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Experimental Rodent and Human *In Vitro*-Models for Balanced Activity of Oncogenes and Tumor-Suppressor Genes

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ABSTRACT

The importance of coordinated oncogenes and tumor-suppressor genes activity in malignancies and death control has been examined in different points of view. Abilities about safe in vitro-differentiation of embryonic and adult stem cells in different directions, were tested. Normal mouse embryonic stem cells (mESCs) and adult progenitor cells, as well as malignant cells HeLa and RIN-5F were used. The absence of phagocyte differentiation of myeloid progenitors in co-cultivation with mESCs, containing additional oncogene, differed from the data, observed in co-cultivation with RIN-5F and HeLa cells. These results suggested a preserved normal/non-malignant characteristics of the mESCs, and have been confirmed by similarity with immortalized normal human trophoblasts. These data also proposed analogy of both malignant cell lines, supported by the established analogous role of tumor-suppressor proteins SCGN and HACE1 by similar cascade regulation mechanisms. In confirmation of that was the established HeLa nuclear proteins as common with the involved in nurturing of chondrocytes in the cartilage tissue synovial fluid proteins. Improved in vitro-propagation capacity of human limbal progenitor cells were observed on chemically-modified vitelline membranes. In this way, novel abilities for application of stem cells for different goals, with simultaneous prevention of malignant transformations, by incubation in appropriate laboratory conditions, could be revealed.

Keywords: *normal cells, malignant cells, oncogenes, tumor-suppressor genes, cell transfection, cell transfection*

INTRODUCTION As most important in the control of malignancies on the one hand and death on the other, the role of coordinated oncogenes and tumor-suppressor genes activity has been proven [1, 2, 3, 4, 5]. In this aspect, cytokine-activated Janus-kinases (JAKs)–signal transducers and activators of transcription (STATs) pathway has shown an important role in the control of immune responses, and dysregulation of it has been associated with various immune disorders [6]. Signaling strength, kinetics and specificity of this pathway have been shown to be modulated at many levels by distinct regulatory proteins.

Studies on the biology of the stem cells are often focused on their self-renewal and differentiation [2, 3, 4, 7, 8, 9, 10, 11, 12, 13]. A lot of variations of the DNA-repair efficiency have been established to vary greatly among different stem cell types [7, 8]. This high self-renewal potential of the stem cells in *in vitro*-conditions makes them strong candidates for delivering of genes, as well as for restoring organ systems function have been found to be included in these processes [2, 3, 4, 9]. This understanding could be applied toward the ultimate goal of using stem cells not just for various forms of therapy, but rather as a tool to discover the mechanisms and means to bring, reconstituting them from old and young individuals has exhibited indistinguishable progenitor activities both *in vivo* and *in vitro* [3, 11]. The properties of "malignant stem cells", have outlined initial therapeutic strategies against them [4, 12].

As the most important approaches, currently utilizing stem cells, both gene therapy and tissue engineering have been determined [14, 15, 16, 17, 18, 19, 20]. Both have been found to exploit the current knowledge in molecular biology and biomaterial science in order to direct stem cells *in vitro*- and/or *in vivo*-differentiation to desired lineages and tissues. In this aspect, a lot of studies have been directed to revealing of the ability for laboratory cultivation of viruses in cell cultures, with the aim for development of both viral recombinants for malignant immunotherapy and of products for therapy of

these disorders. As such tools can be used both DNA- and RNA-viruses [14, 15, 17, 19, 20], as well as bacterial plasmids [18] and yeasts [16]. As a novel method for identification of target proteins, protein protein interactions and regulation pathways, label-free mass-spectrometry has been developed and applied in the last years [21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32].

In this connection, the main goal was connected with establishment of novel safe pathways for experimental balanced activity of oncogenes and tumor-suppressor genes, as well as of their protein products, in both normal and malignant cells with human and rodent origin.

