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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TABLE OF CONTENTS

LIST OF ABBREVATIONS

INTRODUCTION

MAPK	mitogen-activated protein kinase
PI3K	phosphatidylinositol 3-kinase
SOS	son of sevenless
GDP	guanosine-diphosphate
GTP	guanosine-triphosphate
Raf1	v-raf-1 murine leukemia viral oncogene homolog 1
MAPKK or MEK	MAPK kinase
ERK 1/2	extracellular signal-regulated kinase 1 and 2
CREB	cAMP response element-binding protein
NF-ĸB	nuclear factor immunoglobulin chain enhancer-B cell
RTK	receptor tirosine kinase
PIP3	phosphatidylinositol 3,4,5-triphosphate
PIP2	phosphatidylinositol 4,5-biphosphate
PDK	phosphoinositide-dependent kinase
mTOR	mammalian target of rapamycin
PTEN	phosphatase and tensin homolog
GEF	guanine nucleotide exchange factor
GAP	GTPase activating protein
RasGRF	Ras guanyl-nucleotide releasing factor
RasGDS	Ras guanyl-nucleotide dissociation stimulator
RasGRP 1 2 3 4	Ras guanyl-nucleotide releasing peptide
REM	Ras exhange motif
SH3	Src-homology-3
C1	C1 domain
PKC	protein kinase C
DAG	diacylglycerol
GLA	glycine-lysine-arginine cluster
PPP	proline cluster
PLC	phospholipase C
siRNA	small interfering RNA
HGF	hepatocyte growth factor
HGFR	HGF receptor
AML	acute myeloid leukemia
ALL	acute lymphocytic leukemia
WHO	World Health Organization
DCIS	ductal carcinoma in situ
IDC	invasive ductal carcinoma
ER	estrogen receptor
PR	progesterone receptor
HER-2	human epidermal growth factor receptor type 2
ERE	estrogen responsive element
EGF	epidermal growth factor
IGF-I	insulin-like growth factor
Ki67	Kiel clone 67
SERM	selective ER modulator
FDA	Food and Drug Administration
MUC4	mucin 4
IGF-IR	IGF-I receptor
EGFR	EGF receptor
C1-C4	conserved domains 1-4
V1-V4	variable domain 1-5
cPKC	classic PKC isozyme
nPKC	novel PKC isozyme
aPKC	atypical PKC isozyme
PS	pseudosubstrate
PMA	phorbol-myristate-acetate
PE	phorbol ester
IP3	inositol triphosphate
TPA	12-o-tetradecanozl-phorbol 13-acetate
TNF	tumor necrosis factor
TRAIL	TNF-related apoptosis inducing ligand
IP6	hexaphosphate
Rb	retinoblastoma
HIF	hypoxia-inducible factor
RMS	rhabdomyosarcoma
ERMS	embryonic RMS
ARMS	alveolar RMS
PRMS	pleomorphic RMS
PAX 3 and 7	paired boksz 3 and 7
FOXO	forkhead bokx protein
MYCN	v-myc avian myelocytomatosis viral oncogene
SCID	severe combined immunodeficiency
Q-PCR	quantitative polimerase chain reaction

MATERIALS & METHODS

EMEM	Eagle's minimal essential media
DMEM	Dulbecco's minimal essential media
L-15	Leibovitz 15 media
FBS	fetal bovine serum
RD	rhabdomyosarcoma
HRP	horseradish peroxidase
DAB	diaminobenzidine
HE	hematoxylin-eosin
PBS	phosphate buffered saline
AOI	area of interest
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
shRasGRP3	RasGRP3 shRNA sequence
shSCR	scrambled shRNA sequence
nPKCδ	novel nPKCδ
DN-nPKCδ	dominant-negative mutant of nPKCδ
pεMTH	empty vector
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
PI	propidium jodide
DilC	1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindocarbocyanine Perchlorate
P	phospho group

RESULTS

Tu	tumor
Co	control
GI-III	grade I-III
PC	positive control
NC	negative control
NeA	necrotic area
CYCS	cytochrome c
CTSD	cathepsin D
LOR	loricrin

DISCUSSION

DLC1	dynein light chain

BEGINNING THOUGHTS

Remarkable discoveries regarding how Ras proteins function as key regulators of signal transduction and drivers of oncogenesis have been emerged over the past three decades, with thousands of scientific articles published on the subject. The implication of Ras proteins in pathological processes such as cancer has always been at the leading edge of molecular oncology, currently showing a rate of 300 articles published per month related to this topic. The knowledge regarding Ras biology has accumulated since their discovery, however, been remarkable, but despite the intensive efforts by the pharmaceutical industry, these findings have not been translated into clinically effective anti-Ras therapies. This has prompted renewed interest in search of new targets revealing what is beyond of Ras: „upstream" and „downstream" proteins having the ability to modify Ras-related oncogenic signaling. Among these regulator proteins, the RasGRPs and PKC family members have been the subject of intensive study in the field of cancer since their initial discovery as major cellular receptors for the tumor promoting phorbol esters. However, despite these efforts, the search for a direct link between these proteins and oncogenical Ras-related signaling is still in progress. We aimed this thesis to experimentally investigate and support the potential oncogenic function and significance of RasGRP3 and nPKCδ in the formation of human cancer.

INTRODUCTION

RAS-RELATED SIGNALING - A SHORT OVERVIEW

The study of oncogenic and normal cells has helped to define the numerous genes that regulate proliferation and division. The genes of many signal transduction proteins were first discovered in their role as *oncogenes*- genes that lead to cell transformation when mutated- before their functions in normal cells were elucidated. The *ras* oncogenes were among the first to be discovered [Harvey, 1964; Kirsten and Mayer, 1967]. 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. Despite the intense investigations, the mechanisms of Ras activation and signaling in response to external stimuli as well as the molecules that participate in the upstream and downstrearn signaling events and regulations are still not fully undersood.

The members of the Ras GTPase family are crucial players in many signaling networks connecting a great variety of upstream signals to an even wider set of downstream effector pathways linked to the functional control of cellular outcomes including nuclear events and in signal transduction pathways that control proliferation, migration, apoptosis and senescence [Barbacid, 1987; Chang and Karin, 2001; Starr et al., 2003]. Studies during the last quarter century have characterized the Ras proteins as essential components in regulating oncogenic pathways [Cooper et al., 1980]. 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 [Perucho et al., 1981; Bos, 1989]. Due to its central role in intracellular signal transduction and malignant transformation, drugs targeting Ras proteins or Ras effector pathways have been developed with the aim to either correct or eliminate aberrant Ras signaling.

