SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PhD)

INVESTIGATION OF RAS ONCOGENIC SIGNALING MODULATORS IN THE FORMATION AND PROGRESSION OF CANCER

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The Examination takes place at Department of Biophysics and Cell Biology (Room 2.306), Faculty of Medicine, University of Debrecen
at 11:00 on 14th of November 2014

Head of the **Defense Committee**: Szöllősi János, DSc
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The PhD Defense takes place at the Lecture Hall of In Vitro Diagnostic Building, Faculty of Medicine, University of Debrecen
at 13:00 on 14th of November 2014

INTRODUCTION

The implication of Ras proteins in pathological processes such as cancer has always been at the leading edge of molecular oncology. The aim of this thesis is to experimentally investigate and support the potential oncogenic significance of two, tumor-promoting phorbol-ester sensitive protein family: Ras guanil nucleotide releasing peptids and protein kinase C, as upstream and downstream regulators of Ras-related signaling. The thesis is divided into two parts: (1) investigation of the role of the newly discovered Ras upstream regulator, Ras guanil nucleotid releasing peptid 3 in human breast cancer formation and (2) the investigation of the role of Ras downstream modulator protein kinase C δ in a rare form of human skeletal muscle cancer, rhabdomyosarcoma.

RASGRP3 CONTRIBUTE TO THE FORMATION AND PROGRESSION OF HUMAN BREAST CANCER

The protein products of *ras*, collectively referred to as Ras, are present in every cell and are involved in the transmission of signals from the cell surface to the nucleus, Ras GTPase family are crucial players in many signaling networks. Ras is the common upstream molecule of several signaling pathways including Mitogen-Activated Protein Kinase (MAPK) and PhosphoInositide 3-Kinase (PI3K) cascades linked to the functional control of cellular outcomes including nuclear events and in signal transduction pathways that control proliferation, migration, apoptosis and senescence. Amplification of *ras* proto-oncogenes and mutations that lead to the expression of constitutively active Ras can be found in a variety of tumor types.

Ras is able to fulfilI diverse functions through a common molecular switch that cycles between GTP-associated active state (RasGTP) and GDP-bound inactive state (RasGDP). The tight regulation of the guanine nucleotide bound state is critical for the regulation of normal cellular proliferation. One class of regulatory proteins is the guanine nucleotide exchange factors (GEFs), whose physiological function is to convert Ras from a GDP- to a GTP-bound

state. Several mammalian guanyl-nucleotide exchange factors have been identified, in 1998, Ebinu et al. have introduced a new RasGEF called Ras Guanyl nucleotide Releasing Peptide or RasGRP.

A unique feature of these proteins is the presence of conserved domain-1 (C1) domain serving as a binding site for phorbol-estres and its physiological counterpart, diacylglycerol (DAG) regulating the translocation of these proteins from the cytoplasm to membranes such as cell membrane, nuclear membrane or Golgi –complex. RasGRP1 and RasGRP3 activity is also regulated by phosphorylation, very likely occurs through PKC kinases, which themselves can get recruited to the membrane by DAG. Phosphorylation appears to have an enhancing effect to stimulus-dependent Ras activation.

RasGRP3 has recently emerged as a player contributing to prostate cancer and melanoma. It was shown that RasGRP3 mRNA levels are elevated in patients with cancer as compared to non-diseased individuals. *In vitro* experiments using RasGRP3 gene-silencing revealed that down regulation of RasGRP3 expression decreased basal RasGTP levels, interfered with cell growth, and increased apoptosis. Similar results were obtained *in vivo*; using SCID mouse model, the decreasing levels of RasGRP3 expression inhibited growth of RasGRP3 gene-silenced prostate cancer and melanoma cell lines. Reduction of RasGRP3 levels caused diminished levels of growth factor-induced phospho-Akt (pAkt) and to lesser extent phospho-ERK ½ (pERK ½).

PROTEIN KINASE Cδ PROMOTES *IN VITRO* AND *IN VIVO* PROLIFERATION OF RHABDOMYOSARCOMA CELLS

Protein kinase C family members are serine/threonine-related protein kinases that sit at the crossroads of many signal transduction pathways and are implicated in a wide range of growth factor-dependent cellular responses including proliferation, apoptosis, and differentiation. PKCs contain a C1 domain responsible for DAG binding, generated as a

product of PLC activation. Subsequently, different isoforms have been discovered among PKCs; grouped into three subclasses based on structural and regulatory characteristics: (1) the classical isoforms (cPKCs; α, βI, βII and γ); (2) novel PKCs (nPKCs; δ, θ, ε and η) and (3) atypical PKCs (aPKCs; ζ ι and λ). Main PKC downstream events include the activation of MAPK and PI3K cascades.