MATERIALS AND METHODS

Stem cells, isolated from mouse Balb/c embryos, were cultivated for 48 – 72 hours on previously formed monolayers of feeder primary MEFs after their previously treatment by Mitomycin-c (mm-c) (Sigma-Aldrich) and/or 3T3 fibroblasts. After tripsinization, they were transfected by electroporation (5 X 10^6 cells/ml). For this aim, recombinant DNA-genome from *adeno-associated virus* (*AAV*) (*Parvoviridae*) [17], containing promoter for gene, coding Elongation Factor 1-alpha (*EF1-α*); gene *Dcn1*, isolated from 3T3 fibroblasts of laboratory mice Balb/c, as well as gene for neomycin resistance, isolated from bacterial DNA-plasmid, are used. For this goal, electroporator for cell transfection (BioRad) was 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. On the other hand, cell cultures of the derived from human cervical carcinoma cell line HeLa were also prepared. All cells were incubated at 37^0C 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). Malignant cells from cell lines RIN-5F and HeLa with rat and human origin, respectively, were also used and similarly proceeded. Sub-populations of both cell lines, containing and non-containing additionally-inserted copy of the respective tumor-suppressor gene were applied.

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 previously isolated nuclear DNA of them 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).

Fixed light microscopic slides 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).

Recombinant rat protein SCGN, isolated from transfected with recombinant DNA-vectors *E. coli* bacteria strains, was incubated in previously prepared rat pancreatic and brain lysates, respectively, as anatomical organs, which are known as the most actively expression of *SCGN* gene in building cells. Control probes with incubated SCGN protein in PBS were also similarly prepared and proceeded. For this goal, 10mg recombinant rat protein SCGN, was diluted in 5ml buffer (containing 0.1M $NaHCO_3$ and 0.5M NaCI, pH 8.3), after which 2.5 ml CnBr-Sepharose solution in 1mM HCI was added. In this way, 10 mg recombinant SCGN protein on 2.5 ml Sepharose volume was obtained, it was blocked by 0.1M Tris-Base and washed several times with Acetate and Phosphate buffers with low and high pH-values. Rat brain and pancreas were isolated mechanically homogenized, sonicated and centrifuged, and after pick up the supernatants. Subsequently, the prepared sepharose solution described above, was separated on 3 equal parts, for approximately 3.3mg recombinant SCGN protein on each probe be received, and each one part was added to each probe, planned to be tested, by shaking for 2 hours for connection of the proteins from them with the recombinant protein SCGN. After centrifugation, the supernatants were taken off and the pellets, containing sepharose and protein material, were washed 3-4 times with PBS, after which the proteins were eluted by two times washing with 0.1M Glycine buffer (pH 2.8) and after that - with 0.1M Diethanolamine (pH 11). The transfection of cells RIN-5F with recombinant gene constructs, containing *SCGN* gene, as well as the subsequent isolation of recombinant SCGN protein and its incubation with brain and pancreatic lysates were performed with mass-spectrometry assay (LC-MS/MS), downstream informatics platform nuclear extracts (NE) of non-transfected human malignant

cells HeLa and protein extracts from synovial fluid (SF) from human knee joint. Protein concentrations were measured by using of Bradford assay and individual samples, containing 20µg HeLa cell nuclear protein extract; 20µg HeLa nuclear extract with 60µg SF protein extract and 60µg SF were prepared. Similarly, samples, containing 5, 10, 20 and 40µg protein extract, were prepared from each of the probes, containing recombinant rat SCGN. 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 centrifugation, the supernatants were turned off and the pellets were diluted in LDS (lithium dodecylsulphate) buffer. The supernatants from the probes, containing SCGN, were precipitated with 3 volumes of cold 100% EtOH, after which the so prepared pellets were subjected on the described procedure. After SDS-PAGE and consequent 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 LC-MS/MS. For analysis, peptides were dissolved in 20µl solvent, containing 5% acetonitrile and 0.1% formic acid. Peptide mixtures were analysed on an LTQ-Orbitrap, hyphenated with an Agilent 1100 LC system. Peptides from the HeLa NE and HeLa NE + SF were analyzed in triplicates, while the peptides from the SF, SCGN-brain pull-down and SCGN-pancreas pull-down were analyzed once. The obtained raw files were processed with the MaxQuant software, using the label-free quantification option. Data was searched against respective protein databases (IPI Human 3.72 for HeLa/SF study and IPI Rat 3.72 for SCGN study), concatenated with reversed 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. Protein identifications and label-free quantification (LFQ) results were processed by the Perseus-program. Briefly, details regarding protein identification (number of identified peptides, IPI/Uniprot accession number, gene name) and label-free quantification results were extracted and loaded into Perseus. Proteins determined to be contaminants, reverse database hit or protein identified by less than 2 peptides were removed. LFQ intensity was log2 transform and the data were imputed in order to remove missing values and fit them in a normal distribution (Width = 0.3; Shift = 1.8). T-test was performed on the transformed, imputed LFQ values with p-values threshold of either 0.05 or 0.001 and the S0 value (artificial within group variance) was switched to either 0 or 1.

