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RESEARCH ARTICLE

Investigation of the molecular and intra-molecular mechanisms, determining the cell differentiation direction in appropriate incubation conditions, on cellular and organism levels

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Abstract

The main goal of the current study was directed to derivation of one or another type of normal mature cells from partially-differentiated cells, by incubation in different laboratory conditions, but also to reveal the molecular mechanisms, underlining these maturation variations. In short-term (2-3 days) pre-cultivation of mouse embryonic 3T3 fibroblasts, in cultural fluid, in which mouse malignant myeloma cells, signs of early myeloid differentiation were noted. After freezing in the presence of the cryo-protector Dymethylsulfoxide (DMSO), subsequent thawing and in vitro-re-incubation, appearance of separated sub-populations of multi-nuclear osteoclast-like cells was observed. When the cultural fluids from the so derived osteoclast-like cells, were added to de novo-formed confluent monolayers of 3T3 embryonic cells, signs osteoblast-like cell differentiation was noticed, as appearance of dark-staining mineral depositions. In co-cultivation of cells from both cell types derived, zones of destructed osteoblast-like cell monolayer were seen. These data could be explained with eventual existence of separated stem/progenitor cells in the general 3T3 cell line, which are able to differentiate in respective directions depending of the respective incubation conditions. On the other hand, the role of 3T3 embryonic fibroblasts as feeder cells, supporting the proliferation of stem/progenitor cells from both human ocular cornea limbus and oral mucosa, was also indicated, which supported the literature findings about the importance of embryonic mouse fibroblasts as feeder cells, supporting stem cell proliferation, for the needs of clinical practice. This application of the embryonic mouse fibroblasts was proved for embryonic stem cells in our previous studies. Because epithelial stem/progenitor cells from human oral mucosa were proved to express limbal epithelial stem cell markers, they were in vitro-cultivated in similar laboratory conditions as limbal epithelial stem/progenitor cells. These results were in agreement with respective literature data, and could be explained with eventual existence of separated stem/progenitor cell sub-populations, which are able to differentiate in respective directions in appropriate cultivation conditions. In this way, any role of the extra-cellular matrix components on the cell differentiation activation and direction, as well as the influence of DMSO and other organic detergents on the processes of intra-cellular fusion in the formation of osteoclast-like cells, could be suggested. For study of the underlying events, which determine the differentiation of cell progenitors to one or another direction in respective incubation conditions, investigations on the direct and/or indirect intra-molecular (protein-protein, protein-DNA, protein-RNA, etc.) interactions, was necessary.

INTRODUCTION

Studies on the biology of the stem/progenitor cells are often focused on their self-renewal and differentiation [Molofsky et al., 2004]. Their abilities of these cells to differentiate into different cell lineages, suggest their usability as appropriate cellular vectors for treating of different injuries, including different types of malignancies. These properties make them strong candidates for delivering of genes and restoring organ systems function, which have been established to be included in these processes. In this way, by identification, as well as by ultimate manipulation, genes that regulate stem cells' number, replication rate and self-renewal capacity, might have important clinical benefits, as strong candidates for different therapeutic procedures. Undifferentiated stem/progenitor cells could circulate in the body and contribute to repair if needed. On the other hand, they could be contributing to other tissues in the event of transplantation or repair.

According many literature data, cytoskeleton components are able, are very important in the control of cell growth, proliferation and differentiation, by different intra- and extra-cellular inter-molecular interactions in cascade regulatory mechanisms [Aamodt and Culotti, 1986; Buchwitz et al., 1999; Jaumot et al., 1994; Nguyen-Ngoc et al., 2007; Oakata et al., 1995; Savage and Chalfie, 1991]. Furthermore, the deformed cell cytoskeleton, besides the spontaneous chromosomal fragility [Daniel, 1986], has been proposed as one of the main reasons, leading to for high frequency of chromosomal aberrations because of abnormal cell division, due to abnormal cell spindle structure [Zheng et al., 1993].

In this aspect, the main goal of the current study was connected with investigation on the direction of cell maturation of partially-differentiated cells, depending on the concrete incubation conditions, but also on the molecular mechanisms, underlining those processes in the normal cell, which at the same time prevent its malignant transformation.