The discovery of PKC as the major intracellular receptor for the tumour promoting phorbol esters suggested that the activation of PKC is involved in cell transformation. In cancer cells, PKC isozymes are involved in cell proliferation, survival, migration, angiogenesis and anticancer drug resistance through their increased or decreased participation in survival or proliferation-associated signaling pathways, such as Ras/Raf/MEK/ERK ½ or PI3K/Akt/mTOR pathways. In general, overexpression of PKC isozymes is closely related to poor prognosis, poor response to chemotherapy and poor patient survival. Among PKC isozymes, PKCα, β, ε, and δ have been the most broadly studied isozymes in relation to cancer, this study is focussed on the investigation of the roles of PKC δ in rhabdomyosarcoma (RMS). RMS is a rare form of pediatric soft tissue cancer affecting muscles throughout the body. It accounts for approximately 50% of all pediatric soft-tissue sarcomas and for 7–8% of all childhood malignancies. The etiology and pathogenesis of RMS are still poorly understood. The clinical outcome of RMS is generally poor, therefore, understanding the molecular pathways that contribute to the pathogenesis and developing molecularly targeted therapies is urgently needed.

Extremely limited information is possessed about the isoform-specific involvement of the PKCs in the regulation of physiological and pathological *in vitro* and *in vivo* growth of skeletal muscle cells. It has been previously shown that nPKCδ plays a pivotal and exclusive role in mediating the in vitro growth of human skeletal muscle cultures and in the mouse C2C12 skeletal muscle myoblast cell line. nPKCδ is a key player in promoting cellular

growth and inducing malignant transformation. Overexpression of the constitutively active nPKCδ stimulated *in vitro* growth of C2C12 myoblasts. nPKCδ overexpresser C2C12 cells, initiated the development of large and malignantly transformed rhabdomyosarcomas in SCID mice. These findings introduced this isoform as a promising target for the treatment of skeletal muscle malignancies.

Although only few reports are available on describing the expression profile of the PKC family in RMS, involvement of individual PKC isozymes and their use as therapeutic targets are beginning to be explored.

AIMS

In light of the potential oncogenic effect of RasGRPs the overall aim of the study was to collect valid and reliable information on the change of expression and potencial function of RasGRP3 in breast-derived ductal adenocarcinoma.

Since nPKCδ is reported to play a pivotal role in mediating the malignant transformation of C2C12 myoblasts, an attempt was made to further dissect the role of nPKCδ in the regulation of *in vitro* and *in vivo* growth of human rhabdomyosarcoma cells.

MATERIALS AND METHODS

HUMAN STUDY

The human study was approved by the Institutional Research Ethics Committee of the University of Debrecen (Debrecen, Hungary) and by various authorities of the Hungarian Government. Samples were obtained as part of routine diagnosis from surgical specimen. The tumor samples were divided into two parts. One part was processed to routine haematoxylin-eosin staining-based grading, the second part was frozen in liquid nitrogen and processed for Q-PCR and Western blot analyses.

CELL CULTURING

During the RasGRP3 study, we employed a human primary breast derived ductal adenocarcinoma cell line (BT-474), five different metastatic ductal adenocarcinoma cell lines (MDA-MB-453, MCF7, SK-BR-3, JIMT-1, T-47D) and as a positive control, a prostate derived carcinoma cell line (PC-3). PC-3, BT-474 and T-47D cells were maintained in RPMI-1640 Medium, MCF7 cells were maintained in Eagle's Minimum Essential Medium (EMEM), JIMT-1 cells were maintained in 1:1 Dulbecco’s Modified Eagle Medium (DMEM)/Ham’s F12 Medium, SK-BR-3 cells were maintained in Dulbecco’s Modified Eagle Medium (DMEM) and MDA-MB-453 cells were maintained in Leibovitz’s 15 Medium. All medium were supplemented with 10% (v/v) fetal bovine serum (FBS), 2 mM Glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin. In addition, medium for T47-D cells was supplemented with 0.2 U/ml bovine insulin, medium for MCF7 cells was supplemented with 0.01 mg/ml bovine insulin and medium for JIMT-1 cells was supplemented with 60 U/l bovine insulin. Medium was changed every other day and cells were sub-cultured at 80% confluence at 37 °C in a humidified atmosphere with 5% CO_2.

Human rhabdomyosarcoma (RD) cells were maintained in DMEM supplemented with 10% (v/v) FBS, 2 mM Glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin. Medium was changed every other day and cells were sub-cultured at 80% confluence at 37 °C in a humidified atmosphere with 5% CO_2.