RESULTS AND DISCUSSIONS

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

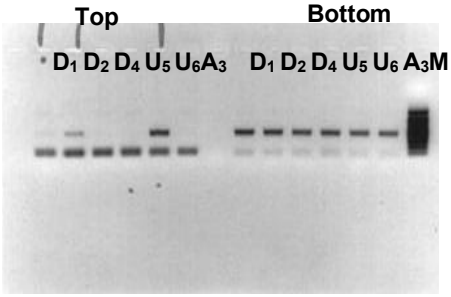

Figure 1: Agarose gel electrophorhesis for prove of the presence and/or the absence of additionally-inserted copy of the oncogene *Dcn1* in cell clones, derived from transfected by electroporation *in vitro*-cultivated mESCsIn *in vitro*-co-cultivation of the so derived myeloid and lymphoid precursors with positive and negative on the additionally-inserted copy of oncogene *Dcn1* transfected cells, no immune reaction is observed against both genetically-manipulated cell types, which could be accepted as a proof for their safety. On the other hand, signs of increased degree for both myeloid and lymphoid differentiation in the presence of transfected cells, positive on additionally-inserted copy of oncogene *Dcn1*, is indicated. Myeloid differentiation and suppression on the cell proliferation has also been established in cultivation of cell sub-populations in the presence of Doxicyclin **(Fig. 2 - a)**. These effects could be explained with the

described in many literature sources activation effect of this substance on the tumor-suppressor genes of *STAT*-family [6]. 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 variation *Cdk2ap1*, by a mechanism of *Oct2/4* promoter methylation [33].

