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Full Length Research Paper

IN VITRO- and *IN VIVO*- experimental models for balanced activity of oncogenes and tumor-suppressor genes in normal and malignant cells

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Gene transfer in laboratory-cultivated mouse embryonic stem cells (mESCs) was made by appropriate recombinant DNA-constructs. Electrophorhetic profiles of genetic material from wild type (WT) on oncogene *DCN1* and "knock-down" (KD) on it inbred lines of experimental mice differed not only on it, but also on the 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. These data were confirmed by the differences noticed 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 laboratory co-cultivation with non-treated human cervical carcinoma Hela cells, as well as with derived normal mESCs, containing additional copy of the oncogene *DCN1* as a result of their transfection with recombinant DNA-constructs. On the other hand, the derived normal cells with inserted additional copy of oncogene indicated safety, immunogenity, and they also indicated preserved normal cell characteristics.

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, 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 vitro* makes them strong candidates for delivering of genes, as well as for restoration of organ and systems functions, has been found to be included in these processes (Liang and Van Zant, 2003; Vaziri and Benchimol, 1998; Vogelstein and Kinzler, 2004). Taking in consideration of this understanding, stem cells could be applied not just for various forms of therapy, but also 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). On the other hand, the properties of "malignant stem cells" have outlined initial therapeutic strategies against them (Smith

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and Boulanger, 2002; Vogelstein and Kinzler, 2004). In many tumor tissues and cultivated cell lines, a broad expression of oncogene *DCUN1D3* (*Dcn1*) has been detected (Colaluca et al., 2008; Ma et al., 2008; O-charoenrat et al., 2008). Functions of this gene have also been found to be sufficient for cullin neddylation in a purified recombinant system, as well as a contribution of its over-expression to malignant disorders, as a potential marker for metastases progression (Bowerman, 2007; Colaluca et al., 2008; Eferl et al., 2003; Gartner et al., 2007; O-charoenrat et al., 2008). 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 – to ensure proper cell cycle regulation of the DNA-replication process. 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 (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, utilizing stem cells, 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 stem cell 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, 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- and RNA-viruses (Barrette et al., 2000; Borysiewicz et al., 1996), as well as bacterial plasmids and yeasts (Brachmann et al., 1998; 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 – 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). In this way, genes, coding cell receptors, cytokines, enzymes, complement activators, apoptosis activators and/or inhibitors, surface antigens, tumor markers, have been proven to be inserted as well. 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 supplying 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, which were isolated from mouse Balb/c embryos, were cultivated for 48 to 72 h on previously formed monolayers of feeder primary MEFs after their previous treatment by Mitomycin-c (mm-c) (Sigma-Aldrich) and/or 3T3 fibroblasts. After tripsinization, they were 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. For this goal, electroporator for cell transfection (BioRad) were used. Separate sub-populations of non-transfected mESCs were cultivated in the presence of 2 µg/ml Doxicyclin (Sigma-Aldrich) for suppression of cell proliferation and eventual stimulation of myeloid cell differentiation by activation of genes from *STAT*-family. 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 bacteria *Escherichia coli* strains, were also cultivated and supported in analogical conditions. On the other hand, cell cultures of the derived from human cervical carcinoma cell line Hela were also prepared. 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), and observed by inverted light microscope (Leica), with addition of leukemia inhibition factor (LIF) in mESCs cultivation and propagation.

After tripsinization of the transfected cells and their consequent treatment with mixture of phenol-chlorophorm-isoamil alcohol (PCI) (Sigma-Aldrich), the so isolated nuclear material was treated with lysis buffer (Sigma-Aldrich) for isolation of genomic DNA. The last was subjected on polymerase chain reaction (PCR) of their previously isolated nuclear DNA and its consequent 1% agarose gel (Sigma-Aldrich) electrophorhesis, in the presence of DNA-primers against the inserted DNA-fragment (Sigma-Aldrich), mixture of the four types deoxy-nucleosid-tri-phosphates (dNTP - Sigma-Aldrich), enzyme Taq-polymerase (Sigma-Aldrich). For differentiation in myeloid and lymphoid precursors, populations of non-transfected mESCs were also cultivated in medium, containing GM-CSF (Sigma-Aldrich) and complement proteins, respectively, by addition of 10% non-inactivated FCS (Sigma-Aldrich) in the last case. Consequently, to the cell sub-populations of both populations of non-transfected cells were added malignant antigens. The last were derived by cultivation of Hela cells in serum-free DMEM