IMMUNOHYSTOCHEMISTRY

Histological parameters were determined by a horseradish-peroxidase (HRP) based method with diaminobenzidine (DAB) as a chromogene. Tissues were fixed overnight in 4% paraformaldehyde, followed by paraffin infiltration and embedding. Sections were de-waxed and subjected to heat-induced epitope retrieval in 10 mM citrate buffer (pH 6.0) at 750 W in microwave oven for 10 mins prior to treatment with 3% hydrogen peroxide in absolute methanol. Sections were then incubated in blocking buffer containing 0.6% Triton X-100 and 1% bovine serum albumin for 30 min and probed with the appropriate primary antibodies. Sections were then stained for 30 minutes with the appropriate horseradish peroxidase-labeled polymer conjugated secondary antibodies. Immunoreactions were visualized using DAB substrate for 3-5 minutes and the sections were counterstained by Mayer's hematoxylin and cover-slipped for microscopic examination. To assess specificity of the immunostaining antibodies were either omitted from the procedure or were pre-absorbed by control blocking peptides provided (along with appropriate protocols) by the manufacturers. In addition, to assess the number of proliferating cells, sections were immunostained against the nuclear marker Ki67 using a streptavidine-biotin-complex three-step immunohistochemical technique according to the instructions of the manufacturer.

MICROSCOPY AND IMAGE ANALYSIS

Immunohistochemical images were captured and digitalized using an RT Spot Colour CCD camera integrated on a Nikon Eclipse 600 fluorescence and light microscope.

Digitalized images were then analyzed using Image J (NIH, Bethesda, MD) image analysis software. The averaged number of proliferating (Ki67 positive) cells was measured by counting the total number of Ki67 positive cells at five randomly placed, equal areas of interest (AOI) using a light microscope, and the values were normalized to the total number of cells measured at the fields. Results obtained in each tumor of the same group were then averaged and the mean values were calculated.

MOLECULAR BIOLOGY

Western blotting

Tissues and cells were homogenized in lysis buffer (20 mM Tris-Cl, 5 mM EGTA, pH 7.5 and protease and phospathase inhibitor cocktails) and the protein content of samples was measured by the BCA protein assay kit. Total cell lysates were mixed with 2x SDS-PAGE sample buffer and boiled for 10 min at 100 ^{o}C. The samples containing 20 μg total protein were separated by electrophoresis on 7.5 % or 10 % SDS-polyacrylamide gels and transferred onto BioBond nitrocellulose membranes. After the membranes were blocked with 5% dry milk in PBS and labeled with the appropriate primary and secondary antibodies, the immunoreactive bands were visualized by SuperSignal West Pico or Femto Chemiluminescent Substrate-enhanced chemiluminescence using a Gel Logic 1500 Imaging System. To obtain an endogenous control, membranes were re-probed with an anti-GAPDH or anti-actin β antibodies followed by a similar visualization procedure as described above.

Quantitative real-time PCR (Q-PCR)

Quantitative real-time PCR was performed on an ABI Prism 7000 sequence detection system by using the 5′ nuclease assay, as we have previously described. Total RNA was extracted with TRIzol reagent and reverse transcribed into cDNA by using High Capacity cDNA Reverse Transcription Kit. PCR amplification was performed by using the TaqMan

primers and probes using the TaqMan Universal PCR Master Mix Protocol. The threshold cycle (Ct) of the given protein was determined and normalized to that of human GAPDH to obtain a ΔCt value (Ct_{GAPDH}-$Ct_{protein}$) from each sample. The Q-PCR experiments were performed in triplicate.

Transfections

shRNA construct for RasGRP3

Gene knockdown was achieved by stable transduction with a retroviral-based pRNA-H1.1/Retro Vector system (GenScript) containing a RasGRP3-specific hairpin RNA (shRasGRP3). Scrambled control plasmid consisted of a sequence (shSCR) which has no specific targets in mammalian cells. The two host cell lines, MCF7 and T-47D were infected with each retroviral supernatant and subjected to Hygromycin B (400 μg/ml) selection for 3 weeks. Studies were carried out on the cloned, antibiotic-resistant cells.

Generation of PKC constructs

The cDNA sequences of nPKCδ, and the kinase (dominant)-negative (DN-nPKCδ) were subcloned into a metallothionein promoter-driven eukaryotic expression vector (pεMTH). RD cells were transfected by either the empty pεMTH vector (control cells) or by the vectors encoding the cDNA sequences of nPKCδ, or DN-nPKCδ using a Lipofectamine anionic detergent according to the protocol suggested by the manufacturer. Cells were selected in Geneticin for 3 weeks. Studies were carried out on the cloned, antibiotic-resistant cells.