Materials and Methods

Laboratory in vitro-incubation of stem/progenitor cells and tissue explants with human origin from two sources, in the presence of metabolites from mouse embryonic fibroblasts in the role of feeder cells

Isolated cells and tissue explants from human ocular cornea limbus and oral mucosa were incubated in different combinations of the growth media Dulbecco's Modified Minimal Essential Medium (DMEM) and Ham's and of DMEM and F12, respectively (Sigma-Aldrich). Those media mixtures were supplemented with 10% Fetal Bovine Serum (FBS) and antibiotic mixture (100 UI/ml Penicillin, 0.25 mg/ml Streptomycin and 0.25 mg/ml Amphotericin-B). Subsequently, L-Glutamine, 10 ng/ml Epidermal Growth Factor (EGF - Sigma-Aldrich), 5 μg/ml Insulin, 0.4 μg/ml Hydrocortisone, 24 μg/ml Adenine, as well as 2% ml/ml conditioned cultural fluid of previously cultivated in it 3T3 feeder cells (fibroblasts from embryos of Balb/c experimental mice), on monolayer of 3T3 cells on vitelline membrane, previously treated by Glutamine-Glutaraldehyde [Bratanov et al., 2011; Valkova et al., 2013], respectively. The so prepared cultures of cells and tissue explants were cultivated at 37^0C, in incubator with 5% CO_2 and 95% air humidity, and observed as native preparations by inverted light microscope (Leica), supplied with mega-pixel CCD-camera.

Derivation of different mature cell types from partially-differentiated normal embryonic mouse cells/fibroblasts, by concrete laboratory in vitro-incubation techniques

Separate population of normal 3T3 mouse embryonic fibroblasts (1 x 10^6 cells/ml), were incubated at 37^0C in incubator with 5% CO_2 and 95% air humidification, in DMEM (high glucose) (Sigma-Aldrich), supplemented with 10% FBS (Sigma-Aldrich), 100 UI/ml Penicillin, 0.25mg/ml Streptomycin and 0.25mg/ml Amphotericin-B (Sigma-Aldrich). 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 trypsinized (by treatment with trypsin/EDTA solution), and tested for viability by Trypan Blue Dye Exclusion Test. A part of the so prepared 3T3 cell cultures were subsequently cultivated in the presence of supplemented cultural fluid, in which mouse malignant myeloma cells were previously cultivated, after its centrifugation and filtration. Separated sub-populations the so prepared mixed cultures were freezed at -80^{0}C for 2-4 weeks after addition respective volume from the cryo-protector Dymethylsulfoxide (DMSO). After thawing, the received cell suspension was centrifuged, the supernatants, containing DMSO, were taken off, the pellets, containing the cells, were resuspended, and the cells were re-cultivated by application of the cultivation techniques, described above, but a part of supernatants were subsequently added to de novo-formed confluent monolayers of mouse embryonic 3T3 fibroblasts.

Ex vivo-preparations of human cells from different sources and maturation stages

Pieces from human skin were sliced, treated with trypsin/EDTA solution and put at 37^{0}C for 5-10 minutes, for separation of the cells of each one piece. The so tripsinized material was then treated by PBS (for stopping of the trypsin action) and the cells were resuspended. The so derived cell suspension was then used for preparation of fixed light microscopy slides, which were dried, fixed with 95% Ethanol, stained by Hematoxylin/Eosin, washed with tapping water, dried at room temperature, and observed by inverted light microscope, supplied with mega-pixel CCD-camera.

Ex vivo-preparations of blood smears from human and mouse origin, containing cells from different types and different maturation stages

Smears from peripheral blood of human and mouse were prepared, and subsequently stained and observed by inverted light microscope, supplied with mega-pixel CCD-camera. The prepared light microscopy slides were fixed, stained, washed, and observed by inverted light microscope, supplied with mega-pixel CCD-camera.