Ras is the common upstream molecule of several signaling pathways including Mitogen-Activated Protein Kinase (MAPK) and PhosphoInositide 3-Kinase (PI3K) cascades (**Figure 1**). Four Ras proteins have been identified, namely H-Ras, N-Ras, Ki-Ras 4A and Ki-Ras 4B. The closest relatives are the Rap proteins (RaplA, RaplB, Rap2A and Rap2B), which are found in the Golgi-complex and endoplasmic reticulum.

Following binding of cytokines, hormones, growth factors or mitogens to their appropriate receptors, activation of the coupling complex Shc/Grb2 occurs. Upon stimulation by Shc/Grb2, the nucleotide exchange factor Son of Sevenless (SOS) exchanges guanosine-diphosphate (GDP) for guanosine-triphosphate (GTP) on Ras. The GTP bound active Ras undergoes a conformational change that allows it to associate with v-raf-1 murine leukemia viral oncogene homolog 1 (Raf) protein and then recruit to cell membrane. Mitogen-Activated Protein Kinase Kinase (MAPKK or MEK) is positively regulated by Raf phosphorylation. MEK mediates the activation of Extracellular-signal-Regulated Kinases 1/2 (ERK 1/2). ERK 1/2 has many direct and indirect targets: it phosphorylates transcription factors including Ets-1, c-Jun, c-Myc, cAMP response element-binding protein (CREB) and nuclear factors including nuclear factor immunoglobulin κ chain enhancer-B cell (NF-κB) promoting cell proliferation. For many years now, it has been known that the Raf/MEK/ERK 1/2 pathway has a role in the regulation of cell survival. ERK 1/2 can phosphorylate Bad which contributes to its inactivation and subsequent sequestration by 14-3-3 proteins. This allows Bcl-2 to form homodimers and an anti-apoptotic response is generated. Activation of the Raf/MEK/ERK 1/2 cascade can also result in the phosphorylation of the pro-apoptotic Bim protein. Phosphorylation of Bim results in its dissociation from Bcl-2, Bcl-X_L and Mcl-1 preventing Bax activation and the formation of Bax: Bax homodimers.

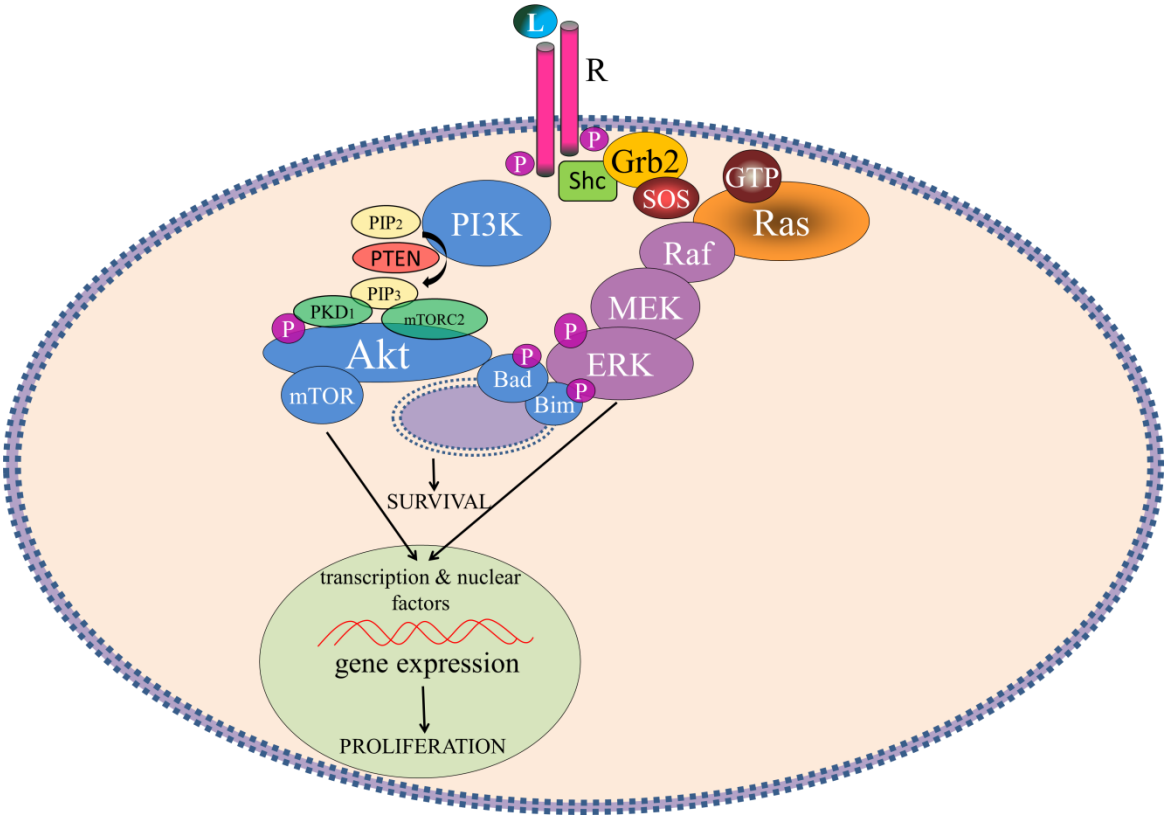


Figure 1. Overview of Ras-related signaling pathways

Signaling is activated by a multitude of mechanisms, including stimulation by growth factors (L) through receptor tyrosine kinases resulting in Ras and PI3K activation. There is a wide range of downstream effectors that mediate key signals for cellular proliferation and survival. A cascade of phosphorylation through Ras/Raf/MEK and ERK 1/2 kinases effects transcription promoting cell proliferation and survival. Signaling via PI3K and Akt increases cell growth and deliver anti-apoptotic signal.

The serine/threonine kinase Akt regulates multiple cellular functions including angiogenesis, metabolism, proliferation and survival. Various growth factors, hormones, and cytokines activate Akt by binding their cognate receptor tyrosine kinase (RTK) or cytokine receptor and triggering activation of the lipid kinase PI3K, which generates phosphatidylinositol (3,4,5)-trisphosphate (PIP_3) from phosphatidylinositol (3,4)-bisphosphate (PIP_2) at the plasma membrane. Akt binds PIP_3 resulting in translocation of Akt to the membrane. Akt is activated through a dual phosphorylation mechanism: (1) phosphoinositide-dependent kinase (PDK) phosphorylates Akt within its activation loop leading to partial Akt activation and (2) phosphorylation within the carboxy terminus is also required for activity

and is carried out by the mammalian target of rapamycin complex 2 (mTORC2). Phosphatase and tensin homolog (PTEN), a lipid phosphatase that catalyzes the dephosphorylation of PIP_3, is a major negative regulator of Akt signaling. Loss of PTEN function has been implicated in many human cancers. Activated Akt phosphorylates a large number of downstream substrates. One of its primary functions is to promote cell growth through regulation of the mTOR signaling pathway. Akt directly phosphorylates and activates mTOR promoting cell growth and G1 cell cycle progression. Akt delivers antiapoptotic signals by phosphorylating Bad therefore maintaining Bcl-X_L function and preventing cytochrome c release from mitochondria. Aberrant Akt signaling is the underlying defect found in several pathologies, Akt is one of the most frequently activated kinases in human cancer. *Akt* gene amplifications have been found in a gastric carcinoma, ovarian, pancreas, breast and stomach malignant tumors.

