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ACTIVATION OF MECHANISMS, MEDIATED BY DCN1-HACE1-, JAK-STAT- AND NF-KB- CASCADE REGULATORY PATHWAYS IN *IN VITRO*- AND *IN VIVO*- EXPERIMENTAL MODELS

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ABSTRACT

Mechanisms for activation of normal cell differentiation, with suppression of malignant cell transformation, at the same time, by development of ways for safe application, were searched and studied. In this connection, role of components from Dcn1-HACE1-, JAK-STAT-, and NF-κB-mediated cascade regulatory pathways in these processes, was particularly investigated. Additional oncogene copy was inserted into normal mouse ESCs, for eventual elongation of life, it was important preserved normal/non-malignant characteristics to be tested and proved. On the other hand, additional tumor-suppressor gene copy was inserted into malignant cells, for eventual decrease of their malignant potential. After propagation of fibroblasts from 3T3 cell line, derived from Balb/c mouse embryos, one part of them was pre-cultivated in cultural fluid, in which mouse malignant myeloma cells were previously cultivated, for a period of 2-3 days. After freezing of separate sub-populations from the so incubated cells for 2-4 weeks in the presence of cryo-protector Dymethylsulfoxide (DMSO), subsequent thawing and re-incubation, osteoclast-like characteristics were assessed. When the cultural fluid from the so-derived osteoclast-like cells was added to *de novo*-received confluent monolayer of 3T3 cells, signs of osteoblast lineage differentiation were noted. In co-cultivation of the so derived from mouse embryonic progenitors osteoblast-like and osteoclast-like cells, zones of destroyed osteoblast-like cell monolayer could be noted.

Keywords Cell differentiation direction, Cell progenitors, Cascade regulatory mechanisms, Extra-cellular matrix components, Organic detergents

INTRODUCTION

Balanced activity in onogenes and tumor-suppressor genes, but also of protein products of both gene types, has been characterized as key in the regulation and prevention of malignant transformation, on the one hand, and ageing, on the other, both on cell and organism levels (Kurz et al., 2005; Kurz et al., 2008; Okuyama et al., 2003; Zhang et al., 2007). In this connection, a possibility for further differentiation of osteoclast cell lineages from derived cells with monocytic characteristics has also been proved (Solari et al., 1996). According another study, large numbers of osteoclasts could be derived from embryonic stem cells in the presence of appropriate growth factors and co-factors (Okuyama et al., 2003; Solari et al., 1996). On the other hand, messages about the role of the cryo-protector Dymethylsulfoxide (DMSO) (Manandhar and Onishchenko, 1995; Norwood et al., 1976), as well as of other organic detergents (Cody et al., 2011; Zhu et al., 2005), in the activation of fusion process between the cells, are also obtained.

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Gene *DCUN1D3* (*Dcn1*), has been proved as 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 (Jin et al., 2006; Kurz et al., 2005; Kurz et al., 2008; Ma et al., 2008; Meyer-Schaller et al., 2009; O-charoenrat et al., 2008; 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; Kurz et al., 2005; Kurz et al., 2008; Ma et al., 2008; Meyer-Schaller et al., 2009; O-charoenrat et al., 2008). Protein product of this oncogene has been characterized as protein-kinase, which makes easier targets for degradation proteins, known as key molecules for processes of ageing and cell death (Jin et al., 2006; Meyer-Schaller et al., 2009). On the other hand, connected with it tumor-suppressor gene *HACE-1* codes protein-kinase,

which, in opposite to kinase protein product of oncogene *Dcn1*, has been proved to make easier targets for degradation key proteins of the malignancy and metastasis processes (Zhang et al., 2007).

Cytokine-activated Janus-kinases (JAKs)–signal transducers and activators of transcription (STATs) pathways have shown important role in the control of immune response, and dysregulation of it has been associated with various immune disorders. Signalling strength, kinetics and specificity of this pathway have been shown to be modulated at many levels by distinct regulatory proteins (Kyba et al., 2003).

Pro-inflammatory influence of NF-κB by the proved activation of the expression of appropriate genes in neutrophils by this molecule, has also been suggested (Cloutier et al., 2007). Signals from Transforming Growth Factor-beta (TGF-β) have been found to up-regulate Wnt5A expression directly through the Smad-complex, as well as through Smad-induced CUX1 and MAP3K7-mediated NF-κB (Okuyama et al., 2003). This pro-inflammatory influence of NF-κB by the proved activation of the expression of appropriate genes in neutrophils by that molecule, has also been suggested (Cloutier et al., 2007; McDonnald et al., 1997). In this connection, Glutathione, and in particular its reduced form (GSH), has been characterized not only as one of the main regulator molecules, involved in the regulation of cell proliferation, differentiation, but also in different types of cell death, inflammatory and pro-inflammatory processes (Ortega et al., 2011). This is a thiol-containing tri-peptide (L-γ-glutamil-L-cysteinyl-glycine), which is ubiquitous in the cells, and its deficiency disrupts the redox-status and upsets the physiological cellular balance between pro-oxidants and antioxidants (Meister, 1983). Lowered cellular GSH levels have been established in different pathological conditions (pre-malignancies and malignancies, inflammations, many neurodegenerative diseases and disorders, AIDS, diabetes and others) (Jahngen-Hodge et al., 1997).