Ex vivo-preparations of chromosomal preparations from mitosis of peripheral human blood leucocytes

Separate population of human peripheral blood (3-5 ml) were ex vivo-incubated, in 4 ml medium RPMI 1640 (Sigma-Aldrich) with added 0.8-1 ml FBS and 0.2 ml phyto-hemaglutinin. The so prepared probes were put at 37^{0}C in incubator with 5% CO_2 and 95% air humidification for 68-70 hours, after which the further cell growth and proliferation was blocked by addition of 0.2 ml Colchicin and put at 37^{0}C for 50 minutes. After careful shaking of the probes, they were centrifuged for 10 minutes at 1000 rpm. The supernatants were taken off, and 0.3 ml from the pellets were resuspended, after which 10 ml warm hypotonic 0.555% KCl solution was added to each suspension, and the probes were put at 37^{0}C for 10 minutes. After centrifugation at 1000 rpm for 10 minutes, the supernatants were taken off, and after resuspendation of 0.3 ml of the pellets, 10 ml fixative mixture of methanol-glacial acetic acid (3:1) were added to each one probe/pellet. After centrifugation at 1000 rpm for 10 minutes, this step was repeated 3-4 times by changing of the fixative mixture. After the last centrifugation procedure, the supernatants were turned off, and the resuspended 0.3 ml of the pellets were put at 4^{0}C used for preparation of metaphase plates on light microscopy slides, put on 30-40 sm, which were dried, stained by G-banding technique and observed as fixed chromosomal preparations by immersion light microscope Amplival.

Isolation of protein material from biological probes with human and rat origin, containing malignant and normal cells in different phases of differentiation

Protein material from human synovial fluid (SF), but also, from nuclear extract (NE) of human malignant cervical carcinoma cells HeLa, were isolated and tested. 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 immature 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 concrete study is directed to find the 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. So, the isolated proteins from both mixtures are subjected on separation by SDS-Polyacrylamide Gel Electrophorhesis (SDS-PAGE), subsequent Comasie-Blue staining of the gel, for identification of protein bands according molecular

weight, washing, chemical treatment of each band for digestion of each protein molecule, and subsequent label-free tandem mass spectrometry, combined with liquid chromatography (LC-MS/MS) assay.

Protein material from anatomic organs (pancreas and brain) of experimental rats was similarly isolated. For remind only of cytoskeleton proteins, two different dilutions the so prepared probes from both organs were pushed from GSH-Agarose columns, by taking in consideration the known affinity of GSH to more of the cytoskeleton components. For protein identification were then separated by SDS-PAGE, stained with Comasie-Blue, and subjected on label-free LC-MS/MS assay after chemical treatment.

Results and Discussion

Laboratory in vitro-incubation of stem/progenitor cells and tissue explants from human ocular cornea limbus and oral mucosa in the presence of metabolites from mouse embryonic fibroblasts in the role of feeder cells

In in vitro-cultivation of corneal epithelial cells and tissue explants on feeder cell monolayer of 3T3 mouse embryonic fibroblasts, cells with different morphology and in different phases of differentiation were established: early epithelial cell progenitors with round shape, oval nucleus and poor cytoplasmic contention, as well as polygonal-shaped mature epithelial cells (Fig. 1). These features were observed in incubation in the presence only of cultural fluid from embryonic 3T3 cells (Fig. 1 – a, b), but also on confluent monolayers of 3T3 fibroblasts, with formation of colonies of stem/progenitor cells (Fig. 1 – c, d), as well as on vitelline membrane, previously treated with Gelatine-Glutaraldehyde (Fig. 1 – e, f). Those results were in agreement with respective literature data about the role of feeder cells and bio-membranes as appropriate bio-substrates for incubation and proliferation of stem/progenitor cells and tissue substrates in laboratory conditions [Anderson et al., 2001; Sudha et al., 2006].