Determination of cell proliferation

The degree of cellular growth of T-47D and MCF7 cells was determined by measuring the DNA content of cells using CyQUANT GR Cell Proliferation Assay Kit according to the protocol of the manufacturer. The assay was performed each day for five days. Fluorescent

signals were quantitated on a Fluorescence Imaging Plate Reader FlexStation^{III} at an excitation wavelength of 485 nm and an emission wavelength of 530 nm.

The CyQUANT assay was employed to assess the chemotherapeutic sensitivity of the cells. After overnight culturing, various concentrations of chemotherapeutics, i.e. Herceptin and Tamoxifen were administered for 72 h to T-47D or MCF7 cells. After the indicated time, CyQuant GR cell proliferation assay was performed and analyzed.

Proliferation of RD cells was determined by MTT assay. Cells were incubated for 3 hours at 37 °C with 0.5 mg/ml MTT reagent [3-(4,5-dimethilthiasol-2-il)-2,5-diphenyltetrasolium bromide]. The supernatant was discarded, and the cells were solubilised with MTT solubilising solution (10% Triton-X 100 and 0.1 N HCl in adhydrous isopropanol) for 30 min and the concentration of formazan crystals was determined colorimetrically at 550 nm (Fluorescence Imaging Plate Reader FlexStation^{III}) according to the protocol of the manufacturer.

Detection of apoptotic and necrotic cells

Cells were harvested, washed and probed for Annexin-V in a final concentration of 1 μM for 20 min at 4 °C in the dark. Propidium iodide (PI) was then added at a 5 μg/ml final concentration 10 minutes before analysis by flow cytometry using the FACScan system. Data were analyzed with FSC Express software.

Mitochondrial membrane potential was also assessed by MitoProbe DilC_1(5) Assay Kit according to the instructions of the manufacturer. T-47D and MCF7 derived cells were incubated with DilC_1(5) working solution and the fluorescence of DilC_1(5) was measured at 630-nm excitation and 670-nm emission wavelengths using Fluorescence Imaging Plate Reader FlexStation^{III}.

XENOGRAFT EXPERIMENTS

Severe combined immunodeficiency (SCID) mice were bred and maintained in the animal facility of the Department of Dermatology. The studies were performed under the current regulations and standards of the Institutional Research Ethics Committee of the University of Debrecen. Cell pellets [4×10^6 viable cells] were injected in a single subcutaneously site of the mice. When a merked tumor growth was observed the animals were sacrificed. The tumors were excised, and the averaged three-dimensional size, the weigh and the haematoxylin-eosin stainig based histological characteristics of the developed tumors were analyzed. To evaluate proliferation Ki67-specific labeling was performed according to the manufacturer instructions. The dissection of the animals and the histopathological evaluations were performed and verified by expert pathologists.

STATISTICAL ANALYSIS

The data are expressed as mean +/- SEM. Significance differences were assessed by a two-tailed un-paired *t*-test ($p < 0.05$ values were defined as significance).

RESULTS

RASGRP3 CONTRIBUTE TO THE FORMATION AND PROGRESSION OF HUMAN BREAST CANCER

RasGRP3 is expressed in human breast derived ductal adenocarcinoma

Using Q-PCR and Western blot, we assessed the expression of the RasGRP3 protein and phosphoRasGRP3, in human breast derived ductal adenocarcinoma samples as well as in normal human breast tissues. We found that the levels of RasGRP3 and phosphoRasGRP3 were significantly higher in the tumor samples compared to the controls. In addition, we also determined the cellular localizations of RasGRP3 and phosphoRasGRP3 by immunohistochemistry. RasGRP3 was localized in the cytoplasm of the cells whereas the phospho form exhibited mostly nuclear immunoreactivity**.** Q-PCR and Western blot analyses also confirmed the expression of RasGRP3 in six different human breast ductal adenocarcinoma derived cell lines, i.e. in BT-474, JIMT-1, MCF7, SK-BR-3, MDA-MB-453 and T-47D cells, with barely detectable levels in the primary BT-474 cells and in one of the metastatic cell lines, MDA-MB-453.

Inhibition of RasGRP3 expression in T-47D and MCF7 cells

To explore the functionality of RasGRP3, we employed the shRNA-interference technique. The cellular levels of the protein in MCF7 and T-47D cells was suppressed by retroviral vectors expressing shRNAs for RasGRP3 (shRasGRP3). During the experiments scrambled control shRNA (shSCR) sequence was used as a control.

RasGRP3 is involved in the regulation of growth of MCF7 and T-47D breast cancer cells

As revealed by growth curve analysis using fluorimetric CyQUANT cellular proliferation assays, down-regulation of RasGRP3 expression resulted in a significant suppression of cell growth in both cell lines compared to the proliferation of cells bearing the non-targeting shSCR.