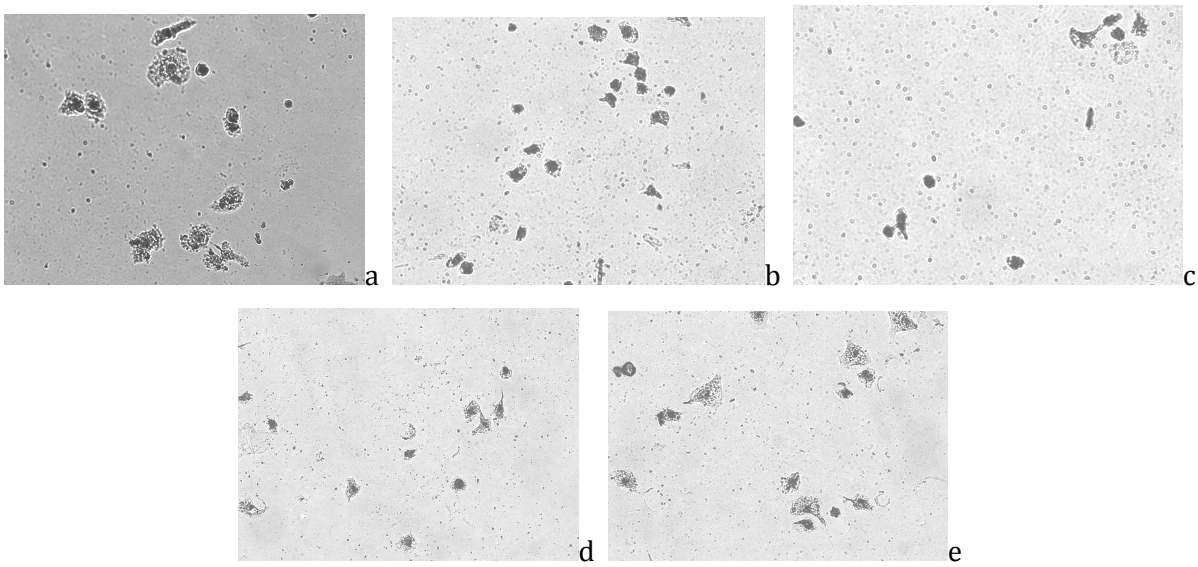

Figure 2: *In vitro*-cultivated sub-populations of non-transfected mESCs, derived by activation of tumor-suppressor genes from *STAT*-family: in the absence of additional external factors of differentiation (a); in the presence of transfected mESCs, containing additional copy of oncogene *Dcn1* (b); in the presence of transfected malignant cells from rat insulinoma cell line RIN-5F, containing additional copy of tumor-suppressor gene *SCGN* (c); in the presence of transfected malignant cells from human cervical carcinoma cell line HeLa, containing additional copy of tumor-suppressor gene *HACE1* (d); in the presence of non-transfected malignant cells from human cervical carcinoma cell line HeLa (e)

The absence of phagocyte differentiation of myeloid cell progenitors, co-cultivated with derived by us transfected mESCs, containing additional oncogene copy **(Fig. 2 - b)**, was different of the results, observed in their co-cultivation in the presence of malignant cells **(Fig. 2 – c, d, e)**. These results could be accepted as a proof for the safety and their non-tumorigenic/normal cell characteristics *in vitro*, as well as an indication about eventual over-expression of the experimentally-activated oncogene in genetically-manipulated normal cells and they were additionally confirmed by the analogy of the so derived cells from animal embryos with normal human trophoblast cells, immortalized by their transfection with recombinant gene vector, based on *SV40* [19, 20, 34, 35]. Furthermore, this immortalization of human trophoblasts has later been proven to be a result of influenced genes from *Cul*-group [34, 35], included in ubiquitination process, in which has also been indicated to be included oncogene *Dcn1*, additional copy of which was inserted by us in mESCs [36, 37, 38]. According these results, taking in consideration literature data antagonistic role between this oncogene and tumor-suppressor gene *HACE1* in the process of ubiquitination was also proposed [20, 34, 36, 37, 38]. On the other hand, the data, obtained in co-cultivation of myeloid progenitor cells with transfected malignant cells RIN-5F and HeLa, containing additional copy of tumor-suppressor genes *SCGN* and *HACE1*, respectively **(Fig. 2 – c and d)**, differed from the data, noticed in co-cultivation with non-transfected HeLa cells, where the strongest phagocyte differentiation signs were observed **(Fig. 2 - e)**, which suggested any eventual decrease of *in vitro*-malignancy of genetically manipulated rodent and human malignant cells. The so observed data in co-cultivation of myeloid progenitors with malignant cells from both lines motivated us to propose analogy of rat insulinoma cell line RIN-5F with human Hela carcinoma cell line, on the one hand, as well as, on the other, between tumor-suppressor genes *SCGN*

and *HACE1*, respectively, which additional copies were inserted in anyone of both malignant lines. The initial experiments aimed at establishing the power of the LC-MS/MS system and MaxQuant software to distinguish artificially mixed protein mixtures from each other. First it had to be determined what amount of HeLa NE presents a good "background", to which SF should be added **(Fig. 3)**.