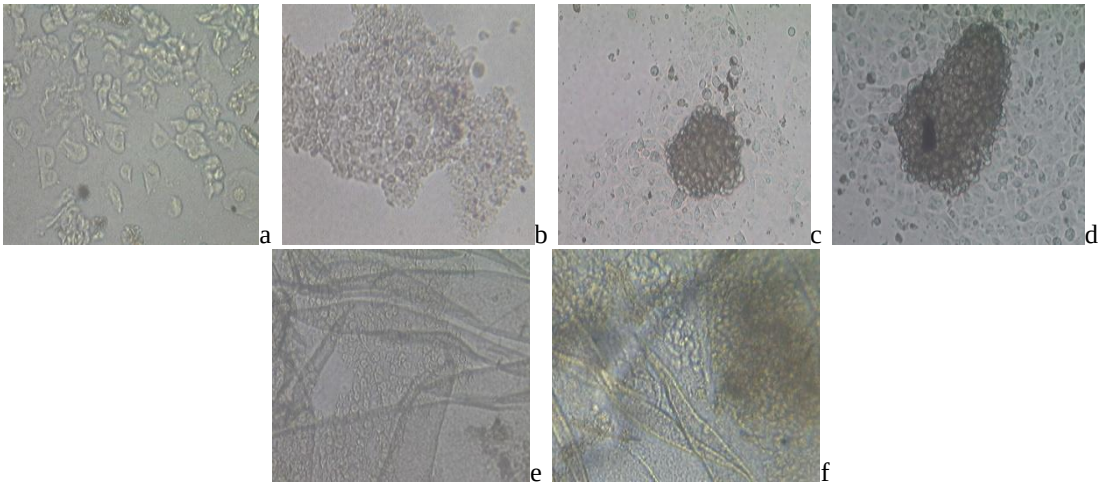


Fig. 1. Epithelial stem/progenitor cells (a, c, e) and tissue explants (b, d, f) from human limbus of ocular cornea. a) stem/progenitor cells, cultivated in growth medium, supplemented with cultural fluid from 3T3 mouse embryonic fibroblasts; b) cell proliferate around tissue explant, incubated in growth medium, supplemented with cultural fluid from 3T3 mouse embryonic cells; c) colony of proliferating stem/progenitor cells, seeded on confluent monolayer of 3T3 mouse embryonic cells; d) colony of proliferating stem/progenitor cells, separated from tissue explants, seeded cultivated on confluent monolayer of 3T3 mouse embryonic fibroblasts; e) stem/progenitor cells, incubated on vitelline membrane, previously treated by Gelatine-Glutaraldehyde; f) proliferating stem/progenitor cells, separated from tissue explants, seeded on vitelline membrane, previously treated by Gelatine-Glutaraldehyde (Native preparations, magnification: x100).

Because of the proved expression of some markers, also proved in limbal stem cells [Collin et al., 1992; Pellegrini *et al*., 1999], epithelial cells (Fig. 2 - a) and tissue explants (Fig. 2 - b) from human oral mucosa were analogically in vitro-cultivated and incubated. Those data were also in agreement with the literature findings in this direction [Grueterich et al., 2002].

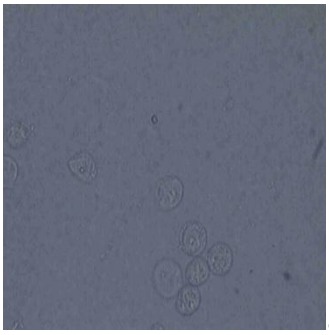
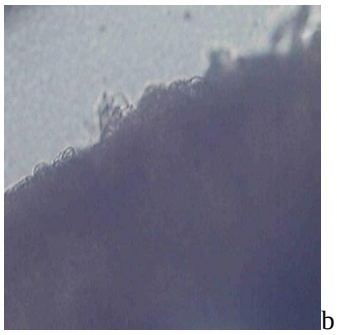
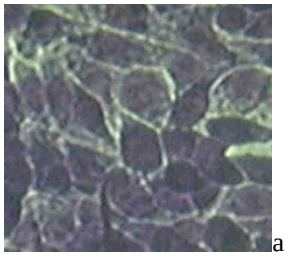
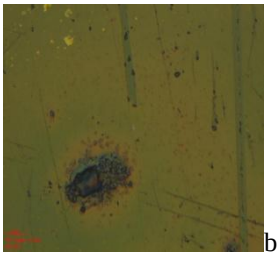
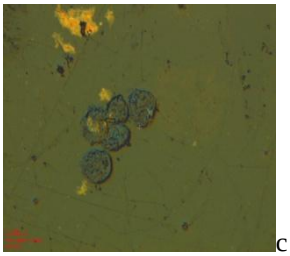
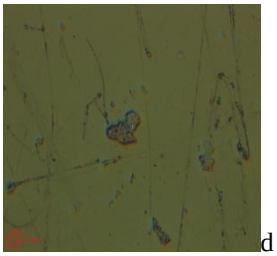
 a  b

Fig. 2. Epithelial stem/progenitor cells (a) and tissue explant (b), both incubated in growth medium, supplemented with cultural fluid from 3T3 mouse embryonic fibroblasts (Native preparations, magnification: x100).