Pathways have been greatly simplified for the purposes of the present work; all interactions are not discussed and illustrated. This section was created on the basis of the following reviews: Ullrich and Schlessinger, 1990; Bollag and McCormick, 1991; Beranger et al., 1991; Rozakis-Adock et al., 1993; Pizon et al., 1994; Moodie, 1995 and Hancock, 2003.

RASGRPs AND PKCs AS POSITIVE REGULATORS OF RAS SIGNALING

Ras is found to be chronically overactivated in tumor cells that lack *ras* gene amplification and mutated Ras. Due to the intense investigations of the last 40 years now we know that many proteins are able to modulate the activity of Ras-related signaling. These proteins can be divided into two groups: (1) “upstream” regulators that mediate the activity of Ras and (2) “downstream” regulators that modulate the activation of downstream Ras kinases.

RASGRPs as „upstream" regulators

The Ras protein sits at the center of a many-tiered cascade of molecular interactions. 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) (**Figure 2**) [Macara et al., 1996; Bos, 1997]. In this way the biological activity of Ras is self-limiting depending upon its guanine nucleotide-bound states [Haubruck and McCormick, 1991]. Only Ras-GTP complex can bind with high affinity to its downstream effectors and thus transmit a signal [Chang and Karin, 2001]. The immediate control of these GTPase-mediated events resides in the proteins that regulate their GTP- or GDP-binding status. Two classes of regulatory proteins have been identified: (1) the guanine nucleotide exchange factors (GEFs), whose physiological function is to convert Ras from a GDP- to a GTP-bound state, and (2) the GTPase activating proteins (GAPs), which turn off Ras by activating its intrinsic GTPase activity [Boguski and McCormick, 1993].

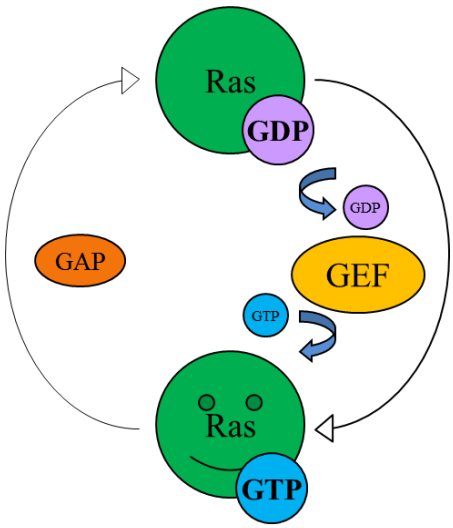


Figure 2. Ras activation cycle

The activity of Ras proteins is tightly controlled by switching between the GDP- and GTP-bound states. Although Ras proteins possess intrinsic GTPase activity, regulatory proteins like GEFs and GAPs profoundly influence the activation of Ras. As the cellular amount of GTP is tenfold higher than GDP, GEFs promote the formation of active RasGTP, while GAPs stimulate the intrinsic rate of GTP hydrolysis and thereby the formation of inactive RasGDP.

The intrinsic conversion of GDP- to GTP-bound Ras, also know as guanylnucleotide exchange is a slow reaction [Feuerstein et al., 1987], this can be enhanced *in vivo* by GEF proteins [Lai et al., 1993]. Several mammalian guanyl-nucleotide exchange factors have been identified: (1) Son of Sevenless (SOS) type proteins [Chardin et al., 1993], (2) guanyl-nucleotide releasing factors (GRFs) [Shou et al., 1992], (3) guanyl-nucleotide dissociation stimulators (GDSs) [Kaibuchi et al., 1991; Albright et al., 1993] and (4) guanyl-nucleotide releasing peptids (GRPs) [Ebinu et al., 1998].

These exchange factors are immediate upstream activators of Ras. Genetic loss of these proteins has biological effects similar to loss of the Ras proteins themselves [Barbacid, 1987]. The tight regulation of the guanine nucleotide bound state is critical for the regulation of normal cellular proliferation. When Ras loses its control it remains switched "on", the message is delivered continuously, giving the cell unchecked permission to proliferate inducing cellular transformation [Barbacid, 1987].

Protein kinase Cs as „downstream" regulators

Protein kinase C (PKC) 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 cellular responses [Takai et al., 1979] including signal transduction, modulation of gene expression, proliferation, apoptosis and differentiation [Nishizuka, 1988 and 1995]. PKCs have been discovered more than two decades ago; it was the first enzyme which was identified as a receptor for diacylglycerol (DAG), generated as a product of PLC activation [Inoue et al., 1977; Kishimoto et al., 1980]. PKC activity represents one mechanism used by cells to regulate Ras signaling specificity. PKCs reside in several major signaling pathways. Two of the main PKC downstream events include the activation of MAPK and PI3K cascades; PKCs signal to downstream effectors such as Rac1/MEK/ERK 1/2 kinases, Bad and

NF-κB modulating the respond of the cells to growth factors and cytokines [Nishizuka, 1988 and 1995].

C1 DOMAIN- A COMMON BIOCHEMICAL FUNCTION

Structure of RasGRPs and PKCs

RasGRP and PKC proteins are of considerable length and contain several conserved regions representing conserved structural domains. Shematic structures of the proteins are shown on **Figure 3 and Figure 4**.

RasGRPs are characterized by the presence of (1) a conserved catalytic core known as GEF which catalyzes the GDP dissociation from Ras [Broek et al., 1987; Ebinu et al., 1998], (2) a sequence which has been termed a Ras exchange motif (REM) [Lai et al., 1993; Ebinu et al., 1998], (3) a prolin cluster which serves as a binding site for src-homolgy-3 (SH3) domain [Pawson, 1995], (4) a pair of EF hands serving as sensors of calcium concentration [Grabarek, 2006; Gifford et al., 2007] and (5) a glycine-lysine-arginine rich region which makes the protein a potential target for N-terminal myristoylation [Lee et al., 2005].

