African Journal of Pharmacy and Pharmacology Vol. 5(6), pp. 786-791, June 2011
Available online http://www.academicjournals.org/ajpp
DOI: 10.5897/AJPP11.222
ISSN 1996-0816 ©2011 Academic Journals

Full Length Research Paper

Experimental model for safe *in vitro*- and *in vivo*- influence of internal and external factors of cell differentiation

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Accepted 23 May, 2011

Gene transfer in laboratory-cultivated mouse embryonic stem cells (mESCs) was made by appropriate recombinant DNA-constructs. Electrophoretic profiles of genetic material from wild type on oncogene Dcn1 and "knock-down" on it inbred experimental mice differed not only in it, but also in tumor-suppressor gene HACE1 between both categories of laboratory rodents. The results obtained were compared with previous data, received from malignant rat insulinoma RIN-5F cells, transfected by recombinant gene constructs with inserted copy of "secretagogin" gene, by their *in vitro*-co-cultivation with malignant cell precursors, derived from populations of non-transfected laboratory-cultivated mESCs in the presence of Doxyciclin, probably by activation of tumor-suppressor genes of STAT-family. Furthermore, the so induced "secretagogin" over-expression could exert protective function on the transfected Rin-5F cells, which was confirmed by noticed differences in the degree of myeloid differentiation of derived precursor cells in their *in vitro*-co-cultivation with containing additional copy of "secretagogin" gene Rin-5F malignant rat insulinoma cells, in comparison with the results, obtained in their co-cultivation with human cervical carcinoma Hela cells in our laboratory. On the other hand, the derived normal cells with inserted additional copy of oncogene indicated good safety and immunogenity.

Key words: Oncogenes, tumor-suppressor genes, myeloid cell precursors, recombinant gene constructs, cell transfection.

INTRODUCTION

Studies on the biology of the stem cells are often focused on their self-renewal and differentiation (Amit et al., 2000; Coulombel, 2005; Cumano et al., 1992; Keller, 1995; Liang and Van Zant, 2003; Molofsky et al., 2004; Rubin, 1997; Smith, 2001; Vaziri and Benchimol, 1998; Vogelstein and Kinzler, 2004). It is important to note that the efficiency of DNA-repair varies greatly among different stem cell types (Liang and Van Zant, 2003). 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 (Liang and Van Zant, 2003; Rubin, 1997; Vaziri and Benchimol, 1998; Vogelstein and Kinzler, 2004). 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* (Smith, 2001; Vaziri and Benchimol, 1998). The properties of "malignant stem cells", have outlined initial therapeutic strategies against them (Smith and Boulanger, 2002; Vogelstein and Kinzler, 2004). In many tumor tissues, and cultivated cell

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lines has also been detected a broad expression of oncogene DCUN1D3 (Dcn1), which has been characterized as a regulator of gene p53 (Colaluca et al., 2008; Kurz et al., 2008; Ma et al., 2008; O-charoenrat et al., 2008). 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 (Bowerman, 2007; Colaluca et al., 2008; Eferl et al., 2003; Gartner et al., 2007; Zhang et al., 2007). Links between DNA-replication, chromatin and proteolysis has been confirmed by the newly discovered cullin-RING E3-ubiquitin ligases, assembled on the CUL4 platform (Jin et al., 2006).

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 ensuring proper cell cycle regulation of the DNA-replication process. Cullin-based E3-ligases have recently been proven as crucial regulators of mitosis (Colaluca et al., 2008; Kurz et al., 2008; O-charoenrat et al., 2008). 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 (Jin et al., 2006). On the other hand, both positive and negative roles of ubiquitin-mediated proteolysis in the regulation of longevity in the eukaryotic organism *Cenorhabditis elegans* by insulin/IGFs–signaling pathways have been established (Bowerman, 2007). As the most important approaches, currently utilizing stem cells, both gene therapy and tissue engineering have been determined (Barrette et al., 2000; Borysiewicz et al., 1996; Brachmann et al., 1998; Chen et al., 2003; Domi and Moss, 2005). 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 for DNA- and RNA-viruses (Barrette et al., 2000; Borysiewicz et al., 1996), as well as bacterial plasmids and yeasts (Chen et al., 2003; Domi and Moss, 2005). 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, RsrII-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 was connected with a design of maximally safe experimental model for providing, on the one hand, of active tumor-suppressor gene for prevention of eventual malignant transformations, and on the other hand, of active oncogene for prevention of early aging and death.