RasGRP3 is involved in the regulation of survival of MCF7 tumor cells

To asses the role of RasGRP3 in the regulation of cell survival, annexin-V/PI labeling was performed. As determined by flow cytometric analysis, inhibition of RasGRP3 expression induced apoptosis in MCF7 cells but not in the T-47D cell line. To further support the role of RasGRP3 in the regulation of apoptosis a quantitative fluorimetric MitoProbe^{TM} $DilC_1$ (5) assay was performed. MitoProbe^{TM} $DilC_1$ (5) is a mitochondrial membrane potential sensitive dye which accumulates in the mitochondria in cells with active membrane potential. The staining intensity decreases in cells with disrupt mitochondrial membrane potential- one of the earliest markers of apoptosis. We found that RasGRP3 silencing significantly decreased mitochondrial membrane potential, in MCF7 cells, while no significant change was observed in T-47D cells.

RasGRP3 expression contributes to resistance to Tamoxifen and Herceptin in the MCF7 and T-47D breast cancer cells

We also investigated the sensitivities of "RasGRP3-silenced" MCF7 and T-47D cells to tamoxifen (Tamoxifen) used in endocrine therapy and to the chemotherapeutic drug trastuzumab (Herceptin). Down-regulation of RasGRP3 increased the sensitivity of T-47D cells to the growth-inhibitory actions of both Tamoxifen and Herceptin; i.e. lower concentration induced comparable effects to those found with higher concentrations on cells

expressing the control shSCR. Interestingly, suppression of RasGRP3 levels did not affect the sensitivity of the MCF7 cells to the actions of the drugs.

Down regulation of RasGRP3 suppressed xenograft tumor formation

To assess the role of RasGRP3 in *in vivo* tumor formation, we employed the SCID mouse xenograft model in which tumors were induced by MCF7 and T-47D cells expressing either shRasGRP3 or shSCR. In both cell lines, down-regulation of RasGRP3 resulted in a marked reduction in tumor growth in comparison of those induced by the shSCR-expressing cells. As revealed by the Haematoxylin-eosin staining based immunohystochemical analysis of the xenograft tumors T-47D cells derived tumors showed „ Pushing-type" of growth reflected in an infiltrative and expansive growth pattern with mechanical pressure to the sorrounded tissues. Necrotic areas (NeA) frequently developed in these tumors. Compared to T-47D cells MCF7 derived tumors are composed of more differentiated tumor tissue with less infiltrative nature. The differential features of the various cells on tumorigenesis were also proven by immunohistochemical analysis of the expression of the proliferation marker Ki67. In MCF7 derived xenograft tumors the expression of Ki67 was significantly lower in RasGRP3 silenced cells compared to scrambled control cells.

RasGRP3 is involved in growth factor-induced Akt, ERK1/2 and estrogen receptor activation

Growth factors such as IGF-I and EGF represent important signaling molecules in breast cancer. We therefore evaluated the role of RasGRP3 in modulating the IGF-I and EGF induced activation of the Ras signaling pathway in both MCF7 and T-47D cells. Cells were treated with IGF-I and EGF, and the activation of ERK 1/2 and Akt kinases were examined by Western blot. We confirmed that in both cell lines the down-regulation of RasGRP3 reduced the IGF-I and EGF-induced ERK 1/2 and Akt phosphorylation. Consistent with the central

role of Ras signaling pathway in the activation of ERα, decreasing the level of pERK 1/2 and pAkt led to a reduction in ERα phosphorylation. According to our results MCF7 cells were more sensitive to the growth factors than T-47D cells and the onset of phosphorilation events was different, with MCF7 derived "RasGRP3-silenced" cells appearing to respond to growth factors induced Akt and ERK1/2 down-regulation earlier.

To validate further a role for RasGRP3 in the modulation of the activation of ERα we examined the effect of RasGRP3 gene-silencing on the expression of several ERα-regulated genes, namely progesterone receptor, cathepsin D, cytochrome C and loricrin determined by Q-PCR. Except loricrin we found a significant reduction in the expression of these genes in „RasGRP3-silenced" MCF7 and T-47D cells.

RasGRP3 modulates the expression of IGF-I and EGF receptors in MCF7 cells

To further clarify the role of RasGRP3 on the activation of the downstream signaling molecules, the effect of RasGRP3 inhibition on the expression of upstream IGF-I, EGF, and HER-2/neu receptors was investigated**.** Suppression of RasGRP3 levels decreased the expression of both IGF-I and EGF receptors in MCF7 cells, whereas it had no effect on the receptor expression of T-47D cells. RasGRP3 down-regulation did not modify the expression of HER-2 receptor of the cells.