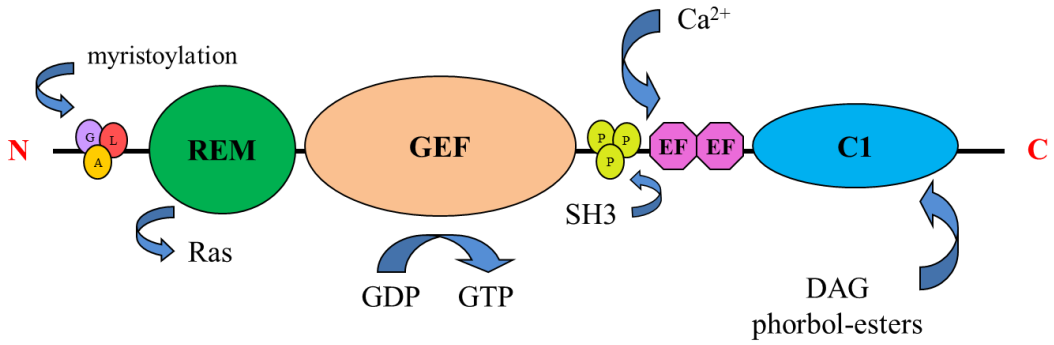


Figure 3. Cartoon highlighting the general protein domains in RasGRP proteins

REM=Ras exchange motif; **GEF**=guanine exchange factor sequence; **C1**= DAG/phorbol-ester-binding C1 domain; **EF**=Ca^{2+}-binding EF hands; **GLA**=glycine-lysine-arginine cluster; **PPP**= proline cluster

PKCs all share four conserved domains (referred as C1–C4) interrupted by five variable regions (referred as V1–V5) [Nishizuka, 1988; Kikkawa et al., 1989]. Each PKC contains a highly homologous N-terminal **regulatory domain** (C1 and C2) and C-terminal **catalytic domain** (C3 and C4). Different isoforms have been discovered among PKCs. PKC comprises 11 phospholipid-dependent serine-threonine kinases [Nishizuka, 1988; Dekker et al., 1994; Parker and Murray-Rust, 2004] grouped into three subclasses; this dissertation is focused on the novel isoforms. The novel PKCs (nPKCs, which can be further subdivided based on structural features into the related δ, θ, ε and η isoforms) contain all regions of regulatory and catalytic domains.

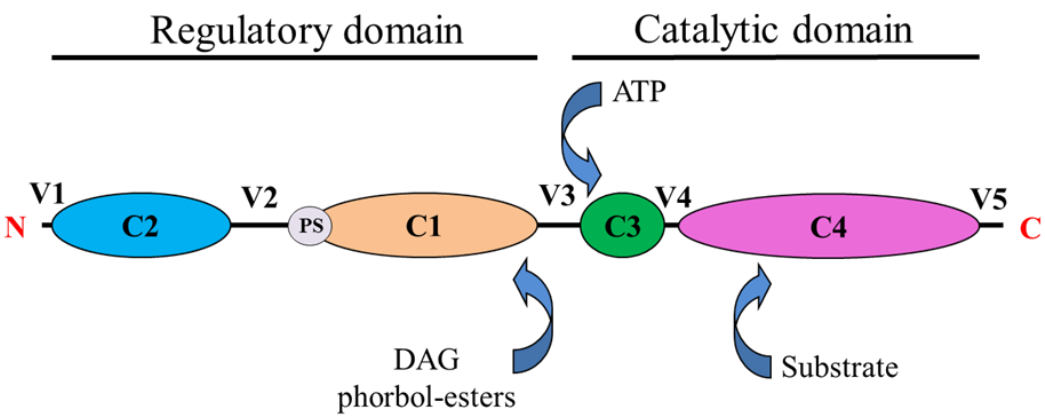


Figure 4. Schematic representation of the structure of the nPKC family

C3= ATP-binding C3 domain; **C4**= substrate-binding C4 domain; **C1**= DAG/phorbol-ester-binding C1 domain; **Ps**= autoinhibitory Ps sequence.
Compared to other PKC isoforms, the C2 domain of nPKCs lack the calcium-coordinating acidic residues, the determinant for calcium binding. An autoinhibitory pseudosubstrate (Ps) sequence is located at the C1 domain. In the absence of stimuli, the Ps motif maintains the enzyme in an inactive state by sterically blocking the catalytic domain. Cofactor binding results in a conformational change that releases the Ps motif and increases the catalytic activity of the enzyme [House and Kemp, 1987; Blumberg, 1991].

A unique feature of RasGRPs and PKCs is the presence of a C1 domain [Hurley et al., 1997]. This domain serves as binding site for phorbol-esters and its physiological counterpart, DAG [Ebinu et al., 1998; Lorenzo et al, 2000]. These molecules have very important roles in the life of both PKCs and RasGRPs by causing the activation and translocation of these proteins from the cytoplasm to membranes such as cell membrane [Ebinu et al., 1998 and 2000; Lorenzo et al., 2001; Reuther et al., 2002; Yang et al., 2002; Teixeria et al., 2003] or nuclear membrane [Bivona et al., 2003; Daniels et al., 2006].

Activation mechanisms of RasGRPs and PKCs

RasGRPs and PKCs are viewed as lipid-sensitive proteins that are activated by DAG via their C1 domains. The intracellular DAG binds to the C1 domain and increases the affinity of the proteins for membranes [Griner and Kazanietz, 2007; Steinberg, 2008; Kang et al., 2012]. Translocation to the plasma membrane generally has been considered as the hallmark of activation [Steinberg, 2008]. Once anchored to membranes, RasGRPs and PKCs enhance Ras-related signaling (**Figure 5**).

RasGRPs and PKCs are also pharmacologically activated by tumor-promoting phorbol esters such as phorbol 12-myristate 13-acetate (PMA) that anchor these proteins in their active conformations to membranes (**Figure 5**) [Couturier et al., 1984; Arcoleo and Weinstein, 1985; Ashendel, 1985] without receptor stimulation.