In this aspect, the main goal of the current study was directed to a possibility for derivation of mature cells from osteoblast and osteoclast lineages from common progenitors with different origin and sources, by appropriate conditions *in vitro*-incubation.

MATERIALS AND METHODS

Normal cells from embryonic mouse Balb/c 3T3 line (1 x 10^6 cells/ml) were incubated at 37^0C in incubator with 5% CO_2 and 95% air humidification Dulbecco's Modified Minimal Essential Medium (DMEM) (high glucose), supplemented with 15% Fetal Calf Serum (FCS), 100 UI/ml Penicillin, 0.25mg/ml Streptomycin and 0.25mg/ml Amphotericin-B. The media were changed twice a week, and during this time the cell growth and proliferation was followed by observation at every 24 hours. When the formation of confluent cell monolayers was observed, the cells were trypsinised (by treatment with trypsin/EDTA solution), and tested for viability by Trypan Blue Dye Exclusion Test. Both primary and/or fibroblasts from the derived from them permanent cell line 3T3, of Balb/c experimental mouse embryos, were also used as feeder cells for seeding of mouse embryonic stem cells (ESCs). In all cases, after determination of the cell viability by Trypan Blue Dye Exclusion Test and subsequent seeding in initial density 5 x 10^6 cells/ml, the feeder cells were scaled-up by cultivation in Dulbecco's Modified Eagle's Medium (DMEM) with high glucose, supplemented with antibiotics mixture of 100 U/ml Penicillin and 100 μg/ml Streptomycin, as well as 15% Foetal Bovine Serum (FBS), previously inactivated by heating at 65^0C. The so prepared cell cultures were incubated at 37^0C, in incubator with 5% CO_2 and 95% air humidity. The so prepared cell cultures were observed at each 24 hour as native preparations, by Inverted light microscope. Formation of confluent mono-layers was usually observed around the 5^{-th} day after seeding. However, for prevention of ability for growth and proliferation of eventually seeded normal mouse ESCs, further growth and proliferation of the feeder cells from the so formed confluent monolayer should be stopped. For this aim, the cultural fluid of the confluent mono-layer was changed with cultivation medium, containing Mitomycin-c (mm-c), for 2-3 hours at 37^0C. Subsequently, the confluent cell monolayer was washed with Phosphate Buffered Saline (PBS) and stem cell medium was added, which besides of the described components, was additionally supplemented with 20% FBS; 0.5% beta-Mercapto-ethanol; Leukemia-Inhibiting Factor (LIF) and Colonia-Stimulation Factor (CSF). Mouse ESCs are similarly isolated and proceeded, but in earlier studies of pregnancy, and they were seeded in the same initial density (5 x 10^6 cells/ml), after counting and determination of cell viability by Trypan Blue Dye Exclusion Test. Formation of stem cell colonies was observed around the 3^{-rd}-4^{-th} day after seeding, and they were seen by phase-contrast light microscope. After scaling-up of the so proliferated mouse ESCs, sub-population of

them was determined to be subjected on transfection by recombinant DNA-constructs by electroporation, for insertion of additional mouse *Dcn1* oncogene copy in them. Cell cultures from malignant human cervical carcinoma cells HeLa were also used and analogically proceeded. Previously designed commercial recombinant gene constructs were used, which were based on *adeno-associated virus* DNA, containing promoter of *Eukaryotic Elongation Factor-1 alpha* (*EF-1α*) gene from *adeno-associated virus*; copy of mouse oncogene *Dcn1* of 3T3 mouse embryonic fibroblasts, as well as marker gene, determining resistance to Neomycine, isolated from bacteria plasmid DNA, received by treatment with respective restrictases and subsequently – with lygases. The gene, determining antibiotic resistance, was necessary for the selection of positively-transfected mouse ESCs, and it was located in immediate nearness to the gene of interest. For this goal, all transfected mouse ESCs were cultivated in the presence of the synthetic analogue of Neomycine – G418, on confluent monolayer of feeder cells, with previously suppressed further proliferation. Around the 28^{-th} day after the seeding in the presence of G418, more of the seeded mouse ESCs died and only those, which possessed the gene, connected with resistance to Neomycine, survived, proliferated and gave colonies. After picking-up the so formed clones of transfected mouse ESCs, possessing the antibiotic resistance gene and scaling-up of each one of them, they were subjected on genomic assay, by isolation of total DNA and RNA from all ESCs derived clones, which were subjected on transfection, but also of the used recombinant gene constructs, followed by standard Polymerase Chain Reaction (PCR) and Reverse Transcriptase PCR (RT-PCR), with application of appropriate 3'- and 5'-DNA-primers, complementary to the used recombinant gene constructs, respectively, with subsequent electrophorhesis in 1% Agarose gel with previously added Ethidium bromide solution. Malignant HeLa cells were similarly proceeded, but the main difference in the presence of tumor-suppressor gene *HACE-1* in the used recombinant DNA-constructs, this gene was further tested, and malignant cells, positive on additionally-inserted copy of it, were further selected, tested and applied according to the respective experimental goals. However, because of the mouse origin of the inserted gene *HACE-1*, the possibility of the used recombinant gene constructs and transfected by them positive on additional copy of mouse tumor-suppressor gene HeLa cells was provided by insertion of tag for human FLAG protein. For comparison with appropriate *in vivo*-experimental models, similar assays were performed from mature cells from the skin of two inbred experimental mice lines: wild-type (WT) homozygotes on oncogene *Dcn1* (*Dcn1+*/*Dcn1+*) and partially knocked-down (KD) heterozygotes on it (*Dcn1+*/*Dcn1-*). In this case, appropriate 3'- and 5'-primers, complementary with the cell nuclear DNA, were used.