MATERIALS AND METHODS

Stem cells, isolated from mouse Balb/c embryos, were cultivated for 48 to 72 h on previously formed monolayers of feeder primary MEFs after their previously treatment by Mitomycin-c (mm-c) and/or 3T3 fibroblasts, after which they are tripsinized and consequently transfected by electroporation (5×10^6 cells/ml). For this aim, recombinant DNA-genome from adeno-associated virus (AAV) (Parvoviridae) (Chen et al., 2003), 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, were used. On 8 to 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 was made by their cultivation in medium, containing diluted substance G-418, known as a synthetic analogue of neomycin. Genomic assay of the so derived cell clones was made by PCR and consequent 1% gel electrophorhesis with primers against the inserted DNA-fragment. The results were compared with the data from PCR and 1% gel electrophorhesis of the recombinant gene construct with the same primers against it. Separate sub-populations of non-transfected mESCs were cultivated in the presence of 2 µg/ml Doxycycline (Sigma-Aldrich) for suppression of cell proliferation and eventual stimulation of myeloid cell differentiation by activation of genes from STAT-family.

For differentiation in myeloid and lymphoid in precursors, population of non-transfected mESCs were also cultivated in medium, containing GM-CSF and complement proteins, respectively, as well as malignant antigens. All cells were incubated at 37°C in incubator with 5% CO_2 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). Despite of the inactivated gene expression in the mature normal cells, the presence of the tested gene in them was confirmed by electrophorhesis against the cell genome in the same conditions of genomic DNA from epithelial cells, isolated from skin of adult experimental mice. At the same time, malignant cells from rat insulinoma cell line RIN-5F, containing additional copy of the "secretagogin" gene, inserted by their transfection with recombinant gene construct pGEX-1λT (Amersham Pharmacia Biotech) of *Escherichia coli,* were also

Figure 1. Native light-microscopy preparations from transfected mESCs, negative (a) and positive on additionally-inserted copy of the oncogene Dcn1, respectively (b).

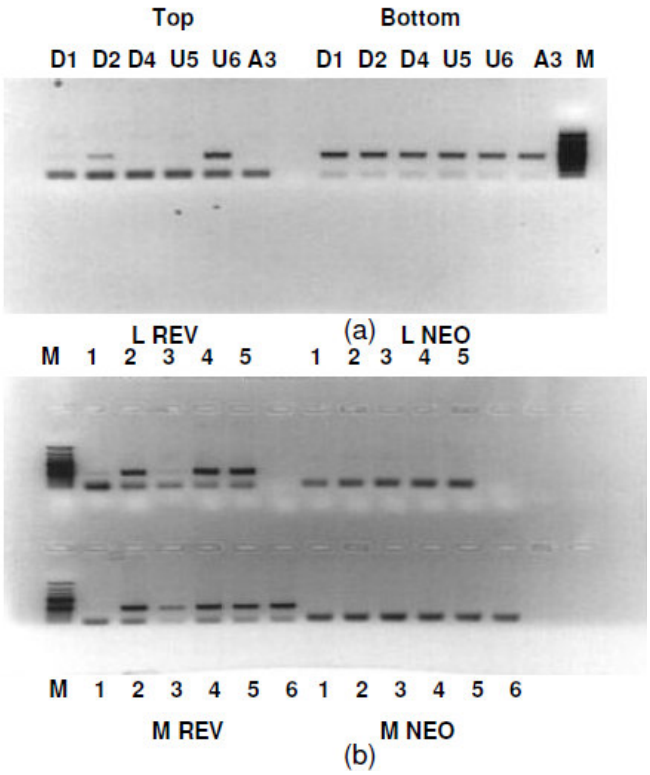

Figure 2. Agarose gel electrophorhesis for prove of the presence and/or the absence of additionally-inserted copy of the oncogene Dcn1 in cell clones, derived from transfected electroporation *in vitro*-cultivated mESCs (a) and in use for cell transfection recombinant gene constructs, respectively (b).

cultivated and supported in analogical conditions.