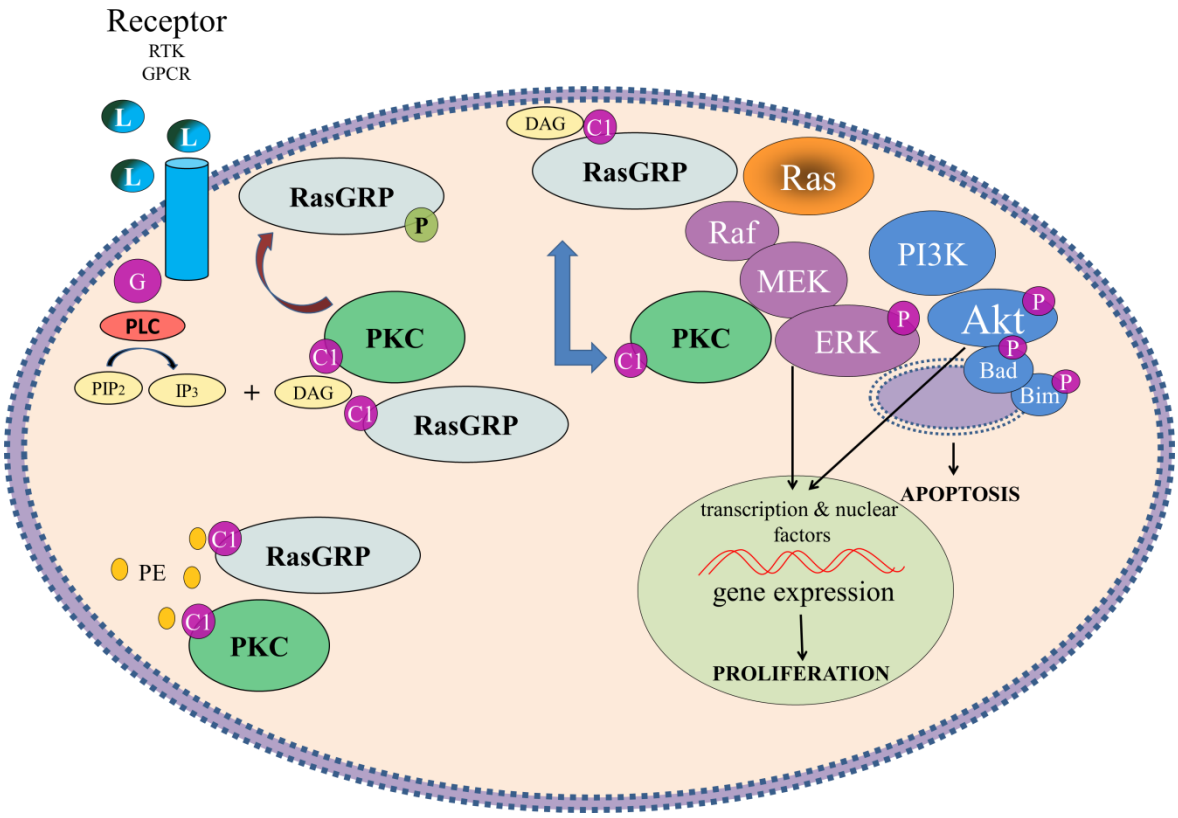


Figure 5. A schematic representation of the activation of RasGRPs and PKCs

External stimuli activate receptor tyrosine kinase (RTK) or G protein-coupled (GPCR) receptors which are coupled to phospholipase C (PLC). PLC cleaves PIP2 into DAG. DAG binds to the DAG responsive C1 domains of RasGRPs and PKCs resulting in their activation and translocation. Phorbol-esters (PE) are also involved in RasGRP and PKC activation. Subsequent RasGRP and PKC downstream events include activation of the MAPK and PI3K pathways.

RasGRP1 and RasGRP3 activity is also regulated by phosphorylation, triggered in an indirect way by DAG [Aiba et al., 2004; Roose et al., 2005; Zheng et al., 2005]. It was demonstrated that such phosphorylation very likely occurs through PKC kinases, which themselves can get recruited to the membrane by DAG [Aiba et al.,2004; Roose et al., 2005; Zheng et al., 2005]. It is unknown how, at the molecular level, phosphorylation of RasGRP1 and RasGRP3 regulates their exchange activity. Phosphorylation appears to have an enhancing effect to stimulus-dependent Ras activation (**Figure 5**) [Aiba et al.,2004; Zheng et al., 2005]. All pathways are considered to have a role in tumorigenesis; with the overexpression and overactivation of these proteins a constantly activated Ras signaling can be formed.

THE ROLES OF RASGRPs AND PKCs IN CANCER FORMATION

The discovery of RasGRPs and PKCs as major intracellular receptor for the tumour promoting phorbol esters suggested that the activation of these proteins is involved in cell transformation and carcinogenesis. In cancer cells, RasGRPs and PKC isozymes are involved in the modulation of cell proliferation, survival, migration and anticancer drug resistance through their increased or decreased participation in survival or proliferation-associated signaling pathways, such as Ras/Raf/MEK/ERK 1/2 or PI3K/Akt/mTOR pathways [Yang et al., 2010; Yang et al., 2011; Marshall, 1996; Griner and Kazanietz; 2007].

RasGRPs and Cancer

Fibroblast screening assays that measure anchorage-independent growth are standard assays to measure the transformative potential of a molecule. RasGRP1 was cloned in a classical fibroblast transformation assay [Ebinu et al., 1998], suggesting amongst the first on that RasGRPs may play a role in cancer. After the discovery of the proteins RasGRP research evolved in immunbiology investigating its role in lymphocyte development and created false impression that RasGRPs are selective to the hematopoietic system. Recent studies on RasGRPs and cancer have revisited this concept and started to characterize the mechanisms of oncogenic RasGRP signaling [Yang et al., 2010; Yang et al., 2011]. Patricia Lorenzo and her team were the first to describe oncogenic RasGRP1 function in primary mouse keratinocytes [Rambaratsingh et al., 2003]. Characterization of RasGRP1 transgenic mice, which overexpress RasGRP1 in epidermal keratinocytes, showed that almost 50% of the mouse colony spontaneously developed skin tumors. The majority of those tumors were classified as benign papillomas and an only small fraction as malignant squamous cell sarcomas [Oki-Idouchi and Lorenzo, 2007].

RasGRP family members contribute to blood neoplasms. RasGRPs have been implicated in the cell biology of B cell lymphomas, acute myeloid leukemia (AML) and T cell acute lymphoblastic leukemia/lymphoma (T-ALL). The first indication suggesting that RasGRP proteins are contributing to the development of hematological malignancies came from retroviral mutagenesis screens, which looked for new genes involved in B cell lymphoma [Suzuki et al., 2002]. RasGRP4 was isolated from patients with AML [Reuther et al., 2002; Watanabe-Okochi et al., 2009]. More importantly, transplantation of RasGRP4-transduced bone marrow progenitors resulted in the development of AML-like disease in xenograft model [Watanabe-Okochi et al., 2009]. The first study implicating RasGRP1 in T-ALL pathogenesis came from retroviral mutagenesis screens also [Kim et al., 2003]. It was reported that overexpression of RasGRP1 in thymocyte leads to thymic lymphomas [Klinger et al., 2005]. An independent group made very similar observations by using retroviral overexpression of RasGRP1 in a bone marrow transfer model to induce leukemia [Oki et al., 2011].