After the derivation of ESCs, containing additional oncogene copy, it is necessary to be investigated if these cells have preserved their normal/non-malignant characteristics, as well as if they possess properties to support adequate immune response on organism level. The main reason of this necessity is that in arising of eventual need of transplantation of these cells with eventually elongated life, or even immortalized, in the live organism, which in any way are malignantly transformed in the work process, and, hence, they could cause the death of the organism much faster and much earlier than any disorder, disease, even cancer. So, investigation on the derived transfected cells, is necessary, and if they show any sign(s) of malignancy, all the procedures, described above, should be repeated, till the phase of their proliferation on confluent feeder cell mono-layers, until enough numbers of non-transfected mouse ESCs' colonies are available. For general initial cell differentiation of the scaled-up transfected mouse ESCs, containing additional copy of oncogene *Dcn1*, they were pre-incubated in the absence of feeder cells for one night, in the presence of Gelatine solution in PBS, which is necessary to form thin film for cell adhesion. For further differentiation of the so derived early progenitors in myeloid direction, methods for their pre-cultivation in conditions, in which activation of tumor-suppressor genes in them could be achieved at the same time, were applied. For this goal, first the derived myeloid cells were pre-incubated in the described cultivation medium, containing 2 μg/ml Doxicyclin. The aim of comparatively small concentrations of this substance was connected with maximal escape of its toxicity for the cells, but on the other hand, in particular of this antibiotic, with further myeloid differentiation by activation of genes from *STAT*-family, proved by Kyba et al. (2003). Those genes are known as tumor suppressors, in particular in the process of myeloid cell differentiation.

Despite the received proofs about successful myeloid cell *in vitro*-differentiation of normal cells, on the one hand, as well as of tumor-suppressor genes activation, on the other, the application of Doxicyclin for these goals isn't a good way for

achievement of myeloid cell differentiation. The negative effects of this substance on the cell and organism levels, both *in vitro* and *in vivo*, are connected mainly with its known unwished, characteristic about all antibiotics side effects, in particular, toxicity for the cells on its influence. A proof about that is the observed high mortality in all cell cultures, pre-cultivated in the presence of Doxicyclin, despite of the low concentration used (Moore et al., 1998). By taking all that in consideration, it could be concluded that safer ways and strategies for induction of tumor-suppressor gene(s) over-expression in normal cells, as well as for such, directed to provide of adequate immune reaction, are necessary to be searched, developed, tested and applied.

Sub-populations from 3T3 fibroblast cell line, derived from Balb/c mouse embryos, were consequently pre-incubated in the presence of supplemented cultural fluid, in which mouse malignant myeloma cells were previously cultivated, after its centrifugation and filtration, for a short-time period of 2-3 days. Subsequently, new 3T3 cell sub-populations were *de novo*-pre-cultivated in the cultural fluids, supplemented of the cell cultures, described above, for a longer time period of 7-10 days. Subsequently, one part of the so pre-cultured cells was resuspended and freezed at -80^{0}C for 2-4 weeks after addition respective volume from the cryo-protector Dymethylsulfoxide (DMSO), taking in consideration the proved enhanced inter-cellular fusion on the influence of many organic detergents by their action to change properties of cell membranes (Cody et al., 2011; Manandhar and Onishchenko, 1995; Norwood et al., 1976; Zhu et al., 2005). After subsequent thawing, the cells were re-incubation by the procedures, described above. Fixed light microscopic slides from each one of the cells, derived from normal mouse embryonic fibroblasts 3T3, incubated by each one of the described techniques, but also from mixed cultures from both received cell types, were prepared. For this goal, the respective preparations were fixed with 95% Ethanol, washing with PBS, after which they were stained by mouse anti-GSH-antibody, Giemsa and Hematoxilin/Eosin, respectively, and then washed and dried at room temperature. The so prepared slides were observed by inverted light microscope, supplied with mega-pixel CCD-camera.