PROTEIN KINASE Cδ PROMOTES *IN VITRO* AND *IN VIVO* PROLIFERATION OF RHABDOMYOSARCOMA CELLS

Overexpression of nPKCδ in human rhabdomyosarcoma cells

To explore the functionality of nPKCδ in the regulation of *in vitro* and *in vivo* proliferation we have stably transfected human rhabdomyosarcoma derived RD cells either with empty MTH (pεMTH) vector or with vectors encoding the active (nPKCδ) or dominant-negative (DN-nPKCδ) nPKCδ isoforms.

nPKCδ is involved in the regulation of cellular growth of RD cells

As revealed by growth curve analysis using MTT assays, the overexpression of nPKCδ significantly increased the proliferation of RD cells compared to the proliferation of cells expressing either the control (empty MTH) vector or DN-nPKCδ mutant. Furthermore, DN-nPKCδ overexpresser RD cells exhibited a significantly suppressed growth rate when compared to control.

nPKCδ is involved in IGF-I-induced ERK1/2 activation

The insulin-like growth factor pathway are involved in sarcomas, including RMS. Therefore, we investigated the role of nPKCδ in modulating the IGF-I induced activation of the Ras-related ERK 1/2 signaling in RD cells. Cells were treated with IGF-I, and the activation of ERK 1/2 kinase was examined by Western blot. According to our results the overexpression of nPKCδ enhanced the IGF-I-induced ERK 1/2 phosphorylation compared to the control (empty MTH) vector transfected or DN-nPKCδ mutant cells. Overexpression of nPKCδ had no measurable effect on the total ERK1/2 expression level.

Overexpression of nPKCδ increased xenograft tumor formation

To establish the relevance of nPKCδ in RMS tumorigenesis, we assessed the role of the nPKCδ isoform in the *in vivo* tumor formation of RD cells. For this, tumors were induced by RD cells overexpressing either the empty MTH (pεMTH) vector or vectors encoding nPKCδ or its dominant-negative (DN-nPKCδ) mutant. As expected, injection of all RD cell types resulted in tumor development in immunodeficient mice, among them, unless the difference is not significant tumors induced by nPKCδ overexpressors were characterized by the largest three-dimensional size. As revealed by the haematoxylin-eosin staining based immunohystochemical analysis these tumors were diagnosed as malignant rhabdomyosarcomas with high cell division rates (number of mitosis) and infiltrating (malignant) growth properties, very often destructing the neighboring adipose and skeletal muscle tissues. Immunohistochemical analyses of the expression of the proliferation marker Ki67 revealed that in tumors induced by nPKCδ overexpressors were characterized by the highest percentage of Ki67 positive (i.e. proliferating) cells within the sarcomas, the latter value was significantly different from those measured in tumors induced by control (pεMTH) or DN-nPKCδ overexpressing cells.

DISCUSSION

RasGRP3 CONTRIBUTE TO THE FORMATION AND PROGRESSION OF HUMAN BREAST CANCER

Previous studies have clearly identified the existence of RasGRP3 on metastatic prostate cancer and melanoma. However, we are the first to describe and quantitatively asses the expression of RasGRP3 and its active form on human breast ductal adenocarcinoma cells. Q-PCR, Western blot and immunohistochemical analyses revealed that the expression of RasGRP3 and the phosphoRasGRP3 are elevated in numerous human breast tumor samples as well as in multiple breast derived ductal adenocarcinoma cell lines. We have also presented that the relative level of RasGRP3 and phosphoRasGRP3 expressions in the tumor tissues was markedly higher compared to the normal tissues. Of further importance, we are the first to show that the subcellular localization pattern of RasGRP3 and its active form are markedly different; i.e., RasGRP3 is localized mostly in the cytoplasm whereas phosphoRasGRP3 showed prominent nuclear staining in the breast cancer cells. Both *in vitro* and *in vivo* studies were carried out to define the exact functional role of RasGRP3 using RasGRP3 gene silencing in ductal adenocarcinoma derived MCF7 and T-47D cell lines. Down-regulation of RasGRP3 inhibited cell proliferation of both cell lines and induced apoptosis in MCF7 cells. Suppression of RasGRP3 expression reduced the ability of both T-47D and MCF7 cells to form tumors in SCID mice, moreover we were the first to show that in the case of MCF7 cells RasGRP3 down regulation decreased Ki67 positivity, well-known to be important in molecular classification of breast cancers. Although it was confirmed that modulation of RasGRP3 contributes to Akt and ERK1/2 activation in prostate cancer and melanoma cells, here we are the first to demonstrate that RasGRP3 contributed to signaling downstream of IGF-I and EGF by modifying the level of IGF-I and EGF induced phosphorylation of Akt and ERK 1/2. Namely, down-regulation of RasGRP3 decreased the level of phosphorylated Akt