Roles of RasGRP3 in human cancers

RasGRP3 has recently emerged as a player contributing to prostate cancer and melanoma [Yang et al., 2010; Yang et al., 2011]. It was shown that RasGRP3 mRNA levels are elevated in patients with prostate cancer as compared to non-diseased individuals [Yang et al., 2010]. Evaluation of RasGRP3 expression in human prostate cell lines showed that androgen-independent cell lines expressed almost 10 times more RasGRP3 as compared to androgen-dependent cell lines. *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 cell lines [Yang et al., 2010]. To understand the molecular mechanism by which RasGRP3 promotes proliferation, the authors investigated the activation of signaling molecules downstream of hepatocyte growth factor (HGF), a growth factor know to be important in prostate cancer. Reduction of RasGRP3 levels caused diminished levels of HGF-induced phospho-Akt (pAkt) and to lesser extent phospho-ERK 1/2 (pERK 1/2). These results suggest that RasGRP3 may promote growth and survival through the activation of Akt and ERK 1/2 pathways in the context of prostate cancer, either in response to HGF stimulation or in a constitutive manner when mutations result in active PI3K signaling. The authors also performed additional experiment where they overexpressed RasGRP3 in an androgen-dependent cell line, which had low endogenous RasGRP3 protein levels. Elevated levels of RasGRP3 caused phenotypic changes and turned androgen-dependent line into androgen-independent, which is interesting and suggests that deregulated levels of RasGRP3 may facilitate progression from androgen-dependent to more aggressive, androgen-independent phenotype [Yang et al., 2010].

The Blumberg group also explored the contribution of RasGRP3 to melanoma development and demonstrated that samples from melanoma patients expressed RasGRP3 [Yang et al., 2011]. The authors also show that many of the melanoma cell lines but not normal melanocytes express various amounts of RasGRP3. To assess the role of RasGRP3 in those cell lines, the investigators decreased RasGRP3 expression levels in some of them using gene-silencing. With few exceptions, in most cell lines interfering with RasGRP3 levels decreased cell proliferation *in vitro* and *in vivo*. The effects of RasGRP3 ablation on signaling pathways were also examined. Knockdown of RasGRP3 expression caused a reduction of HGF-induced pAkt levels, suggesting that RasGRP3 can contribute to the activation of the HGF pathway in melanoma [Yang et al., 2011]. The expression of the hepatocyte growth factor receptor (HGFR) was diminished, which is an interesting result since HGF can

contribute to the resistance to various kinase inhibitors [Straussman et al., 2012; Wilson et al., 2012]. Therefore, co-targeting RasGRP3 could potentially overcome this resistance mechanism.

PKCs and Cancer

PKC isoform roles in cancer cells largely depend on the type of cancer being investigated, but in general, overexpression of PKC isozymes is closely related to poor prognosis, poor response to chemotherapy and poor patient survival. These results are likely caused by the high levels of cancer cell migration, invasion, survival, and anticancer drug resistance stimulated by PKC isozymes [Kang, 2014]. PKC isozymes that are specifically overexpressed in certain types of cancer can be used as diagnostic or therapeutic targets. Thus, understanding the role and expression of individual PKC isozymes in each type of cancer may help to elucidate important cues for discovering novel drugs and for developing diagnostic or therapeutic tools.

PKCs have been studied as targets for the treatment of cancer for many years [Fields and Murray, 2008]. PKCα isozyme-specific or broad, nonspecific inhibitors have been developed and applied in phases II and III clinical trials. However, since specific inhibitors and oligonucleotides inhibiting classical PKCs have entered clinical trials, satisfactory results have not yet been obtained [Villalona-Calero et al., 2004; Paz-Ares et al., 2006; Ritch et al., 2006]. PKC isozymes that are overexpressed or hyperactivated in cancer tissues can be used as immunohistochemical biomarkers during cancer diagnosis by comparing the expression in cancer tissues to that in normal tissues. For example, PKCθ has been used as a biomarker for gastric cancer, PKCι has been used as a diagnostic biomarker for non-small cell lung cancer [Regala et al., 2005], while PKCα and PKCβII have been used for breast cancer [Assender et al., 2007; Lønne et al., 2010] and diffuse non-Hodgkin's lymphoma [Schaffel et al., 2007; Riihijärvi et al., 2010].

Among PKC isozymes, PKCα, β, ε, and δ have been the most broadly studied isozymes in relation to cancer. This study is focused on the roles of PKC δ in tumor biology.

Roles of nPKCδ in human cancers

nPKCδ is a key PKC isozyme expressed in breast cancer cells, mediating cell proliferation via ERK 1/2 activation [Allen-Petersen et al, 2014]. In estrogen-dependent breast cancer cells, phorbol ester TPA (12-o-tetradecanoyl-phorbol 13-acetate)-mediated nPKCδ activation leads to activation and nuclear translocation of ERα and enhanced ER-dependent reporter gene expression thereby suggesting a role of PKCδ as a proliferative factor [de Servi et al., 2005]. nPKCδ shows prosurvival and proapoptotic functions as well. nPKCδ promotes survival of breast cancer cells through the inhibition of TNF-related apoptosis-inducing ligand (TRAIL)-induced caspase activation [Zhang et al, 2005; Yin et al., 2010], inhibition of Notch1 activity [Kim et al., 2012], and through the activation of Akt and NF-$_K$B [Díaz Bessone et al., 2011]. On the other hand, proapoptotic effects of nPKCδ have also been reported; treating MCF-7 cells with inositol hexaphosphate (IP_6) causes an increase of nPKCδ activation upregulating the retinoblastoma protein (Rb), resulting in G_1 arrest and increased apoptosis [Vucenik et al., 2005]. In addition, nPKCδ also plays a critical role in breast cancer cell migration and chemotherapeutic resistance stimulated by the MAPK signaling pathway [Nabha et al., 2005; Lin et al., 2008; Park et al., 2009].

Similar to breast cancer, in colon cancer cells, nPKCδ mediates proliferation and survival as well. nPKCδ can inhibit cell growth by enhancing p53 expression [Perletti et al., 2004 and 2005], and the activity of cyclins D1, D3 and E [Kim et al., 2007]. nPKCδ expression also acts as a proapoptotic regulator in these cells by enhancing Bax levels, in contrast nPKCδ may have antiapoptotic functions as well; apoptosis is prevented by NF-$_K$B activation [Cerda et al., 2006].

nPKCδ is also reported in gastric cancer cells by positively controlling cisplatin-induced cell death correlating with overexpression of p53 and enhanced caspase-9 production. In addition, enhanced nPKCδ expression and phosphorylation can increase cell motility and invasion by integrin α2 or α3 activation [Lee et al., 2005].

nPKCδ has been identified in multiple malignant glioma cell lines and tissues [Misra-Pess et al., 1992; Mandil et al., 2001]. nPKCδ mediates glioma cells proliferation via EGFR transactivation by enhanced nPKCδ/c-Src signaling [Amos et al., 2005]. nPKCδ can influence both proapoptotic and antiapoptotic effects. Cytosolic and nuclear nPKCδ increases p38 phosphorylation and decreases Akt phosphorylation resulting in enhanced apoptotic effects. However, nPKCδ localized in the endoplasmic reticulum leads to antiapoptosis of glioma cells [Gomel et al., 2007]. Activation of nPKCδ also stimulates glioma cell invasion and motility and increases the sensitivity of cells to cisplatin [Sarkar and Yong, 2010].