The first question in this aspect was connected to a detailed study on the molecular mechanisms, underlying the cell proliferation, their differentiation to one or other direction, changes in the cells in the presence of different substances and/or in co-cultivation with other cells, etc. For this goal, one of the main sub-tasks was connected with following and investigation of intra- and extra-cellular molecules, which support cell growth and proliferation of the normal stem/progenitor cells, on the one hand, but also, which are also responsible for the control of those processes, not-allowing changes, and in this way, could lead to malignant cell transformation, on both cellular and organism levels. For this aim, identification of proteins, which are common for both normal cells in early stages of maturation, as well as in malignant cells, was necessary. One of the hypotheses was connected with eventual changes of the cascade mechanisms, in which these intermediate molecules participate, on the influence of tumor-suppressor genes and coded by them protein products, allowing this way stopping cell growth, proliferation and/or eventual malignant transformation. Participation of HACE-1 tumor-suppressor protein in such regulatory pathways was proved and cited in the literature (Meyer-Schaller et al., 2009).

Individual samples, HeLa cell nuclear protein extract (NE); protein extract from synovial fluid (SF), as well as from both samples, were prepared. In all cases, the isolated protein fractions were precipitated in cold 100% EtOH overnight, after which the so formed precipitate were washed with cold 80% EtOH and after centrifulation, the supernatants were turned off and the pellets were diluted in SDS buffer. After SDS-Electrophorhesis on Polyacrylamide Agarose Gel (SDS-PAGE), followed by Comasie-Blue staining, the gel was washed and sliced with an in-house tool. Gel slices were washed with water and acetonitrile, followed by reduction and alkylation of cysteine residues by DTT and iodoacetamide. Following overnight trypsin digestion, peptides were extracted by acetonitrile and 5% Formic acid, and subsequently concentrated in a speed-vacuum centrifuge In this way, all probes were prepared for tandem mass spectrometry assay, combined with liquid chromatography (LC-MS/MS). For analysis, peptides were dissolved in 20 pi solvent, containing 5% Acetonitrile and 0.1% Formic acid. Data were searched against respective protein databases, concatenated withreversed copies of the peptide sequences and supplemented with frequently observed contaminants. Methionine oxidations and acetylation of protein N-termini were specified as variable modifications, and carbamidomethylation - as fixed modification, respectively.

RESULTS AND DISCUSSION

Despite the fact that all of the clones, derived from the mouse ESCs after their transfection, contained the gene, connected with resistance to Neomycin, only 3 of them showed a positive signal of the respective gene of interest (oncogene *Dcn1*), in electrophorhetic assay on 1% Agarose gel after standard PCR (Figure 1A). However, in two from these three cell clones, a positive signal of the same gene was established in these conditions, after subjection of the same probes on RT-PCR (Figure 1B). These data could be explained with eventual stable non-reversible insertion of the oncogene in these two ESCs clones. These results could be confirmed with the imaginations of electrophorhetic profiles of the used recombinant DNA-constructs, after their subjection of standard PCR (Figure 1C) and RT-PCR (Figure 1D), respectively, by application of the same 3'- and 5'-DNA-primers. In application of similar assays of genetic material from adult laboratory mice from two inbred lines (wild type homozygotes on oncogene *Dcn1* and partially knocked-down mutant heterozygotes on the same gene), differences could be noted not only in the elctrophorhetic profiles of RT-PCR of oncogene *Dcn1* in the two categories of inbred animals, but also in these of the connected with it tumor-suppresser gene *HACE-1* between them (Figure 1E, Figure 1F).

tumor-suppressor gene copy in malignant cells, was directed to eventual decrease of their malignant potential, which was the other main accent of the further studies. The results obtained confirmed the literature data, connected with those two genes, (Jin et al., 2006; Meyer-Schaller et al., 2009; O-charoenrat et al., 2008; Zhang et al., 2007), including our previous results (Sainova et al., 2011), but also similar influences between other genes as *NUMB* and *p53* (Colaluca et al., 2008). Besides on genetic level, a possibility about influence on the expression levels of different genes by protein products to each other, as well as on the genes, has also been established (Bauer et al., 2000; Bellosta et al., 2005). The same genes, as well as coded by them proteins, were established to be involved in the process of immortalization of the human trophoblasts, on the one hand (Graham et al., 1993; Southern and Berg, 1982; Takao et al., 2011). However, on the other hand, their preserved normal/non-malignant cell characteristics were proved by identification of similar signs, as in the transfected mouse ESCs, containing additional oncogene copy (Ali et al., 2004; Jaumot et al., 1994). In this way, the so derived ESCs are characterized as appropriate experimental model, representing suitable analogue to similarly-manipulated human cells. The noted cytoplasmic pseudopodia and intra-cellular contacts in co-cultivation of the derived from mouse ESCs myeloid progenitors, in the

