EXPERIMENTAL MODEL FOR SAFE GENE TRANSFER BY RECOMBINANT GENE CONSTRUCTS

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ABSTRACT

For gene transfer in laboratory-cultivated mouse embryonic stem cells (mESCs), previously designed recombinant gene constructs with respective genes inserted in them are necessary. For this aim, recombinant DNA-genomes from adeno-asssociated virus (AAV) (Parvoviridae), containing promoter of gene, coding Elongation Factor 1-alpha (EF1-α); isolated from 3T3 fibroblasts of adult laboratory mice Balb/c inserted oncogene Dcn1, (in its role of regulator on the tumor-suppressor gene p53 by specific pathways of indirect inhibition), as well as gene for neomycin resistance, isolated from bacterial DNA-plasmid, are used for gene transfaction by electroporation. On the other hand, eventual subsequent super-activation of tumor-suppressor genes both in vitro and in vivo is also necessary.

Keywords: embryonic stem cells (ESCs), immune cell precursors, recombinant gene constructs

Introduction

Studies on the biology of the stem cells are often focused on their self-renewal and differentiation (1). It is important to note that the efficiency of DNA-repair varies greatly among different stem cell types. This high self-renewal potential of the stem cells in in vitro-conditions makes them strong candidates for delivering of genes, as well as for restoring organ systems function have been found to be included in these processes. This understanding could be applied toward the ultimate goal of using stem cells not just for various forms of therapy, but rather as a tool to discover the mechanisms and means to bring, reconstituting them from old and young individuals has exhibited indistinguishable progenitor activities both in vivo and in vitro.

In many tumor tissues and cultured cell lines has also been detected a broad expression of oncogene DCUNID3 (Dcn1), which has been characterized as a regulator of gene p53 (7). Function of this gene has also been found to be sufficient for cullin neddylation in a purified recombinant system, as well as, on the other hand – contribution of its over-expression to malignant disorders, as well as a potential marker for metastatic progression (10).

Links between DNA-replication, chromatin and proteolysis has been confirmed by the newly discovered cullin-RING E3-ubiquitin ligases, assembled on the CUL4 platform (4). In this aspect, a conserved component of CUL4-Dbd1 E3-ligase has been found as essential for the replication factor Cdt1 destruction and thus – for ensure proper cell cycle regulation of the DNA-replication process (9). Cullin-based E3-ligases, have recently been proven as crucial regulators of mitosis. A key role of the enzyme CUL7 E3-ubiquitin ligase in the proteolytic targeting insulin receptor substrate-1, which has been proven as a critical mediator for insulin/IGF1-signalling, has been demonstrated. On the other hand, both positive and negative roles of ubiquitin-mediated proteolysis in the regulation of longevity in the one-cell eukaryotic organism Cenorhabditis elegans by insulin/IGFs-signaling pathways, have been established (4).

As the most important approaches, currently utilizing stem cells, both gene therapy and tissue engineering have been determined (2, 3, 6, 8). Both have been found to exploit the current knowledge in molecular biology and biomaterial science in order to direct MSCs to in vivo-differentiation to desired lineages and tissues. In this aspect,
widely studied is the ability for in vitro-cultivation of viruses in cell cultures, with the aim for development of both viral recombinants for malignant immunotherapy and of products for therapy of these disorders. As such tools can be used both DNA- (3, 6) and RNA-viruses (2), as well as bacterial plasmids (8) and yeasts (5). For this aim, an intact gene tk, coding the enzyme timidinkinase (TK), has been found to be necessary, but, on the other hand – the integration of the searched gene(s) out of tk locus of the virus genome, as well as virus promoter, which could provide the expression of the inserted gene(s). Modifications by changes of the promoter and/or in the insertion site, as well as in the target vector repeats in fragments, expressing proteins with immunomodulator functions, have been proven to be possible. In this way have been inserted genes, coding cell receptors, cytokines, enzymes, complement activators, apoptosis activators and/or inhibitors, surface antigens, tumor markers. Besides the respective inserted gene(s), a marker gene has also been found to be necessary, but both gene types are controlled by appropriate promoter sequences. As a next step has been carried out polymerase chain reaction (PCR) of the received construction, by use of oligonucleotide primers for insurance of respective restriction sites – SfiI-site on the 5′-end and, respectively, RsrlI-restriction site on the 3′-end of the PCR-product, which is obtained as a result of digestion by respective restriction enzymes (bacterial restrictases, which are particularly endonucleases), connected with respective early or late promoter in the virus genome or plasmid DNA.

