaluated as the number of positively X-gal stained cells for β-galactosidase expression.
Efficiencies for all conditions are significantly higher for pKM4 than pCH110 (ANOVA, $p < 0.0001$).

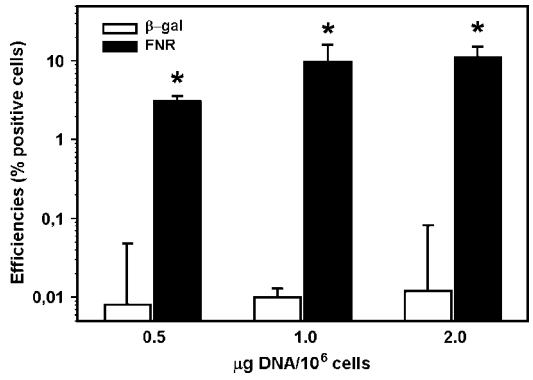

Figure 4. Expression of β-galactosidase (open bars) and FNR (solid bars) after transfection with the pCH110 and pKM4 plasmidic vectors, respectively. The efficiency was assessed as percentage of positive cells. The DNA amount tested increased from 0.5 to 2.0 µg/10^6 cells. Transfection with pKM4 resulted in a significantly higher efficiency at the three DNA amounts tested. Bars represent mean ± SD of at least three different preparations. Please note the log scale on the y-axis. *$p < 0.0001$, ANOVA

The efficiency was however significantly higher for jetPEI than Sigma PEI for each plasmid (Student's t-test, $p = 0.00015$).

Since PEI is reported to be a non-toxic transfection agent [9,27,28], we did not observe either cellular detachment or morphological changes upon transfection of the hepatocytes in culture conditions. We also did not notice appreciable cell loss when cells transfected during cold storage were seeded.

Discussion

One of the primary goals of this work was to achieve an effective non-viral gene transfer in hepatocytes during cold storage. Our results show that the PEI/DNA transfection system is effective during hypothermic preservation in UW solution of rat hepatocytes, a condition normally used for organ preservation until implantation. In line with previous reports on the use of PEI as a transfection agent *in vitro* and *in vivo* [9,27,28], we did not observe any toxic effect either at the cellular or at the recipient level pointing to the safe use of cold PEI/DNA transfection. We have previously reported that isolated hepatocytes can be safely preserved at 0 °C for at least 72 h without affecting their metabolic performance. More interesting was the observation of their functional efficiency upon reperfusion as assessed by functional tests both *in vitro* [22,29–33] and *in vivo* [5,23,24].

Transfection during cold storage presents some advantages over transfection in culture since a culture step would imply an enormous culture surface to obtain the amount of cells required, the loss of a consistent quantity of material on harvest, as well as higher costs and the need for trained personnel. We were successful in transfecting hepatocytes during cold storage; however, the transfection efficiency must be increased to allow the assessment of the potentially beneficial effect of FNR activity when treating hepatic disorders. A higher number of cells expressing FNR is needed given that transfection of primary hepatocytes does not permit a previous selection of the transfected cells.

The percentage of implanted FNR-positive hepatocytes *in vivo* was at least 5 times the transfection efficiency measured *in vitro*, while the number of cells positive for β-galactosidase was comparable both *in vitro* and *in vivo*. Together with the lack of the inflammatory infiltrate which normally surrounds transplanted cells [23], these data point to the possible advantage of FNR-expressing cells in their engraftment ability. Reasons for these results may be related to the advantage of FNR-expressing cells to tolerate the passage from cold anoxia to a normoxic and normothermic environment. The exact mechanism accounting for this behavior needs to be determined although it may be attributed to an antioxidant function of FNR during the first challenge to oxygen after a prolonged period of anoxia [14–17]. Preliminary data indicate that Cos-7 cells expressing pea FNR are less susceptible than non-transfected cells to hydrogen peroxide damage as measured by MTT assay, suggesting that the expression of FNR protects cells from the cellular insult linked to the redox alteration as supposed in this study. Although FNRs are present in all kingdoms, the plant isoforms are 200- to 500-fold more active than their animal or prokaryotic counterparts [13], a property that might explain their remarkable competence as antioxidants in model bacterial systems [14,15]. The antioxidant properties of FNR may be particularly important in counteracting the imbalance of the redox state occurring during reoxygenation.

The transient nature of the transfection method we describe is somehow desirable due to the short duration of the oxidative stress suffered by hepatocytes after reoxygenation. The injury produced by ischemia followed by reperfusion has been attributed to a burst of ROS in the

first moments of reoxygenation. If the transplanted cells or tissues are able to cope with this sudden increment of ROS, the equilibrium could be restored and the damage mitigated until the situation returns to normal. Due to the short time frame of the oxidative stress, it may be deleterious to maintain the overexpression levels of FNR after that period when it is no longer needed and could even perturb the intracellular redox homeostasis.

The disappearance of FNR-positive cells 7 days after transplantation could be due to the silencing of transgene expression and/or death and/or removal of transfected hepatocytes. We are not in the position to answer this important point. However, the finding of a comparable percentage of FNR-positive cells up to 72 h after cell injection together with a comparable number of CFSE-stained cells till 7 days after transplantation, supports the idea that these cells are not undergoing death or removal. Since previous data showed a shutdown in gene expression 7 days after transfection [27,34], it seems possible that the lack of positive cells we observed after 1 week may be linked to either gene silencing and/or plasmid loss. Clearly, this conclusion needs to be experimentally proven.

The utility of a greater FNR expression still needs to be addressed by a higher number of pKM4-transfected cells and, more important, in a suitable model of hepatic disease to answer the relevant question on the clinical effectiveness of hepatocyte transplantation and its applicability to humans.

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