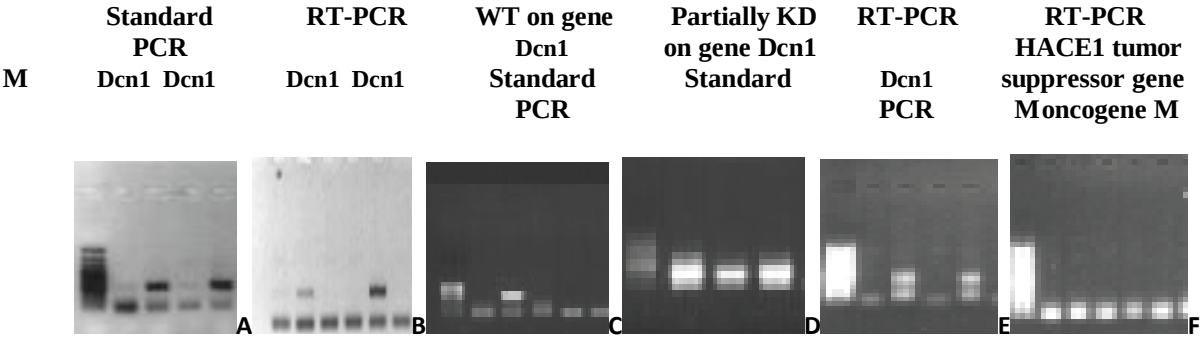


Figure 1. 1% agarose gel electrophorhesis for prove the presence or absence of additionally-inserted copy of mouse oncogene *Dcn1* in transfected mouse ESCs (A,B) and inbred experimental mice lines, from wild type (WT) homozygotes on the oncogene *Dcn1* (*Dcn1+*/*Dcn1+*) and partially knocked-down mutant (MT) heterozygotes on the same gene (*Dcn1+*/*Dcn1-*) (C-D), in application of specific primers, complementary of the used recombinant DNA-construct (A,B) and to the cell nuclear DNA (C-F), respectively. A,C: By standard PCR. B,D-:. By RT-PCR

The main goal of the insertion of additional oncogene copy in normal mouse ESCs, was connected with eventual elongation of life and suppression of changes, connected with the process of ageing. On the other hand, however, normal/non-malignant characteristics should be preserved. Analogically, the insertion of additional

presence of non-containing additional tumor-suppressor gene malignant cells, are in confirmation of literature findings about the appearance of similar changes in result of phagocyte differentiation in similar incubation conditions (Moore et al., 1998). These data also confirmed our previous investigations about the

signs of phagocyte differentiation, as well as for eventual decrease of malignant potential of neo-plastic cells in the presence of additional tumor-suppressor gene, inserted in their genomes (Sainova et al., 2011; Sainova et al., 2013). Despite the noticed signs of phagocyte differentiation again were stronger in co-cultivation with malignant cells, non-containing additional tumor-suppressor gene, these differences were slighter in the current study in then in our previous results (Sainova et al., 2013), which could be explained with the presence of inserted additional oncogene copy in the genome of the applied myeloid progenitors. The results obtained confirmed the eventually decreased malignant potential of human cervical carcinoma HeLa cells, containing additional copy of tumor-suppressor gene *HACE-1*, which protein product has been proved to participate in direct and indirect interactions with cytoskeleton proteins and in this way by cascade regulatory pathways, and in this way – to influence the functions of nuclear proteins, as well as processes as cell growth and proliferation. Because the fact of the mouse origin of the normal cells, containing additional antibody, possessing analogue of normal immortalized human trophoblasts, signs of malignant mouse cells, making them maximally near to human neo-plastic cells should be searched, despite of the proved in previous studies similarities with rat malignant cells (Sainova et al., 2013). The observed signs of phagocyte differentiation in the presence of malignant cells, non-containing additional tumor-suppressor gene copy, were in confirmation with our previous results from studies of cultivation of mouse ESCs, non-containing additional oncogene, in the same conditions. Messages about similar immortalization of normal human trophoblasts by the influence of virus *SV40* have been obtained (Southern and Berg, 1982). Immortalization of these human cells has been achieved by activated expression of genes, which, together with the coded by them protein products, are known to participate in the same cascade regulatory mechanisms (Ali et al., 2004; Kurz et al., 2008; O-charoenrat et al., 2008; Takao et al., 2011). On the other hand, the preserved normal/non-malignant cell characteristics of the so derived immortalized human trophoblasts have been proved by very similar methods, directed to activation of gene(s) with contradictory functions in both trophoblasts tested and cells, co-cultivated in them, as well as to achievement and support of adequate immune response, at the same time (Ali et al., 2004; Kurz et al., 2008; O-charoenrat et al., 2008). All these data suggest many signs of analogy of the described above normal mouse ESCs, containing and/or non-containing additional oncogene(s), with immortalized and non-immortalized normal human trophoblasts. These characteristics suggest a possibility about the usefulness of the normal mouse ESCs, both containing and non-containing additional copy of any oncogene, as convenient experimental model in the role of appropriate analogue of immortalized and non-immortalized normal human trophoblasts. These similarities could allow future experimental, research, pharmaceutical and therapeutic procedures, where and when work with people and/or cells from human origin aren't possible.