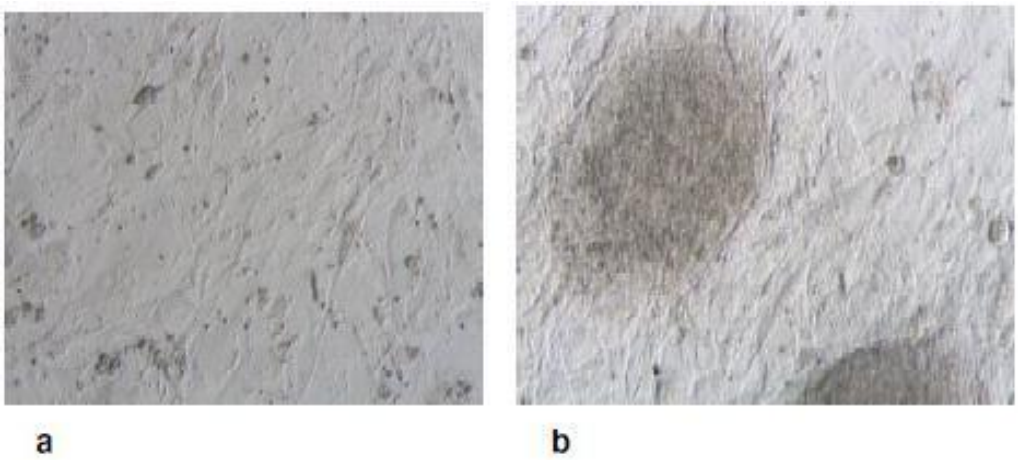

Figure 1. Native light-microscopy preparations from transfected mESCs, (a) negative, and (b) positive on additionally-inserted copy of the oncogene *Dcn1*, respectively.

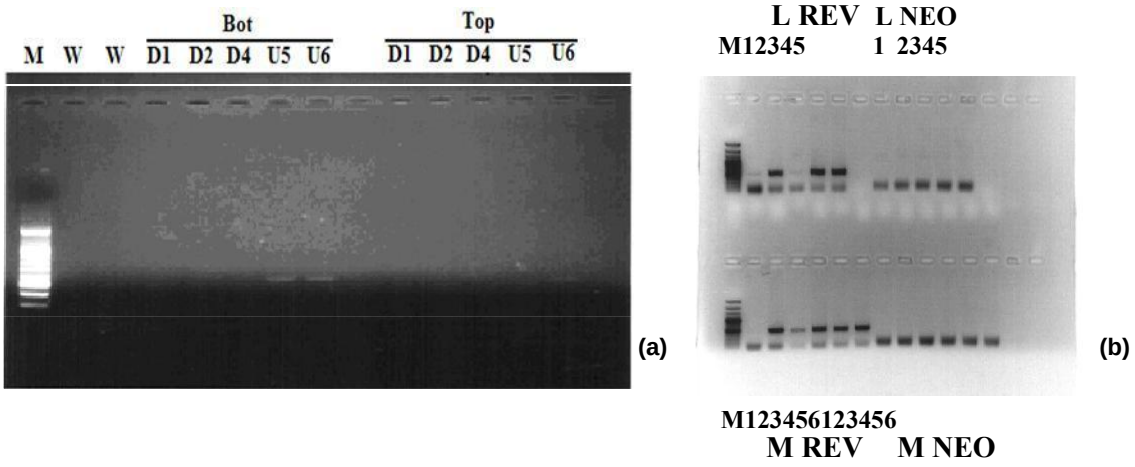

Figure 2. Agarose gel electrophorhesis for prove of the presence and/or the absence of additionally-inserted copy of the oncogene *Dcn1* in cell clones, derived from and transfected by (a) electroporation *in vitro*-cultivated mESCs, and (b) in the use for cell transfection recombinant gene constructs,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

(Sigma-Aldrich) for 24 h, its consequent centrifugation and filtration. Fixed light microscopic preparations were prepared by their consequent fixation by treatment with 95% ethanol (Sigma-Aldrich) or paraphormaldehyde (Sigma-Aldrich), washing with 1:9 diluted PBS (Sigma-Aldrich) and Giemsa-staining (Sigma-Aldrich).

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 positive on the additionally inserted copy of the oncogene *Dcn1* and the other 7 cell clones - negative on it (Figures 2a and b). Decreased cell proliferation level on the one hand and active myeloid differentiation on the other was established in cultivation of cell sub-populations in the presence of 2 µg/ml doxicyclin (Sigma-Aldrich).

According 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*.