The role of the mouse embryonic fibroblasts as feeder cells, supporting stem cell proliferation, has been proved for embryonic stem cells in our previous investigations [Sainova et al., 2011].

Derivation of different mature cell types from partially-differentiated normal embryonic mouse cells/fibroblasts

In short-term (2-3 days) pre-incubation of normal embryonic 3T3 fibroblasts in the presence of cultural fluid from mouse maliganant myeloma cells, round cell shape, as well as light-stained cytoplasmic content with appearance of granules, centrally-located nuclei and changed nuclei/cytoplasm ratio in many of the cells could be noted (Fig. 3 - b), which could be accepted as signs of initial myeloid differentiation, in comparison with the untreated controls (Fig. 3 - a). In subsequent freezing of separate sub-populations of the so pre-incubated cells by addition of the cryo-protector DMSO, subsequent thawing and re-cultivation in standard conditions, appearance of many multi-nuclear osteoclast-like cells could be seen (Fig. 3 – c, d). In addition the so supplemented cultural fluid from the osteoclast-like cells, derived by the described procedure, to de novo-formed confluent monolayer of 3T3 mouse embryonic cells, a tendency about osteoblast-like cell differentiation could be noted, a proof for which is the appearance of dark-stained regions of mineral depositions (Fig. 3 - e). Besides the role of the extracellular matrix components, eventually containing in the supplemented cultural fluids, cell-cell fusion on the influence of DMSO [Norwood et al., 1976], similarly to other organic detergents [Gattei et al., 1992], was proposed, because the proof influence of the cell membrane properties of those substances. 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 could be seen (Fig. 3 – f).

 a  b  c  d

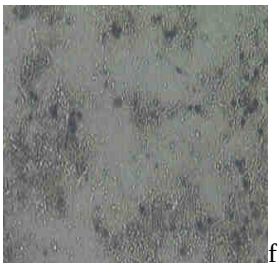
 e  f

Fig. 3. Mouse embryonic cells 3T3, incubated in different conditions: a) control preparation – confluent monolayer of 3T3 fibroblasts (Fixed preparation, stained by Giemsa, magnification: x200); b) myeloid-like cell, derived by pre-

cultivation of 3T3 cells in cultural fluid from mouse malignant myeloma cells (Fixed preparation, stained by Hematoxylin/Eosin, magnification: x200); c) mixed preparation of 3T3 cells, pre-cultivated with mouse malignant myeloma cells, freezed by addition of DMSO and re-cultivated in standard conditions. Cell cluster, but also fusion between some of the cells from it, could be observed (Fixed preparation, stained by Hematoxylin/Eosin, magnification: x200); d) mixed preparation of 3T3 fibroblasts, pre-cultivated with mouse malignant myeloma cells, freezed by addition of DMSO and re-cultivated in standard conditions. Formation of multi-nuclear osteoclast-like cell as a result of the fusion process, could be noted (Fixed preparation, stained by Hematoxylin/Eosin, magnification: x100); e) confluent monolayer of osteoblast-like cells, derived by pre-cultivation of de novo-formed confluent monolayer of 3T3 fibroblasts with cultural fluid of multi-nuclear osteoclast-like cells, described above. Dark-staining calcifications could be noticed (Fixed preparation, stained by Giemsa, magnification: x100); f) 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).

The features observed could be explained with eventual existing of separate stem/progenitor cells in the general 3T3 line, which are able to differentiate in different lineages, when appropriate cultivation conditions are available.

Ex vivo-preparations of human cells from different sources and maturation stages

For a better confirmation the influence between different cell types to each other, ex vivo- probes from biological materials were prepared and assessed, and different types of cells were observed (Fig. 4).