In co-cultivation of sub-populations of the myeloid progenitor cells, derived from transfected mouse ESCs, containing additional oncogene *Dcn1* by incubation in the presence of Doxicyclin and eventual subsequent activated expression of *STAT*-genes, with malignant cells, non-containing additional tumor-suppressor gene separated,

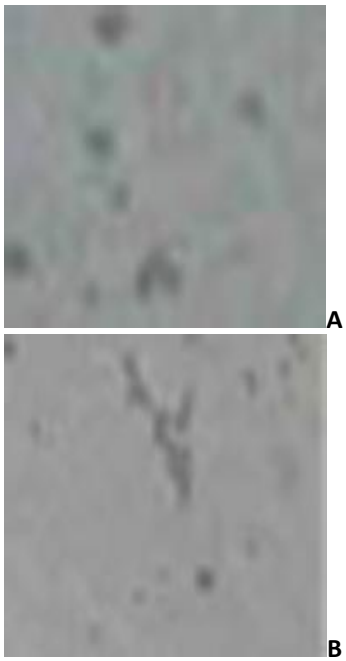


Figure 2. Myeloid progenitors, derived from containing additional copy of oncogene *Dcn1* mouse ESCs sub-populations, by activation of the tumor-suppressor genes from *STAT*-family by cultivation in the presence of Doxicyclin. A: Myeloid progenitors, containing additional oncogene *Dcn1*, co-cultivated with malignant cells HeLa, containing additional copy of tumor-suppressor gene *HACE-1*. B: Myeloid progenitors, containing additional oncogene *Dcn1*, co-cultivated with malignant cells HeLa, non-containing additional tumor-suppressor gene copy (double staining by polyclonal anti-STAT antibody and Giemsa dye, magnification: x100).

forming of pseudopodia and intra-cellular contacts were noted (Figure 2B), unlike in the sub-populations of the same myeloid progenitors, co-cultivated with malignant cells, containing additional copy of such gene (Figure 2A).

In short-term (2-3 days) pre-incubation in the so supplemented, normal embryonic cells acquire round cell shape, but also light-stained cytoplasmic content with appearance of granules, centrally-located nuclei, as well as changed nuclei/cytoplasm ratio in many of the cells (Figure 2B) could be noted, which are signs of initial myeloid differentiation, in comparison with the untreated controls (Figure 2A).

In long-term (7-10 days) pre-incubation of other cell sub-population from the same group treated normal embryonic fibroblasts in those same conditions, subsequent freezing at -80^{o}C for 2-4 weeks after addition respective volume from the cryo-protector DMSO, thawing and re-cultivation in standard conditions, multi-nuclear osteoclast-like were seen (Figure 4A). In addition the cultural fluid from the so derived osteoclast-like cells to new-formed mono-layers of *de novo*-cultivated 3T3 mouse embryonic cells, tendency about osteoblast-like cell differentiation was noted, a proof for which is the appearance of dark-stained regions of mineral depositions (Figure 4B). In co-cultivation of the so derived from mouse embryonic progenitors osteoblast-like and osteoclast-like cells (cell suspension from each one of both cell types plus cultural fluid of the respective cell type), zones of destroyed osteoblast-like cell monolayer were observed (Figure 4C). Bright spots, were probably due to a reaction to anti-mouse GSH antibody and, hence, where these bright zones and more intensive, the levels of GSH are probably higher (Figure 4B, Figure 4C).