and ERK 1/2 induced by both IGF-I or EGF stimulation in both cell lines, but with a different activation profile. MCF7 derived "RasGRP3-silenced" cells appearing to respond to growth factors induced Akt and ERK1/2 down-regulation earlier and more effectively. Furthermore, RasGRP3 not only caused the down regulation of IGF-I and EGF induced downstream signaling, but also decreased the expression of IGF-I and EGF receptors in MCF7 cells. Moreover, we demonstrated the role of RasGRP3 in decreasing the phosphorylation of ERα through the down-regulation of Akt and ERK 1/2 pathways both in T-47D and MCF7 cells. It can be supposed that in T-47D cells by decreasing ERα transactivation RasGRP3 down-regulation contributed to an increased Tamoxifen sensitivity. Besides Tamoxifen we were the first to investigate the role of RasGRP3 in regulation of trastuzumab sensitivity of T-47D and MCF7 cells. Although the down regulation of RasGRP3 had no effect on HER-2 expression of the cell lines, in T-47D cells by decreasing the level of phosphorylated Akt and ERK 1/2 induced by IGF-I or EGF stimulation can contribute to an increased sensitivity of the cells against trastuzumab. Collectively, our current findings identify RasGRP3 as a novel, promising target molecule for pathway directed chemotherapy in the supportive treatment and diagnosis of ductal adenocarcinoma of human breast.

PROTEIN KINASE Cδ PROMOTES *IN VITRO* AND *IN VIVO* PROLIFERATION OF RHABDOMYOSARCOMA CELLS

cPKC and nPKC isozymes play differential roles in regulating the *in vitro* proliferation of myoblasts as well as *in vivo* tumor growth induced by these cells. According to our previous results the overexpression of the constitutively active nPKCδ stimulated whereas the kinase inactive DN-nPKCδ mutant inhibited *in vitro* growth of C2C12 myoblasts. Moreover, nPKCδ overexpresser C2C12 cells, when injected to immunodeficient mice, initiated the development of large and, of great importance, malignantly transformed rhabdomyosarcomas in contrast to control myoblasts which induced benign tumors of much

smaller size. These findings introduced nPKCδ as a significant player in skeletal muscle pathology positively controlling cellular growth. PKC isozymes have been implicated in the pathogenesis of many human cancers including breast, colon, lung, prostate, pancreatic, liver and hematopoietic cancers. Rhabdomyosarcoma is a group of aggressive muscle cancers and the most common soft tissue sarcomas in children. It is demonstrated that phosphorylation levels of PKCα, δ, θ, and ξ are upregulated in human RMS and hence could play roles in the development or progression of the disease. We conducted the first known *in vitro* and *in vivo* studies to define the exact functional role of nPKCδ in RMS tumorigenesis using recombinantly modified RD cells. Similar to those described in case of C2C12 cells, overexpression of nPKCδ enhanced cell proliferation of RD cells compared to control (empty vector transfected) cells or DN-nPKCδ mutant. Of further importance, overexpression of nPKCδ increased xenograft tumor growth as well as the proliferation rate of the developed tumors (Ki67 positivity). Interestingly, the size of tumors induced by cells overexpressing the inactive DN-nPKCδ did not differ from the control tumors suggesting that other factors than nPKCδ may also be involved in promoting the aggressive growth of RMS-derived cells. These data suggest that nPKCδ may play a central role in RMS tumorigenesis. Although it was confirmed that RMS overexpresses IGF-IR, here we are the first to demonstrate that nPKCδ contributed to signaling downstream of IGF-I in RD cells by modifying the level of IGF-I induced phosphorylation of ERK 1/2. Namely, overexpression of nPKCδ increased the level of ERK 1/2 phosphorilation induced by IGF-I. Collectively, these data describe nPKCδ as a novel key player in promoting cellular growth and inducing malignant transformation of RD cells, which findings introduce this isoform as a promising, therapeutically exploitable, novel target for the treatment of skeletal muscle malignancies.