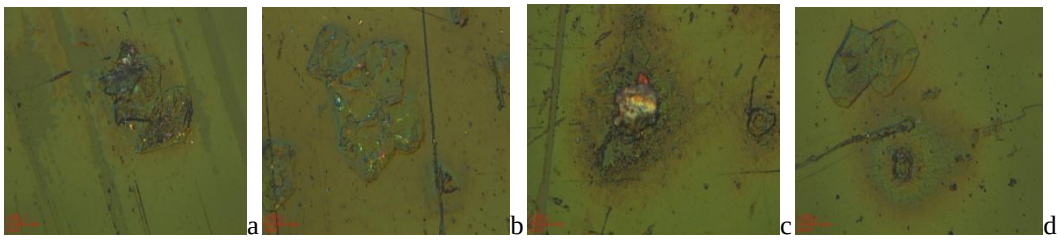

a b c d

Fig. 4. Ex vivo-preparations from human skin material: a) and b) clusters of proliferating epithelial cells; c) myeloid blood cell; d) cluster of two polygonal epithelial cells and myeloid-like blood cell below cells (Fixed preparations, stained by Hematoxylin/Eosin, magnification: x200).

The observed clusters of polygonal cells (Fig. 4 – a, b, d) could be accepted as a proof for early stages of cell differentiation and high proliferation capacity. On the other hand, the presence myeloid cells from different types and cell differentiation phases (Fig. 4 – c, d) might be proposed as one of the protective mechanisms against malignant transformation of cells in early stages of maturation and differentiation on organism level. This protection has been established on both intra- and extra-cellular sublevels, by changes, underlining the activation of normal cell differentiation and maturation, as well as by support of adequate immune reaction, respectively.

Ex vivo-preparations of blood smears from human and mouse origin

The differences, described above, might be confirmed by observation of smears from peripheral blood of human and mouse, respectively, moreover, because the blood is also known as a tissue, composed of cells from different types and maturation stages (Fig. 5).

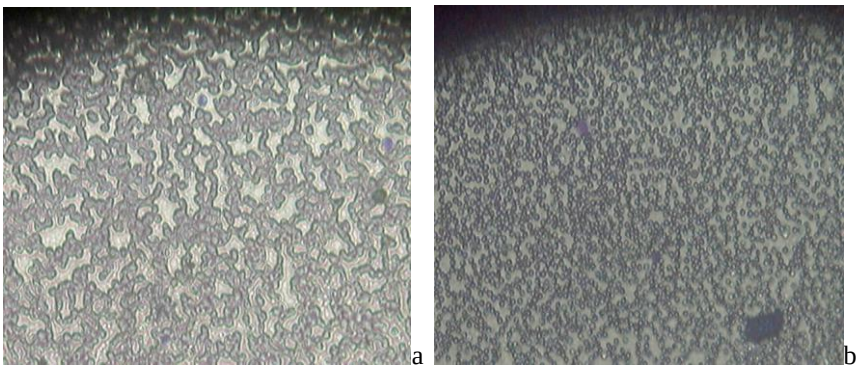

a b

Fig. 5. Fixed light microscopy slides of different normal cell types from peripheral blood smears of human (a) and mouse (b), respectively. Different cell types could be noted, the nuclei could be seen, which possess respective biological characteristics according to the cell type (Fixed preparations, stained by Hematoxylin/Eosin, magnification: x100).

The noted equivalence in the pictures from peripheral blood of human (Fig. 5 – a) and mouse origin (Fig. 5 – b) once more confirmed the analogy between mouse and human cells in respective different phases of maturation and differentiation, and, in this way, the role of the mouse cells as a convenient experimental model, suggested in our previous studies [Sainova et al., 2013], which is also in agreement with the literature data [Smith, 2001].

Ex vivo-preparations of chromosomal preparations from leucocytes smears of peripheral human blood

For a better understanding of the observed maturation variations, depending of the concrete incubation conditions, a necessity for investigation of the molecular mechanisms and intra-molecular (protein-protein, protein-DNA, protein-RNA, etc.) interactions, underlining these processes, arose.