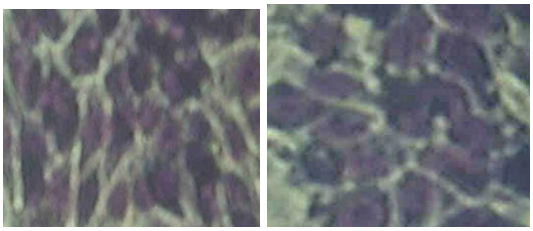
One of the eventual explanations of the possibility of derivation of different types of normal mature cells from 3T3 fibroblasts, depending of the respective incubation conditions, was the hypothesis for existence of stem-like cells in the general embryonic cell line. So, the influence different growth factors and cytokines, their mechanisms of action, but also signaling cascade pathways, have suggested probability for existing of novel therapeutic targets, including not only those molecules, but also their inhibitors and/or antagonists, which could influence their function both directly or indirectly, by targeting specific step of respective signaling mechanisms (Hughes et al., 2006; Zhang et al., 2005). On the other hand, these results proposed eventual positive role of extra-cellular matrix components in the directions of cell differentiation, as well as the role of cryo-protector DMSO as a stimulator of the intra-cellular fusion in the formation of osteoclast-like cells, which, also confirmed some of the negative effects of the organic detergents of the process of osteoblastogenesis and suggested the eventual usability of the applied technique for fusion procedures, as for example, fusion between separated cells, but also of cells with viral particles. The results obtained were in agreement with many literature data about possibility for derivation of large numbers osteoclasts from embryonic stem

A **B** **C**

Figure 4. *In vitro*-derivation of osteoblast-like and osteoclast-like cells from normal mouse embryonic 3T3 fibroblasts. A: Osteoclast-like multi-nuclear cell, derived by long-term co-incubation of normal 3T3 fibroblasts and mouse malignant myeloma cells, subsequent freezing at -80^{o}C in the presence of DMSO, thawing and renovated standard incubation (Hematoxyllin/Eosin staining technique, magnification: x200); B: Osteoblast-like cells, derived by *de novo*-addition of supplemented fluid, picked-up from the derived osteoclast-like cells, described in A, to new-formed mono-layer of normal mouse embryonic 3T3 fibroblasts. Accumulation of dark-stained mineral depositions, could be seen (double staining by anti-mouse GSH antibody and Giemsa dye, magnification: x100); C: mixed culture of co-cultivated osteoblast-like and osteoclast-like cells, derived by the described procedures. Destruction of the osteoblast-like cells monolayer could be seen (Fixed preparation, stained by Giemsa, magnification: x100).

cells in the presence of appropriate growth factors and co-factors (Okuyama et al., 2003). Furthermore, messages about the presence of multi-nucleated osteoclast-like giant cells noted in the presence of tumors in different anatomic organs, as for example in the pancreas have also been received (Kyba et al., 2003). The fusion of monocytic cells in the late stages of differentiation has been characterized as another main mechanism, included in the derivation of osteoclasts and osteoclast-like multi-nuclear cells, both *in vitro* and *in vivo*. In this connection, together with taking in consideration many literature findings, the suggestion about the eventual role of the cryo-protector DMSO as a stimulator of the process of intra-cellular fusion, in particular in stimulation of the osteoclast-like cells arising, has also been confirmed (Calvani et al., 2005; Manandhar and Onishchenko, 1995; Meyer-Schaller et al., 2009). The change in the properties of the cell membrane structures could be accepted as one of the eventual explanations (Manandhar and Onishchenko, 1995). These results could be supported by the established similar effects of other organic solvents (Cody et al., 2011; Kyba et al., 2003; Norwood et al., 1976). Differentiation of malignant myeloid cells in osteoclast-like cells on the influence of autocrine molecules and signals has also been demonstrated (Kung Sutherland et al., 2003). Besides the obtained messages about cell differentiation in osteoclast lineage on the influence of osteoblasts-derived metabolites (Susa et al., 2004; Yu et al., 2011), the opposite phenomenon of differentiation in osteoblast direction in the presence of osteoclasts-conditioned cultural fluid, has also been proposed (Orlandini et al., 1991). Furthermore, a possibility for application of the applied technique for fusion of separated cells, as well as with viral particles, in biotechnological and gene-engineering manipulations, was proposed. The observed morphological changes of cells, derived from 3T3 mouse fibroblasts, could be explained with eventual existence of separate stem-like cells in the general cell line, also taking in consideration its embryonic origin. Those data confirmed some literature data, according which the destruction of osteoblasts has been indicated as one of the main indices for differentiation in osteoclast lineage (Jacob et al., 2006), on the influence of the derived cells. In this way, possibilities for derivation of normal mature cells from partially-differentiated normal cells were proposed, which were also in agreement with literature findings, connected with derivation of normal mature cells from different lineages from partially-differentiated adult cell progenitors, as adult bone marrow mesenchymal stromal/stem cells (MSCs), with both rodent and human origin, respectively (Lisingnoli et al., 2001; Torregianni et al., 2012).

SF is known as a feeder source for different types of adult normal stem/progenitor cells (from synovia, cartilage, bone, muscle and *Hoffa sub-Patella* adipose tissues, as well as separated non-mature bone-marrow and blood cells), in the respective anatomic area, but this liquid is also supplemented of different cellular metabolites. On the other hand, NE is known as rich of proteins, able to connect directly with nucleic acids and, hence, to influence directly the expression on any genes, but suppress others at the same time. The main goal of this investigation was connected with identification of common proteins in both protein mixtures, which is necessary for characterization of molecules, which support of growth and proliferation capacity of cells by appropriate cascade pathways, but also underline in the control and regulation of the same processes by other cascade mechanisms. HeLa NE and SF are two protein mixtures that were proposed to possess some proteins in common - HeLa NE should contain proteins, originating from the cell nucleus and associated with chromatin maintenance, transcription and other biochemical processes (Figure 3).