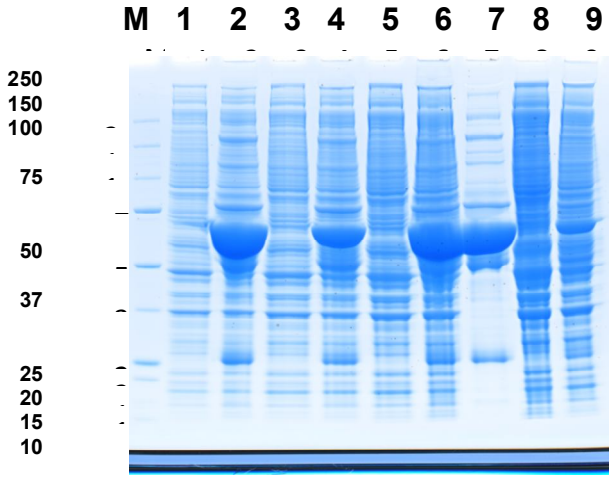

Figure 3: Optimization of HeLa NE and SF protein mixture amounts

A combination of 20µg HeLa NE and 60µg SF was chosen for further analysis (lanes 1, 2 and 7). Gel lanes were fractionated into 23 slices and trypsin in-gel digested. From the gel slice of each lane 5 (3-7) were chosen for further analysis as they contain a number of high and low abundance proteins and are far from the abundant albumin lane. First we looked at the overlap of protein identifications between the HeLa NE and the SF lane. According to the obtained results, almost 700 proteins were identified in HeLa NE and 130 proteins - in SF, with 48 proteins being observed in common for both samples **(Fig. 4)**. These proteins were characterized as important about the nurturing of chondrocytes in the cartilage tissue. Similarly, improved *in vitro*-propagation capacity of human limbal progenitor cells on chemically-modified vitelline membranes have been observed [39].

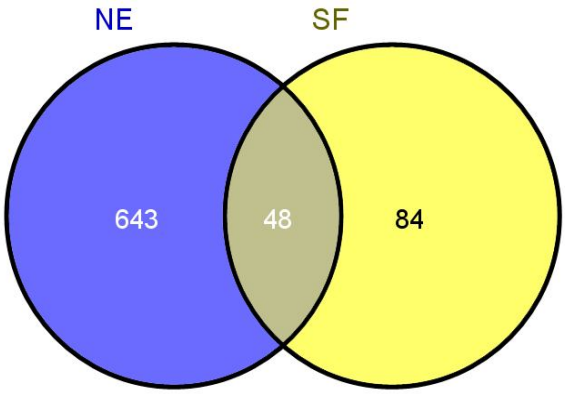

Figure 4: Overlap of the protein identifications between HeLa NE and SF

Protein identifications not identified in the HeLa NE sample were termed unique for the SF and the other 44 protein identifications were manually evaluated for number of peptides, associated with each sample and their intensity. 33 of these 48 common proteins have been characterized as specific for SF extract and the other 15 - either for HeLa NE or for both samples. Those proteins that belonged exclusively to the SF sample were labeled in the MaxQuant output files. They were used further to establish the quantification criteria for LFQ algorithm. Upon processing the LFQ data with Perseus (HeLa vs HeLa+SF), different combinations of p-value and S0 value were tried. During each try, the proteins identified exclusively in the SF sample were used as a reference. **Fig. 5** shows the position of the SF proteins on a graph, generated by plotting the t-test difference on the x-axis vs -Log10 of the p-value on the y-axis. The proteins from the SF sample are almost completely localized on the left side on

the cloud of common protein in the middle of the graph. When applying specific filters (p-value threshold 0.001 and S0 value of 1), almost all of the SF proteins were determined as significantly different between the HeLa and HeLa + SF sample **(Fig. 5 - b)**. Nevertheless, there was a large number of proteins (from the HeLa/HeLa+SF) sample that were shown as significant.