RESULTS

In our experiments 9 transfected by electroporation cell clones were received and derived (Figures 1a and b). According to the genomic assays results, 2 of them were

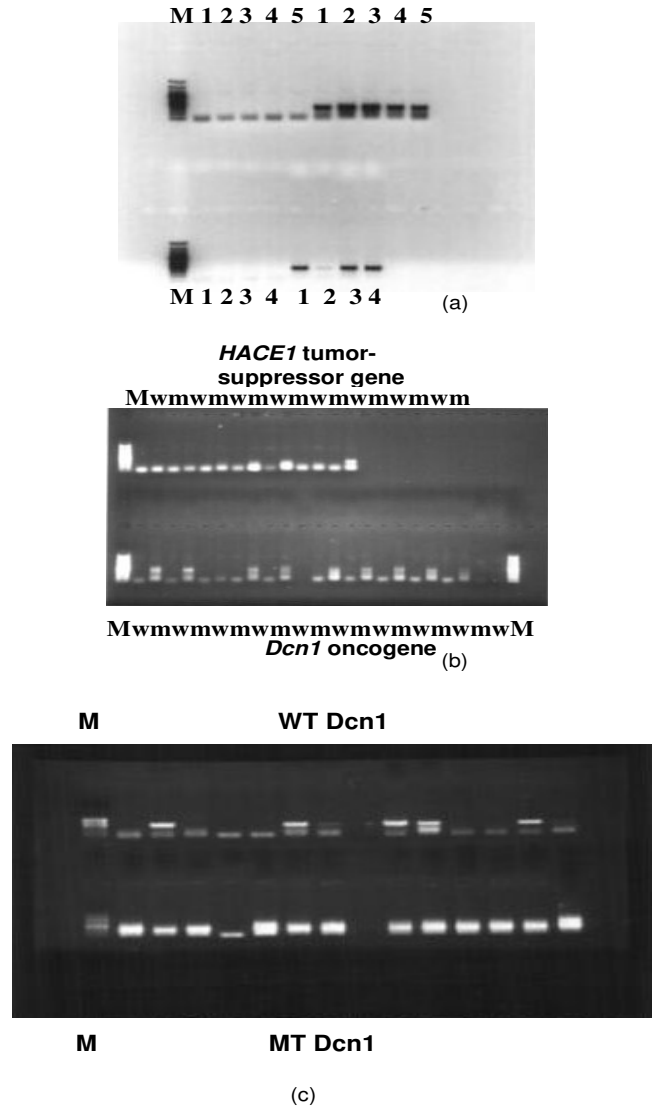

Figure 3. Agarose gel electrophorhesis for prove of the normal presence of the oncogene Dcn1 in mature epithelial cells, isolated from tail skin of adult experimental mice Balb/c with its normal expression (a), as well as from wild type (WT) and partially knock-down mutant (MT) on the same oncogene aboratory mice (b, c). Differences in the electrophoretic profiles both of the oncogene Dcn1 and the opposite tumor-suppressor gene HACE1 could also be noted.

positive on the additionally inserted copy of the oncogene Dcn1 and the other 7 cell clones - negative on it (Figures 2a and b). These results were confirmed by many well known literature data, obtained from PCR and subsequent electrophoresis of the used recombinant vector constructs in the same conditions (Figure 2b), as well as of genomic DNA, isolated from mature epithelial cells from skin of adult Balb/c experimental mice (Figure 3a), and from normal wild type (WT) on the oncogene Dcn1 and partially knock-down on it mutant (MT) adult laboratory rodents (Figures 3b and c). Decreased cell proliferation level on the one hand and active myeloid

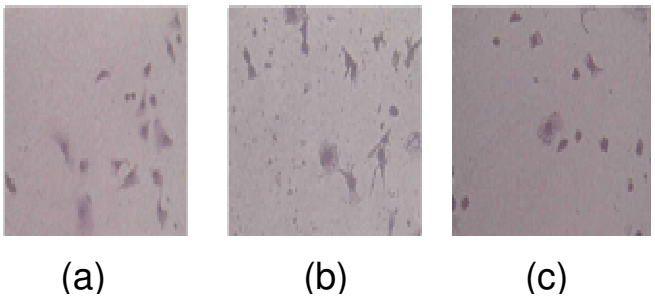

Figure 4. Decreased level of cell *in vitro*-proliferation and activated *in vitro*-differentiation, in particular in myeloid precursor cells, by activation of the tumor-suppressor genes from STAT-family by cultivation in the presence of Doxycycline (2 µg/ml - Sigma-Aldrich): *In vitro*-differentiation of non-transfected mESCs in the presence of complement components and "doxycycline", but in the absence of malignant cells. Immune progenitor cells in different phases of immune differentiation, in particular, in different types myeloid precursors, are seen, most of which contain different types of granules in their cytoplasm (a); *In vitro*-differentiation in the presence of complement components, "doxycycline" and malignant cells Hela. Two large cells with oval form, large excentrically-located dark nuclei, as well as small cytoplasm amount with basophilic and eosinophilic granules, are seen (b); *In vitro*-differentiation in the presence of complement components, Doxycycline and malignant cells RIN-5F, containing gene for "secretagogin". A large cell with located as light nuclei and large cytoplasm amount with eosinophilic and basophilic granules, as well as a smaller cell with excentrically-located as dark nuclei and cytoplasm with neutrophilic granules, are seen (c).