A **B**

Figure 3. *In vitro*-cultivated normal mouse embryonic 3T3 fibroblasts. A: Control embryonic cell culture. B: Mixed cell culture of 3T3 normal embryonic fibroblasts and malignant mouse myeloma cells – signs of early myeloid differentiation, as increased, rounded shape, but also light-stained cytoplasmic content with appearance of granules, centrally-located nuclei and changed nuclei/cytoplasm ratio, could be noted in comparison with the control fibroblasts (stained by Giemsa dye - magnification: x200).

SF, on the other hand, is a bio-fluid, involved in lubricating the knee and nurturing chondrocytes in the cartilage tissue. These data propose information about intermediate molecules (mainly with protein and peptide nature), participation in cascade regulatory pathways (Molofsky et al., 2004; Vogelstein and Kinzler, 2004). As such

molecules are determined mainly cytoskeleton and membrane peptides and proteins, playing role as membrane pumps, enzymes and other (Oakata et al., 1995). Similarly, cytoskeleton components from both rat anatomic organs tested (brain and pancreas, respectively) were established as possessing the highest affinity to GSH, which was observed in both undiluted and diluted probes of them (Figure 5D).

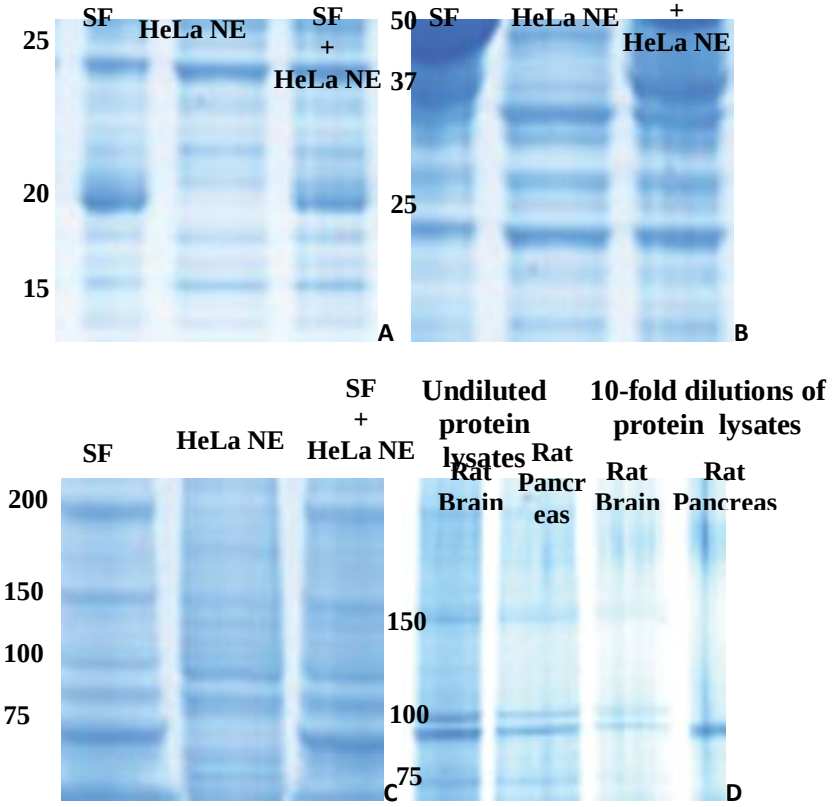


Figure 5. Separation of proteins from protein lysates: A-C - from NE from human malignant cervical carcinoma HeLa cells (line 1) and human SF (line 2), as well as mixture of from both probes (line 3): A: proteins with molecular weight from 15 to 25 kDa; B: proteins with molecular weight from 25 to 50 kDa; C: proteins with molecular weight from 75 to 200 kDa; D: proteins from rat brain (line 1 – undiluted probe and line 3 – 10-folds dilution) and pancreas (line 2 – undiluted probe and line 4 – 10-folds dilution), previously pushed by GSH-Agarose columns (Comasie-Blue staining), M = marker.

According the results, obtained from PAGE and subsequent label-free LC-MS/MS analysis of the probes from protein lysates of brain and pancreas with rat origin, the molecules, determined with high affinity to GSH, which determine the normal/non-malignant differentiation of the cells, building both anatomic organs, were also mainly with cytoskeleton nature (Figure 5D). As such molecules are determined mainly cytoskeleton and membrane peptides and proteins, playing role as membrane pumps, enzymes and other [Nguyen-Ngoc et al., 1997].

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