In this aspect, the main goal is connected with a design of maximally safe experimental model for providing, on the one hand, of active tumor-suppressor gene, necessary for prevention of eventual malignant transformations, and, on the other hand, of active oncogene for prevention of early aging and death, as well as for modulation of the immune response both in vitro and in vivo, mainly by the protein product of the last in its role of active antigen.

Materials and Methods

Used biological material

Feeder cells: as such primary mouse embryonic fibroblasts (MEFs), after their previously treatment by Mitomycin-c (mm-c) for inhibition of their further growth and proliferation, as well as 3T3 cells from Balb/c mice;
Undifferentiated stem cells from embryos of Balb/c mice;
Adult laboratory Balb/c mice;

Recombinant gene constructs: for this aim are used recombinant DNA-genome from adeno-associated virus (AAV) (Parvoviridae) (6), containing promoter for gene, coding Elongation Factor 1-alpha (EF1-a); gene Dcn1, isolated from 3T3 fibroblasts of laboratory mice Balb/c, as well as gene for neomycin resistance, isolated from bacterial DNA-plasmid;
Malignant cells/antigens: cells from the malignant line Hela, isolated from human cervical carcinoma, are used, as well as serum-free filtered cultural fluids after their 24-hour cultivation in it;
Wild type (WT) and partially knock-down mutant (MT) on oncogene Dcn1 experimental adult mice, as well as adult Balb/c mice, male and female animals from all categories are used.

Used chemicals

Feeder media: Dulbecco’s modified Minimal Essential Medium (DMEM) (Sigma-Aldrich) for monolayer cell cultures, is used;
Sera: 20% Fetal Calf Serum (FCS) (Sigma-Aldrich) with previously destroyed own complement protein system by heat at 65°C for 30 minutes; Fetal Calf Serum (FCS) (Sigma-Aldrich) with non-destroyed own complement protein system;
Antibiotics: penicillin (Sigma-Aldrich) - 100 IU/ml; streptomycin (Sigma-Aldrich) - 100 μg/ml; Mitomycin-c (mm-c) (Sigma-Aldrich); synthetic neomycin analogue G-418 (Sigma-Aldrich) – 84 g dry substance in 200 ml liquid feeder medium;
Phosphate Buffered Solution (PBS) (Sigma-Aldrich) (pH 8); Laboratory solution of 0.02% tryspin and 0.05% EDTA (Sigma-Aldrich); Cytokines/growth factors: granulocyte-macrophage – colony-stimulation factor (GM-CSF - Sigma-Aldrich) is used;
Used chemicals for agarose gel electrophorhesis:
- agarose (Sigma-Aldrich);
- DNA-primers (Sigma-Aldrich);
- mixture of the three types desoxy-nucleosid-tri-phosphates (dNTP - Sigma-Aldrich);
- enzyme Taq-polymerase (Sigma-Aldrich);
- lysis buffer for isolation of genomic DNA (Sigma-Aldrich);
- absolute ethanol (Sigma-Aldrich);
- Natrium acetum (Sigma-Aldrich);
- mixture of phenol-chlorophorm-isoamil alcohol (PCI)
- paraphormaldehyde (Sigma-Aldrich), as well as 95% ethanol (Sigma-Aldrich);
- Giemsa dye (Sigma-Aldrich) (solution in PBS in 1:9).

**Used installations:**

- Inverted microscope (Leica);
- Incubator with and 95% air humidification (Heraeus);
- Electroporator for cell transfection (BioRad);
- Machine for Polymerase Chain Reaction (PCR) (BioRad).

Stem cells, isolated from embryos of experimental mice Balb/c, are cultivated for 48 – 72 hours on previously formed monolayers of feeder primary MEFs and/or 3T3 fibroblasts from Balb/c mice, after which they are tripinized and consequently transfected by electroporation (5 X 10⁶ cells/ml). For this aim, recombinant DNA-genome from adeno-associated virus (AAV) (Parvoviridae) (2), containing promoter for gene, coding Elongation Factor 1-alpha (EF1-α); gene Dcn1, isolated from 3T3 fibroblasts of laboratory mice Balb/c, as well as gene for neomycin resistance, isolated from bacterial DNA-plasmid, are used. On 8th – 10th day after their transfection and seeding of the so transfected stem cells on previously formed monolayers of feeder cells, a selection of cell clones, resistant to neomycin is made by their cultivation in medium, containing diluted substance G-418, known as a synthetic analogue of neomycin.