As a proof about what could be accepted the observed effects of early myeloid differentiation and suppression on the cell proliferation in the presence of doxicyclin could be explained with its activation effect on the tumor-suppressor genes of *STAT*-family. The data obtained also slightly differed from the results, obtained in their laboratory co-cultivation with human cervical carcinoma non-transfected Hela cells in the same conditions, as well as in the absence of malignant cells, mainly in the number of formed cytoplasmic excrescences and

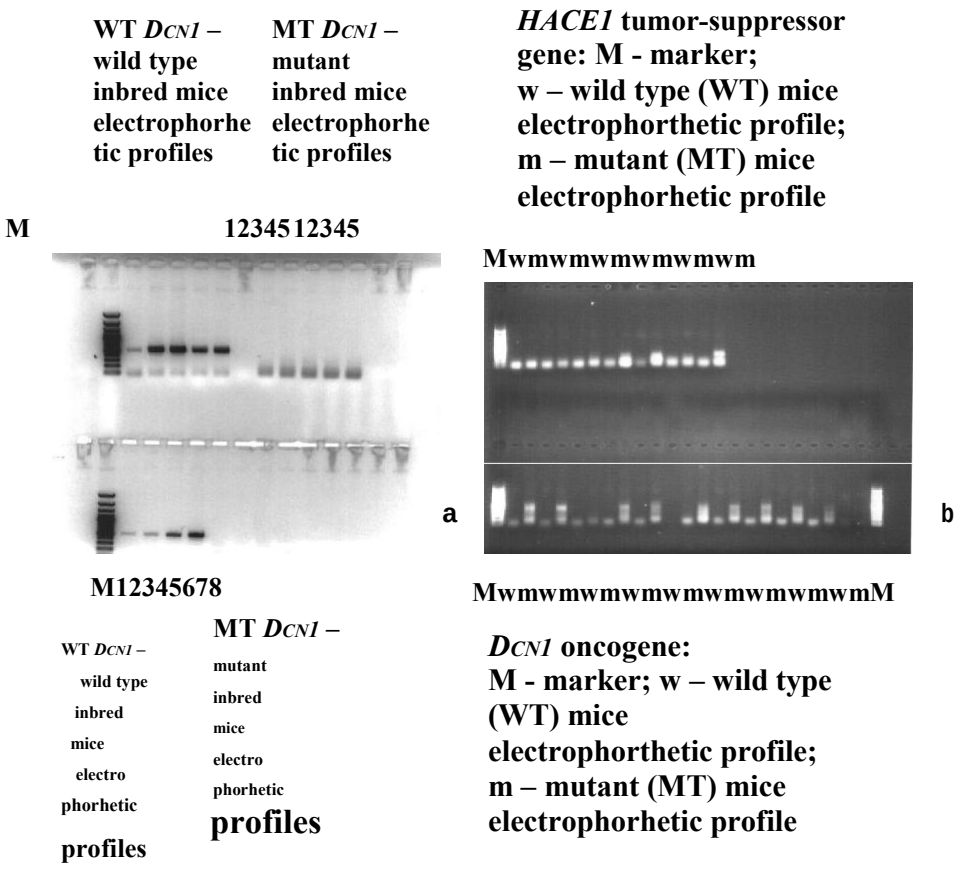

Figure 3. Agarose gel electrophorhesis for prove of the normal presence of the oncogene *Dcn1* in mature epithelial cells, isolated from (a) tail skin of adult experimental mice Balb/c with its normal expression, as well as from (b) wild type (WT) and partially knock-down mutant (MT) on the same oncogene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

contacts between cells by the so formed structures.

DISCUSSION

Despite of the fact that the expression of oncogene *Dcn1* is inhibited in the mature normal cells, the results obtained have supported the presence of this gene in their genomes (Figures 2 and 3). These results were confirmed by previously published 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 (Figure 3b).

Taking in consideration the 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; Zhang et al., 2007), in both WT and knock-down MT on oncogene *Dcn1* in inbred experimental mice lines, respective electrophorhetic 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).

Despite the results of our experiments did not entirely reveal the link between both genes, according to different 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), could be possible. Similar types of correlations 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 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