SUMMARY

The RasGRP3, an activator of H-Ras and R-Ras protein exerts oncogenic effects and the overexpression of the protein is observed in numerous malignant cancer types. The aim of our experiments was to investigate the putative alteration of expression and potential function of RasGRP3 in the formation and progression of human breast cancer. The RasGRP3 and phosphoRasGRP3 expressions were examined in human invasive ductal adenocarcinoma derived samples and cell lines (BT-474, JIMT-1, MCF7, SK-BR-3, MDA-MB-453, T-47D) both in mRNA (Q-PCR) and protein (Western blot; immunohisto- and immunocytochemistry) levels. To explore the biological function of the protein, RasGRP3 knockdown cultures were established. To assess the role of RasGRP3 in the viability of cells, annexin-V/PI staining and MitoProbe ^{TM} DilC1 (5) assay were performed. To clarify the function of the protein in cell proliferation and in the development of chemotherapeutic resistance, CyQuant assay was performed. To observe the RasGRP3 function in tumor formation, the Severe Combined Immunodeficiency (SCID) mouse model was used. To investigate the role of the protein in Ras-related signaling Q-PCR and Western blot experiments were performed. RasGRP3 expression was elevated in human breast tumor tissue samples as well as in multiple human breast cancer cell lines. Down-regulation of RasGRP3 expression in breast cancer cells decreased cell proliferation, induced apoptosis in MCF7 cells, and sensitized T-47D cells to the action of drugs Tamoxifen and trastuzumab (Herceptin). Gene silencing of RasGRP3 reduced tumor formation in mouse xenografts as well. Inhibition of RasGRP3 expression also reduced Akt, ERK1/2 and estrogen receptor alpha phosphorylation downstream from IGF-I or EGF stimulation confirming the functional role of RasGRP3 in the altered behavior of these cells. Taken together, our results suggest that the Ras activator RasGRP3 may have a role in the pathological behavior of breast cancer cells and may constitute a therapeutic target for human breast cancer.

In the second part of the work we investigated the specific role of certain protein kinase C δ (PKCδ) isozyme in the regulation of skeletal muscle growth. We provided the first intriguing functional evidence that nPKCδ is a key player in promoting both in vitro and in vivo skeletal muscle growth. Recombinant overexpression of a constitutively active nPKCδ in rhabdomyosarcoma (RD) cells increased proliferation. Conversely, overexpression of kinase-negative mutant of nPKCδ (DN-nPKCδ) markedly inhibited cell growth. Moreover, overexpression of nPKCδ also stimulated in vivo tumor growth and induced malignant transformation in immunodeficient (SCID) mice whereas that of DN-nPKCδ suppressed tumor formation. Collectively, our data introduce nPKCδ as a novel growth-promoting molecule in skeletal muscles and invite further trials to exploit its therapeutic potential in the treatment of skeletal muscle malignancies.

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List of publications related to the dissertation

1. Czifra, G., Szöllősi, A., **Nagy, Z.**, Boros, M., Juhász, I., Kiss, A., Erdődi, F., Szabó, T., Kovács, I., Török, M., Kovács, L., Blumberg, P.M., Bíró, T.: Protein kinase cδ Promotes proliferation and induces malignant transformation in skeletal muscle.
J. Cell. Mol. Med. "accepted by publisher" (2014)
IF:3.698 (2013)

2. **Nagy, Z.**, Kovács, I., Török, M., Tóth, D., Vereb, G., Buzás, K., Juhász, I., Blumberg, P.M., Bíró, T., Czifra, G.: Function of RasGRP3 in the formation and progression of human breast cancer.
Mol. Cancer. 13, 1-17, 2014.
DOI: http://dx.doi.org/10.1186/1476-4598-13-96
IF:5.397 (2013)

List of other publications

3. Nagy, D., Kosztka, L., Pap, P., **Nagy, Z.**, Rusznák, Z., Csernoch, L., Szűcs, G.: Cytoplasmic Ca2+ concentration changes evoked by muscarinic cholinergic stimulation in primary and metastatic melanoma cell lines.
   Melanoma Res. 21 (1), 12-23, 2011.
   DOI: http://dx.doi.org/10.1097/CMR.0b013e3283414477
   IF:2.187

4. Kosztka, L., Rusznák, Z., Nagy, D., **Nagy, Z.**, Fodor, J., Szűcs, G., Telek, A., Gönczi, M., Ruzsnavszky, O., Szentandrássy, N., Csernoch, L.: Inhibition of TASK-3 (KCNK9) channel biosynthesis changes cell morphology and decreases both DNA content and mitochondrial function of melanoma cells maintained in cell culture.
   Melanoma Res. 21 (4), 308-322, 2011.
   DOI: http://dx.doi.org/10.1097/CMR.0b013e3283462713
   IF:2.187

5. Kőszeghy, Á., Pál, B., Pap, P., Pocsai, K., **Nagy, Z.**, Szűcs, G., Rusznák, Z.: Purkinje-like cells of the rat cochlear nucleus: A combined functional and morphological study.
   Brain Res. 1297, 57-69, 2009.
   DOI: http://dx.doi.org/10.1016/j.brainres.2009.08.041
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Total IF of journals (all publications): 15,932
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