All cells are incubated at 37°C in incubator with 5% CO₂ and 95% air humidification, in Dulbecco’s Modified Minimal Essential Medium (DMEM) (Sigma-Aldrich), supplemented with 10% Fetal Calf Serum (FCS) (Sigma-Aldrich), 100 U/ml penicillin (Sigma-Aldrich) and 100 µg/ml streptomycin (Sigma-Aldrich). The so cultivated cells are observed on every 24 hours by inverted light microscope (Leica), on magnification 10 X 0.10 and/or 10 X 0.25, respectively. The cultural fluids are changed in 48 hour intervals. Malignant cells from line Hela are cultivated and supported in analogical conditions.

The genomic assay of the so derived cell clones is made by PCR and consequent 1% gel electrophorhesis with primers against the inserted DNA-fragment. The results are compared with the data from PCR and 1% gel electrophorhesis of the recombinant gene construct with the same primers against it. Despite of the inactivated gene expression in the mature normal cells, the presence of the tested gene in them is confirmed by electrophorhesis against the cell genome in the same conditions of genomic DNA from epithelial cells, isolated from skin of Balb/c, as well as both wild type (WT) and partially knock-down mutant (MT) on oncogene Dcn1 experimental adult mice.

**Results and Discussion**

In the experiments for transfection of in vitro-cultivated mouse embryonic stem cells (mESCs) with recombinant vector gene constructs, 9 transfected by electroporation cell clones are received and derived, 2 of which are positive on the additionally inserted copy of the oncogene Dcn1 and the other 7 cell clones of them - negative on it (Fig. 1).

![Fig. 1. Agarose gel electrophorhesis for prove of the presence and/or the absence of additionally-inserted copyof the oncogene Dcn1 in clones, derived from transfected by electroporation in vitro-cultivated mESCs](image)

These results are confirmed by the data, obtained from PCR and subsequent electrophoresis of the used recombinant vector constructs in the same conditions (Fig. 2), as well as of genomic DNA, isolated from mature epithelial cells from skin of adult Balb/c experimental mice, by use of primers against the bacterial DNA-genome (Fig. 2) and against the cell genomic DNA (Fig. 3), respectively. These data are confirmed by the results, obtained in use of epithelial cells from wild type (WT) adult experimental mice (Fig. 4 and Fig. 5).
Fig. 2. Agarose gel electrophoresis for prove of the presence and/or absence of additionally-inserted copy of the oncogene Dcn1 in the recombinant gene constructs, used for cell transfection.

On the other hand, according to the results from PCR and subsequent agarose gel electrophoresis against the cell DNA-genome of epithelial cells from wild type (WT) and partially knock-down mutant (MT) on oncogene Dcn1 adult experimental mice, respectively, signs of more active expression of tumor-suppressor gene HACE1 in cells from mutant experimental animals could be noticed (Fig. 5).

Despite of the fact that its expression is inhibited in the mature normal cells, the results obtained support its presence in their genomes (Figs. 3, 4, 5). The observed increased risk for spontaneous cancerogenesis in experimental mice with inactivation of tumor-suppression gene(s) HACE1 and/or p53 has confirmed the importance of coordinate oncogenes and tumor-suppressor genes action in the regulation and prevention of malignant transformation, which could explain the observed increased activity of gene HACE1 from cell genomes of the partial knock-down on the oncogene Dcn1 mutant experimental rodents (Fig. 5). Similar type of correlation of gene p53 has recently been proven with gene NUMB (7).
Fig. 5. Agarose gel electrophoresis of nuclear DNA-material from epithelial cells of normal wild type (WT) on oncogene Dcn1 and mutant (MT) partially knock-down on it adult experimental mice, respectively. Signs of more active expression of tumor-suppressor gene HACE1 in cells from partially knock-down on oncogene Dcn1 mutant experimental mice, could be noticed

Conclusions
The main conclusions, which could be made from the carried out investigations are:

a) Nine transfected by electroporation cell clones are received and derived, from which 2 are positive on the additionally-inserted copy of the oncogene Dcn1 and the other 7 cell clones of them - negative on it, according to the results from genomic assays;

b) Despite of the inactivation of the oncogene Dcn1 in the process of cell differentiation, the results from our experiments confirm its presence in the genome of non-transfected differentiated epithelial cells from adult individuals.

REFERENCES