High expression of nPKCδ in both low- and high grade prostate cancer has been reported [Villar et al., 2007; Castilla et al., 2013] in associacion with apoptosis of prostate cancer cells. Several mechanisms are involved in nPKCδ -dependent apoptosis in these cells triggered by PMA, anticancer drugs, or androgens. These mechanisms include the activation of caspase-8 and -3, p38 and JNK [Gonzalez-Guerrico and Kazanietz, 2005; Gavrielides et al., 2006], and enhanced release of death factors TNFα and/or TRAIL [Xiao et al., 2009; von Burstin et al., 2010]. In addition, nPKCδ is overexpressed in metastatic prostate cancer-derived cell line DU-145 and plays a critical role in migration and invasion [Kharait et al., 2006], involving the upregulation of MMP-9 and activation of NF-κB signaling pathway [Yu et al., 2013]. Furthermore, activation of nPKCδ in mice xenografts injected with PC3 cells can increase angiogenic activity through enhanced NADPH oxidase activity and increased levels of hypoxia-inducible factor (HIF)-1α [Kim et al., 2011].

Different types of renal cell carcinomas show the expression of nPKCδ [Brenner et al., 2004]. nPKCδ was shown to enhance the migration and IGF-1- mediated invasion of these cells by reducing expression and activity of β1-integrin [Datta et al., 2000; Brenner et al., 2008].

Besides these tumor types nPKCδ overexpression is also reported in head and neck squamous cell carcinomas [Forastiere et al., 2001; Haddad and Shin, 2008], non-small cell lung cancer [Symonds et al., 2011], melanoma [la Porta and Comolli, 2000; la Porta et al., 2000], chronic lymphocytic leukemia, [Kazi et al., 2013] acute myeloid leukemia [Jang et al., 2004; Song et al., 2005; Yan et al., 2007] pancreatic cancer and papillary thyroid cancer cell [Ozpolat et al., 2007]. Interestingly nPKCδ is not expressed in primary and recurrent ovarian cancers [Weichert et al., 2003].

BREAST CANCER WITH THE EYE OF A MOLECULAR BIOLOGIST

Breast cancer as a world disease

Breast cancer is the most frequent carcinoma in females and the second most common cause of cancer-related death in women [Bombonati and Sgroi, 2011]. Despite the major advances in breast cancer prevention, the incidence of breast cancer remains high and its clinical treatment remains challenging. Although advances in our understanding of the molecular biology of breast cancer and its progression have aided in the discovery of novel targeted therapeutics, effective new preventive strategies for this disease are still needed for molecular-based, 'patient-tailored' treatment planning.

Histopathological classification of breast cancer

The World Health Organization (WHO) classification divides breast cancer into two histological cathegories: (1) non-invasive/pre-invasive (tumor cells have not spread to nearby breast tissue) carcinomas and (2) invasive (tumor cells have broken out into nearby breast tissue or beyond) carcinomas. Invasive carcinomas are divided into several histopathological subtypes [Tavassoli and Devilee, 2003]; invasive ductal carcinoma (IDC) constitute the majority of all breast cancers worldwide, accounting for 75% of all diagnosed cases [Weigelt et al., 2005 and 2010; Yerushalmi et al., 2009]. IDC is characterized histologically as an infiltrating, malignant and abnormal proliferation of neoplastic epithelial cells that are bounded by the basement membrane of the breast ducts [Espina et al., 2013]. Over the past decade, the molecular interrogation of invasive human breast cancer at the genomic and transcriptomic levels has been one of the most commonly studied disease entities in all of cancer biology.

Prognostic and predictive factors

Several well-established prognostic and predictive factors are universally used to guide the clinical management of women with breast cancer [McGuire, 1991]. Prognostic factors estimate disease outcomes such as disease-free survival, in contrast, a predictive factor estimate the likelihood of response or lack of response to a specific treatment [Cianfrocca and Goldstein, 2004]. The main prognostic and predictive factors in breast carcinoma are: (1) grade of the primary carcinoma, (2) the histologic subtype of carcinoma, (3) hormone receptor status, (4) proliferative rate (Ki67 positivity) of the tumor and (5) *human epidermal growth factor receptor 2 (HER-2)* gene amplification. [Allred et al., 1998; Schnitt, 2001; Lal et al., 2005].

Ki-67

Ki67 (Kiel clone 67) was identified as a nuclear nonhistone protein, shortly after the corresponding antibody was described. The expression of Ki-67 varies in intensity throughout the cell cycle; levels of Ki-67 are low during G1- and early S-phase and progressively increase to reach a maximum during mitosis [Gerdes et al., 1984; van Dierendonck et al., 1989]. A rapid decrease in expression starts during anaphase and telophase. This irregular presence of Ki-67 may represent a handicap to Ki-67 accuracy in identifying cells in this phase. Therefore Ki-67 immunohistochemical staining during the histopathological evaluations of tumors correlates with the mitotic index of the cells which, in turn, are representative of the tumor proliferation rate. The result of this test is reported as the percentage of Ki-67-positive cells. It shows whether a low (<10%), moderate (10% to 20%) or high (>20%) proportion of cancer cells are in the process of dividing. Elevated expression of Ki-67 correlates with higher tumor grade and negative prognosis.