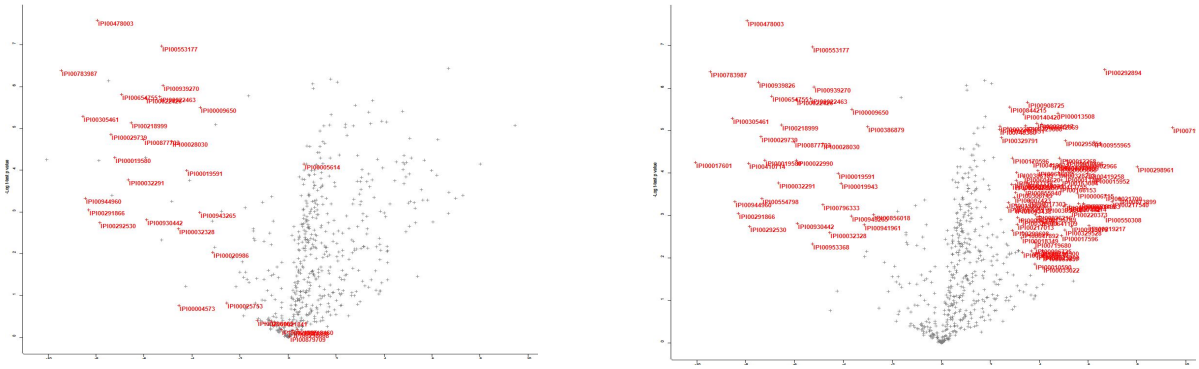

Figure 5: Label-free quantification of HeLa and HeLa+SF sample: Red dots (ID numbers) show proteins that were identified exclusively in the SF sample (a); Red dots (ID numbers) show proteins that were determined to be significantly different between the samples (b)

Similarly, **Fig. 6** and **Fig. 7** show the distribution of proteins from rat brain and pancreas lysates on molecular weight, as well as the distribution of proteins both anatomic organs, to which recombinant rat SCGN protein, incubated lysates of these organs, is specifically or non-specifically connected, respectively.

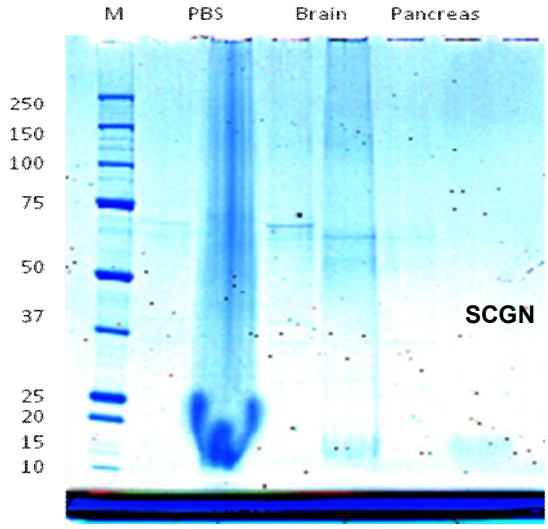

Figure 6: LDS-PAGE of proteins, isolated rat brain and pancreas with incubated recombinant rat protein SCGN in them: lines 2 and 3 - recombinant rat SCGN protein, incubated in PBS (control); lines 4 and 5 - incubated in protein lysate from rat brain; lines 6 and 7 - recombinant rat protein SCGN, incubated in protein lysate from rat pancreas

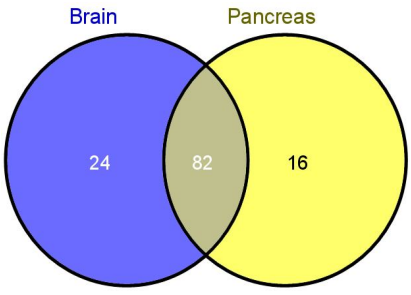

Figure 7: Overlap of the identified proteins between SCGN pull-down in Brain and Pancreas lysates

Studies of the interacting partners of SCGN resulted in much fewer protein identifications (122 for both brain and pancreas sample) (Table 1).

TABLE-1**:** SPECIFIC PROTEINS OF RAT BRAIN AND PANCREAS LYSATES, EVENTUALLY INTERACTING WITH RECOMBINANT RAT PROTEIN SCGN