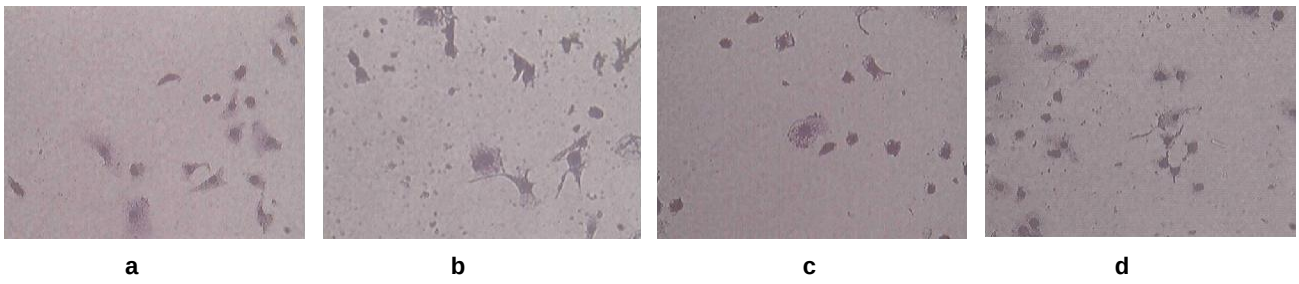

Figure 4. Decreased levels of cell *in vitro*-proliferation and activated *in vitro*-differentiation, in particular in myeloid precursor cells, by activation of the tumor-suppressor genes from *STAT*-family by cultivation in the presence of doxicyclin (2 µg/ml - Sigma-Aldrich). Immune progenitor cells in different phases of immune differentiation, in particular, in different types myeloid precursors, most of which contain different types of granules in their cytoplasm, small cytoplasm amount with basophilic and/or eosinophilic granules, could be seen: (a) *In vitro*-differentiation of non-transfected mESCs in the presence doxicyclin, but in the absence of malignant cells; (b) *In vitro*-differentiation in the presence of doxicyclin and malignant cells Hela. A lot of cytoplasmic excrescences and cell-cell contacts are seen; (c) *In vitro*-differentiation in the presence of doxicyclin and malignant cells RIN-5F, containing tumor-suppressor gene for secretagogin; (d) *In vitro*-differentiation in the presence of doxicyclin and normal transfected mESCs, positive on additional copy of the oncogene *Dcn1*.

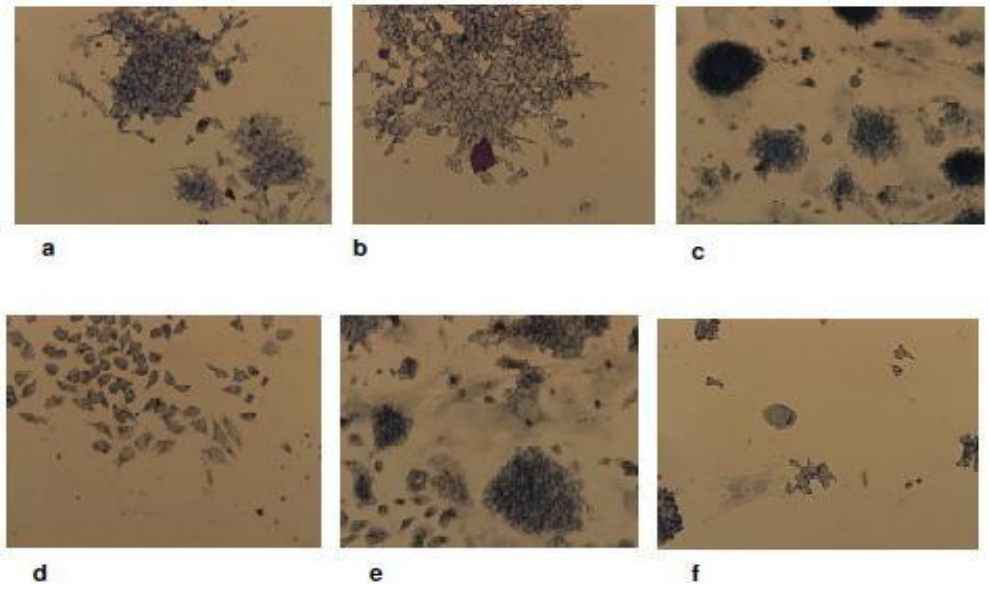

Figure 5. Early stages of myeloid *in vitro*-differentiation of non-transfected mESCs in different conditions: (a) in the absence of differentiation factors and transfected cells; (b) in the presence of GM-CSF, but in the absence of Hela-antigens and transfected cells; (c) in the presence of Hela-antigens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

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*, as well as completely lacked myeloid differentiation potential 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 doxicyclin could be explained with the described 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). The results obtained could also 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 other literature findings, a calcium-dependent SCGN–TAU