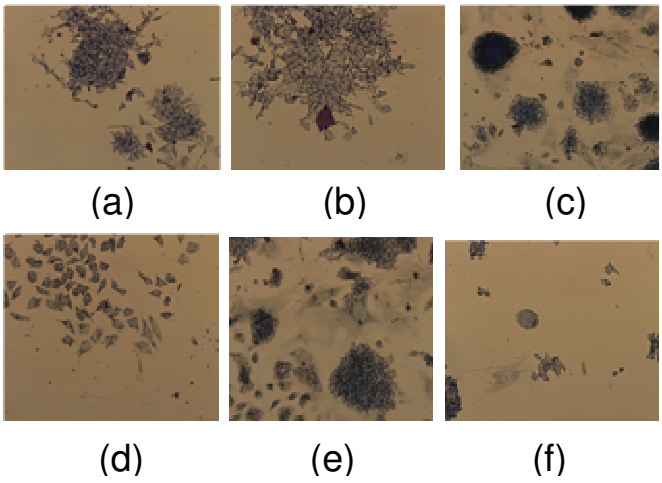

Figure 5. Early stages of myeloid *in vitro*-differentiation of non-transfected mESCs in different conditions: in the absence of differentiation factors and transfected cells (a); in the presence of GM-CSF, but in the absence of Hela-antigens and transfected cells (b); in the presence of Hela-antigens and in the absence of GM-CSF and transfected cells (c); in the presence of both GM-CSF and Hela-antigens, but in the absence of transfected cells (d); in the presence of GM-CSF, Hela-antigens and transfected cells, negative by additionally-inserted copy of oncogene Dcn1 (e); in the presence of GM-CSF, Hela-antigens and transfected cells, positive on additionally-inserted copy of oncogene Dcn1 (f).

differentiation on the other was established in cultivation of cell sub-populations in the presence of 2 µg/ml Doxycycline (Sigma-Aldrich) (Figure 4). These results could be confirmed by the observed signs of early myeloid and lymphoid differentiation in the presence of respective external factors (Figures 5 and 6). According to the results, the tendency for *in vitro*-differentiation in both myeloid and lymphoid precursors is stronger in the presence of transfected mESCs, containing additional copy of the oncogene Dcn1. Hence, the obtained data have also suggested that the derived cells are safe enough, both and on the other hand they have good immunogenic potential.

The results obtained were compared with data, received from malignant rat insulinoma RIN-5F cells, containing additional copy of the "secretagogin" gene, inserted by their transfection with recombinant gene construct pGEX-1λT (Amersham Pharmacia Biotech) of *E. coli*, where a decreased malignant potential of the transfected cells as a result of eventual induced "secretagogin" over-expression, was supposed. As a proof about that, it could be accepted the observed effects of early myeloid differentiation and suppression on the cell proliferation in the presence of 2 µg/ml Doxycycline (Sigma-Aldrich) could be explained with its activation effect on the tumor-suppressor genes of STAT-family.

DISCUSSION

Despite the fact that its expression is inhibited in the mature normal cells, the results obtained have supported its presence in their genomes (Figures 2 and 3). Taking in consideration literature data about the importance of coordinated oncogenes and tumor-suppressor genes action in the regulation and prevention of malignant transformation (Wood et al., 2000), in both WT and knock-down MT on oncogene Dcn1 in experimental mice, respective electrophoretic profiles of this gene, as well as of tumor-suppressor gene HACE1 were made and compared, and the results obtained have indicated certain differences in both genes between the separated categories of laboratory rodents (Figure 3). These data have provided an evidence for the existence of common cell progenitors in the adult individuals. Despite the results of our experiments, did not entirely reveal the link between oncogene Dcn1 and tumor-suppressor gene HACE1, according to other literature findings, genetic interactions between oncogene and tumor-suppressor genes (Bellosta et al., 2005; Etard et al., 2005), as well as influence of the protein product on the one or two genes on the structure and functions of the other of both genes (Bauer et al., 2000; Vogelstein and Kinzler, 2004), have been indicated as possible. Similar type of correlation of gene p53 has recently been proven with gene NUMB, which has been characterized as a cell fate determinant because of its role in the asymmetric cell