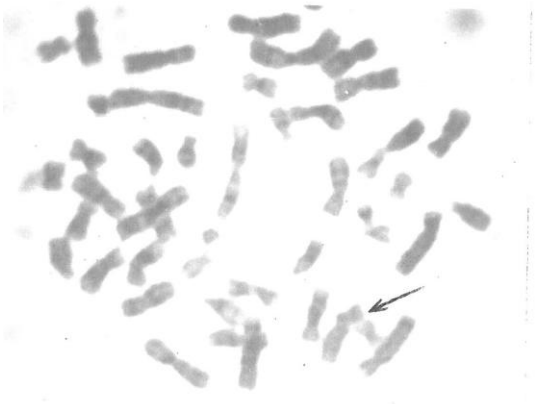


Fig. 6. Chromosomes from mitosis of human peripheral blood leucocyte from patient with polygene disorder. Site with increased spontaneous chromosomal fragility could be seen in centromere region of chromosome 3 (arrow) (Fixed light microscopy preparation, stained by G-banding technique, magnification: x200).

Deformations in the cell cytoskeleton components, as well as the spontaneous chromosomal fragility, in particular in the centromere regions of the chromosomes, have been suggested to lead to number chromosomal aberrations because of abnormal structure of the cell spindle and/or of abnormal segregation during the process of cell division, respectively [Daniel, 1986]. Both phenomena (cytoskeleton deformations and spontaneous chromosomal fragility) have been proved to be very often connected with each other, and, in this way, each one of them could be a reason or a consequence of the other [Zheng et al., 1993].

Determination of proteins, which are common for malignant and normal cells in different phases of differentiation

Protein materials from human synovial fluid (SF), but also, from nuclear extract (NE) of human malignant cervical carcinoma cells HeLa, were isolated and tested. 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 immature 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 concrete study was directed to find the 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 regulatory mechanisms. So, the isolated proteins from both mixtures are subjected on separation by SDS-PAGE, subsequent Comasie-Blue staining of the gel, for identification of protein bands according molecular weight, washing, chemical treatment of each band for digestion of each protein molecule, and subsequent label-free tandem LC-MS/MS assay (Fig. 7 - a). 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].

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 (Fig. 7 - b). These results were in agreement with many literature findings in this direction [Aamodt and Culotti, 1986; Oakata et al., 1995; Peng and Gygi; Savage and Chalfie, 1991]. These cytoskeleton components are proved to play mainly enzyme and secretory functions in the pancreas, and as neuro-filaments in the neuro-transmission processes in the brain, respectively, by respective cascade regulatory pathways [Takeda et al., 1989; Putaala et al., 2001].

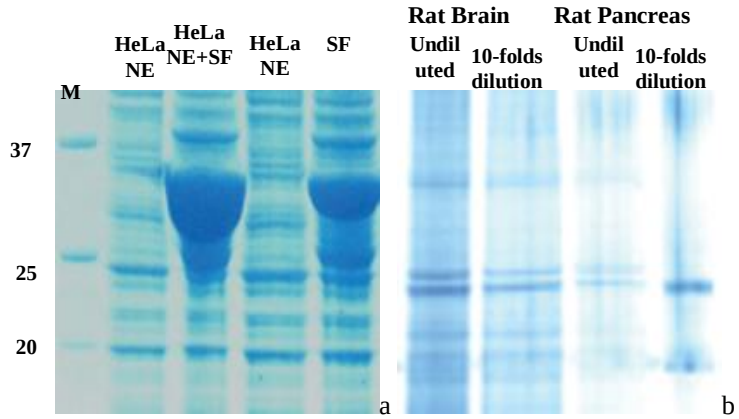


Fig. 7. Separation of proteins from protein lysates: a) from NE from human malignant cervical carcinoma HeLa cells (lines 2 and 4) and human SF (line 5), as well as mixture of from both probes (line 3) marker (M) (line 1); b) from rat brain (line 1 – undiluted probe and line 2 – 10-folds dilution) and pancreas (line 3 – undiluted probe and line 4 – 10-folds dilution), previously pushed by GSH-Agarose columns.

These data confirmed the usability of the laboratory rodents as appropriate experimental models in their role as analogues of humans in investigation of cascade regulatory pathways, proposed in our previous studies [Sainova et al., 2013].

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