2-phospho-D-glycerate hydro-lyase	1 Cys peroxiredoxin	1,4-alpla-D-glucan glucanohydrolase
Aal249	56 kDa selenium-binding protein	14-3-3 protein zeta/delta
Actin-like protein 3	Actin, aortic smooth muscle	37 kDa laminin receptor precursor
Adenine-nucleotide translocator 2	ADP-sugar pyrophosphatase	38 kDa BFA-dependent ADP-ribosylation substrate
Alpha-amylase	Anionic trypsin I	40S ribosomal protein S3
Alpha-tubulin	Beta-MPP	60S ribosomal protein
ATP synthase subunit alpha, mitochondrial	Cationic trypsin III	Annexin A
ATP synthase subunit beta, mitochondrial	Chromodomain helicase DNA-binding protein 3	Arginase-1
Ba1-667	Chymotrypsin B chain A	ATP-dependent RNA helicase eIF4A-2
Beta-catenin	Chymotrypsin-like elastase family member 1	Bleomycin hydrolase
Cellular myosin heavy chain, type A	Chymotrypsin-like elastase family member 2A	Calgizzarin
Cofilin, non-muscle isoform	Cytokeratin-1	Catalase
Complement component 1 Q sub-component	Dual specificity phosphatase 14	Cornifin-A
Cyclophilin A	LRRGT00066	Cytovillin
Cytokeratin-39	Proteasome component C3	Dermcidin
Fatty acid-binding protein 3	Similar to transglutaminase E3 (Predicted)	Dsp protein
Glutathione-binding 13 kDa protein	Secretory lectin ZG16	Elongation factor 2
Heat shock 86 kDa		Epidermal TGase
Hemopexin High sulfur protein B2E		H1d HBPZ3
Inosine phosphorylase		Heat shock protein
LOC367586 protein		Histon H4
LOC500183 protein		Junction plakoglobin
Myl6 protein		Lamin-A
Pyruvate kinase isozymes M1/M2		LDH muscle sub-unit
Rho GDP-dissociation inhibitor 1		Malectin
Ribonuclease/angiogenin inhibitor		Plakophilin 1
Superoxide dismutase Cu-Zn		desmoglein 1 beta
Transgelin-2		Histone H3
Tubulin beta-5 chain		Tubulin beta-2 chain
Type II keratin Kb25		Ubc protein

These data supported the usefulness of the used method for precise establishment of novel protein-protein molecular interactions, but also for the establishment and investigation of different influences of bio-molecules by cascade pathways. In this way, target proteins, which could regulate functions of other proteins, could be identified, and thus, novel regulatory mechanisms on different functions of proteins with oncogenic, as well as of such with tumor-suppressor actions, might be revealed. Some proteins, such as the intercellular enzyme ADP-sugar pyrophosphatase and some proteolytic enzymes in pancreas lysate, as well as with some of the cytoplasmic cytoskeleton proteins, were specifically identified in each sample (Table 1). ADP-sugar pyrophosphatase has been found to be a nucleoside-diphosphate hydrolase enzyme, that catalyzes the hydrolytic breakdown of ADP-glucose, linked in this way with glycogen biosynthesis and eventually, with intercellular communications [40]. Such proteins have also been established to be important about cleaning of the cells of deleterious endogene metabolites eventually by nucleotide recycling from free ADP-ribose molecules might probably be connected with suppression of NAD on the process of T-lymphocyte activation [41]. In accordance with other literature data, a lot of analogical functions of proteins SCGN and HACE1 in as tumor suppressors and members of similar cascade mechanisms have been observed [18, 24, 32, 41, 42, 43, 44, 45, 46, 47]. These data could be supported by the established calcium-dependent SCGN–TAU interaction, and subsequent co-appearance of both proteins [43, 45, 47]. According other literature data, a lot of analogical functions of proteins SCGN and HACE1 in as tumor suppressors and members of similar cascade mechanisms have been observed [24, 40, 42, 44]. Data about the analogical role of both proteins in the processes of Golgy-transport of proteins during cell division have also been received [24, 46, 48]. Both molecules have been indicated to be able to connect with cytoskeleton elements of the cell cycle as microtubule proteins [46, 47, 48, 49, 50] and cyclins [40, 44], which on the other hand are known to be able to connect with histones and histone-like nuclear proteins [42].

CONCLUSIONS

Future investigations about the eventual abilities of proteins SCGN and HACE1 to participate in direct and/or indirect intra- and extracellular interactions with nuclear proteins as eventual members of

cascade regulatory mechanisms, are necessary. In this way, regulatory functions of both proteins on the mechanisms of cell division could be revealed.

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