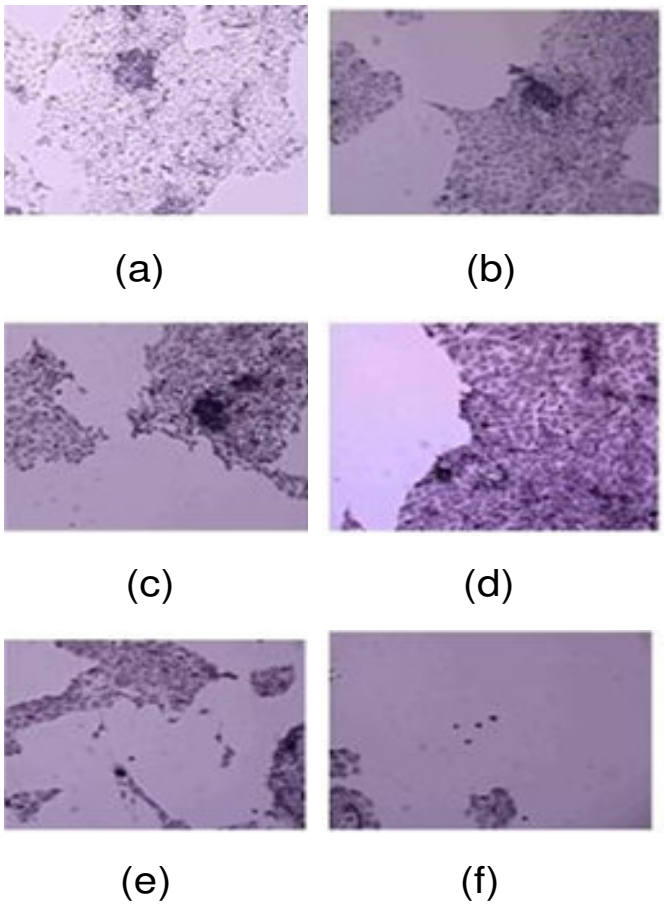

Figure 6. Early stages of lymphoid *in vitro*-differentiation of mESCs indifferent conditions: in the absence of differentiation factors and transfected cells (a); in the presence of complement components and absence of Hela-antigens and transfected cells (b); in the presence of Hela-antigens, but in the absence of complement proteins and transfected cells (c); in the presence of both complement components and Hela-antigens, but in the absence of transfected cells (d); in the presence of complement proteins, Hela-antigens and transfected cells, negative by additionally-inserted copy of oncogene *Dcn1* (e); in the presence of complement proteins, Hela-antigens and transfected cells, positive on additionally-inserted copy of oncogene *Dcn1* (f).

division in the mitosis process, as well as between gene Oct4, which is known as regulator of the processes of stem cell self-renewal and differentiation and gene variation Cdk2ap1, by a mechanism of Oct2/4 promoter methylation.

On the other hand, a rapid lymphoid-restricted (T, B and NK) reconstitution capacity *in vivo* and completely lacked myeloid differentiation potential of both *in vivo* and/or *in vitro* has been reported in stem cells from bone marrow material of adult laboratory mice (Kobari et al., 2000). The observed effects of early myeloid differentiation and suppression on the cell proliferation in the presence of 2 µg/ml doxycycline (Sigma-Aldrich) could be explained with the description in many literature sources activation effect of this substance on the tumor-suppressor genes of STAT-family (Figure 4) (Fitzgerald et al., 2005, 2008, 2009; Kyba et al., 2003; Poehlmann et al., 2005; Suman et al., 2009).

Conclusion

The observed cytoplasmic excrescences and cell-cell-contacts in co-cultivation with malignant cells are well known signs about phagocyte cell differentiation. In this connection, the observed highest degree of the formed structures in the presence of non-transfected malignant Hela cells could be accepted as a proof for eventual decrease of the oncogene potential in malignant cells, containing additional copy of tumor-suppressor gene, in *in vitro*-conditions. The absence of the aforementioned features in the process of myeloid differentiation in the presence of the received positive on additional copy of the oncogene Dcn1 normal transfected cells could be accepted as a proof for the safety of these so derived transfected cells, which have preserved their non-tumorigenic/normal cell characteristics *in vitro*.

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