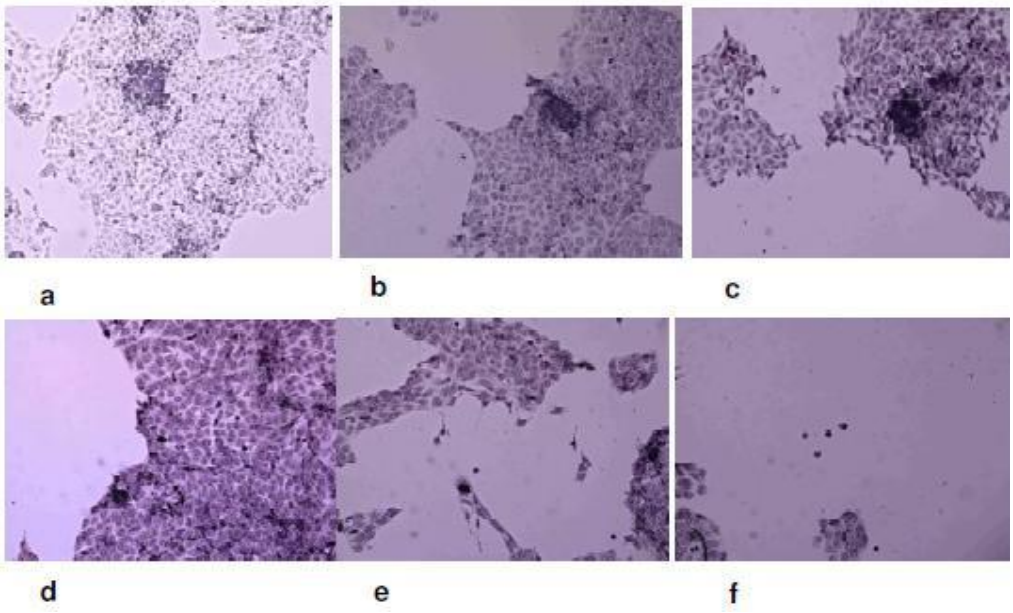

Figure 6. Early stages of lymphoid *in vitro*-differentiation of mESCs indifferent conditions in the: (a) absence of differentiation factors and transfected cells; (b)presence of complement components and absence of Hela-antigens and transfected cells; (c) presence of Hela-antigens, but in the absence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

TTTCTGTGATCTTGCTGGTAGAATTGTATCTGTGCATTGATGTTGGGAACACAGTGGGCAAACTCACAAATGGT
GTGCTATTCTTGGGCAAGAGGGCAAGAGGGACGCTAGGGCCTTCCTTCCACCGGCGTGATCTATCCCTGTCTC
ACTGAAAGCCCCTGTGTTGTTTTCCCTTGACCCTGGGCTTTCCTATCCTCCCAAAGACTCAGCTCCCCTGTTAG
ATGGCTCTGCCTGTCCTTCCCCAGTCCACCAGGGTGGGGGGGACAGGGGCAGCTGAGTGCATTCATTTTGTG
CTTTTGTTGTGGGCTTTCTGCTTAGTCTGAAAGGTGTGTGTGGCATTCATGGCAATCCTGTAACTTCAACATAG
ATTTTTTTTGTGTGTGTGGAAATAAATCTGCAATTGGAAACAACCG

Figure 7. cDNA sequence of the human calcium-binding protein termed secretagogin.

GCGGACGCGTGGGGCCTGTGTTGGGATCAGCCTGAAGATGGTGGTTTA GGTGATCAGGTCAGTTGCTGGCAG
GGAGTCTGCAAAGGGATGTTGGCTTGTAAGCTTAAATGAGGACAATCCCCCCAGAGCTGGCACACC
CCCACCCGTTTCCTCCAGAATCGGTGTCCCTCATCCACCTGGTCCCCTCAAGCTCTCCACTCACTTTATCAATA
ATTCCATTTAAATTGACTTCAGTGGTGAGACCGTATCCTGTTTGCAATTGCTTGTTGTGTTCAAGGGGGGAGGG
GGGAGGAACCTGTAAGATAGTTAAACCACTGGGCAA

Figure 8. cDNA sequence of the human microtubule-associated protein TAU.

interaction, as well as co-appearance of both proteins are shown (Gartner et al., 2007; Maj et al., 2010; Wagner, 2000), despite the fact that two different genes code them (Figures 7 and 8). The noticed cytoplasmic excrescences and cell-cell-contacts in co-cultivation with malignant cells are known as signs of phagocyte cell differentiation. In this connection, the observed highest degree of the formed structures in the presence of non-transfected malignant Hela cells with no induced tumor-suppressor gene over-expression, 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 and immunogenity of these so derived transfected cells,

which have preserved their non-tumorigenic/normal cell characteristics *in vitro*. In this way, the obtained data have also suggested that the so 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 bacterial *E. coli* strains, where a decreased malignant potential of the transfected cells as a result of eventual induced secretagogin over-expression, was supposed.

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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