Hormone receptor status and endocrine therapy

Hormon receptor status is a main factor in planning breast cancer treatment. Estrogen receptors were the first biological markers evaluated in breast cancer [Jensen and Jacobson, 1962]. Estrogen receptors are members of the nuclear receptor superfamily that mediate the pleiotropic effects of the steroid hormone estrogen (**Figure 6**) [Mangelsdorf et al.,1995]. Although estrogens are important physiological regulators in the reproductive system, they have also been associated pathologically with an increased risk for cancer [Sommer and Fuqua, 2001]. The most important clinical implication of ER status in patients with IDC is the resulting role for hormone therapy. Current endocrine therapies for ER-positive breast cancers are primarily designed to target either estrogen or ER levels and/or activity. Estrogen binded ER exerts its transcriptional effects through binding to specific DNA sites, termed estrogen response elements (EREs), located in the promoter and/or enhancer regions of target genes (classical signaling) [Klinge, 2001]. ER regulation is not limited to direct ligand binding, because it can also be modulated by several other pathways (**Figure 6**). Growth factors such as epidermal growth factor (EGF) and insulin-like growth factor-1 (IGF-I), bind to and activate their receptors, which in turn activate the MAPK cascade and the PI3K pathways [Smith, 1998; Driggers and Segars, 2002; Segars and Driggers, 2002]. The activated kinases then phosphorylate and activate ER resulting receptor dimerization and target gene transcription in a ligand-independent manner [Kato et al., 1995; Bunone et al., 1996]. The existence of transcription-independent ER functions increases the potential diversity of ER signaling.

Different types of treatment are available for patients with breast cancer. Systemic treatment options include hormone therapy. The concept of manipulating the endocrine system in the treatment of breast cancer was developed more than 100 years ago [Beatson, 1896] and has led to the development of effective and relatively safe drugs, such as

Tamoxifen [Osborne, 1998]. The drug was approved by the Food and Drug Administration (FDA) in 1977 for the treatment of women with breast cancer [Osborne, 1998; Wickerham, 2002]. Tamoxifen is a first-generation non-steroidal selective ER modulator (SERM), it acts as an antagonist in the breast, mediated by its competitive inhibition of estrogen binding to ER so that transcription of estrogen-responsive genes is inhibited (**Figure 6**) [Jordan and Dowse, 1976].

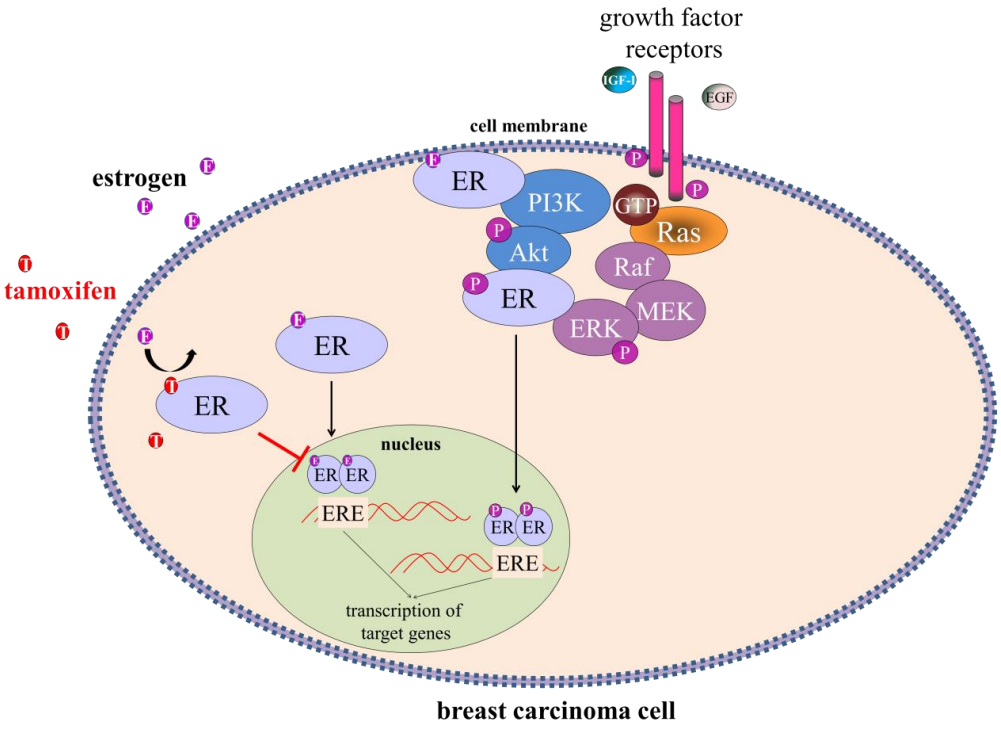


Figure 6. Representation of estrogen receptor signaling pathways and the action of Tamoxifen

Estrogen receptor (ER) mediates transcription of its target genes using two types of mechanisms, these are known as "classical" and "nonclassical" signaling. "Classical" signaling initiates with the binding of estrogen (E) to estrogen receptor, causing it to bind directly to EREs, which subsequently activate transcription of downstream genes. There are several mechanisms of "nonclassical" signaling. The first of these mechanisms is mediated by the signaling of growth factors (such as IGF-I and EGF), through downstream signaling molecules to estrogen receptor. These pathways mediate estrogen receptor state of post-transcriptional modification by affecting its phosphorylation, thus its activity is independent of estrogen binding. It is likely that crosstalk of these pathways not only results in estrogen-independent activation of estrogen receptor but also endocrine resistance. Tamoxifen (T) disrupts estrogen–ER binding, thus the ER mediated signaling is downregulated. The drug blocks cell proliferation and induce apoptotic cell death.

HER-2 and immunotherapy

HER-2 (c-*erb*-B2, HER-2/*neu*) is a member of the HER family [also called the ErbB or human epidermal growth factor receptor family]. After dimerization, HER-2 can signal through at least three different pathways including PI3K, MAPK and PLCγ pathways and can induce cell proliferation, decreased apoptosis, cellular migration and angiogenesis (**Figure 7**) [Rubin and Yarden, 2001]. As a key gene for cell survival, *HER-2* gene amplification and protein overexpression lead to malignant transformation [Neve et al., 2001]. HER-2 is amplified and overexpressed up to 30% of IDCs [Slamon et al., 1989]. HER-2 overexpression in IDC is associated with a negative prognosis; it is always associated with more aggressive tumor phenotypes (higher tumor grade, increased proliferation rate) and increased resistance to endocrine therapy [Slamon et al., 1987; Engel and Kaklamani, 2007]. HER-2 positive breast cancers can benefit from the expression of the receptor, almost immediately after the discovery of its role in tumorgenesis, HER-2 was targeted for cancer therapy.

The humanized monoclonal antibody Trastuzumab (trade name **Herceptin**™) was the first agent developed for HER-2 targeting. Herceptin binds to the extracellular segment of the HER-2 receptor to suppress its dimerization with other HER family members and induces some of its effect by downregulation of HER-2 leading to disruption of receptor dimerization and signaling through the downstream MAPK and PI3K cascades (**Figure 7**) [Nahta et al., 2006; Cameron and Stein, 2008]. The successful development of Herceptin has had a major impact on the treatment of breast cancer. Since then, a variety of HER-2 specific antibodies and small molecular inhibitors have been assessed in clinical trials.

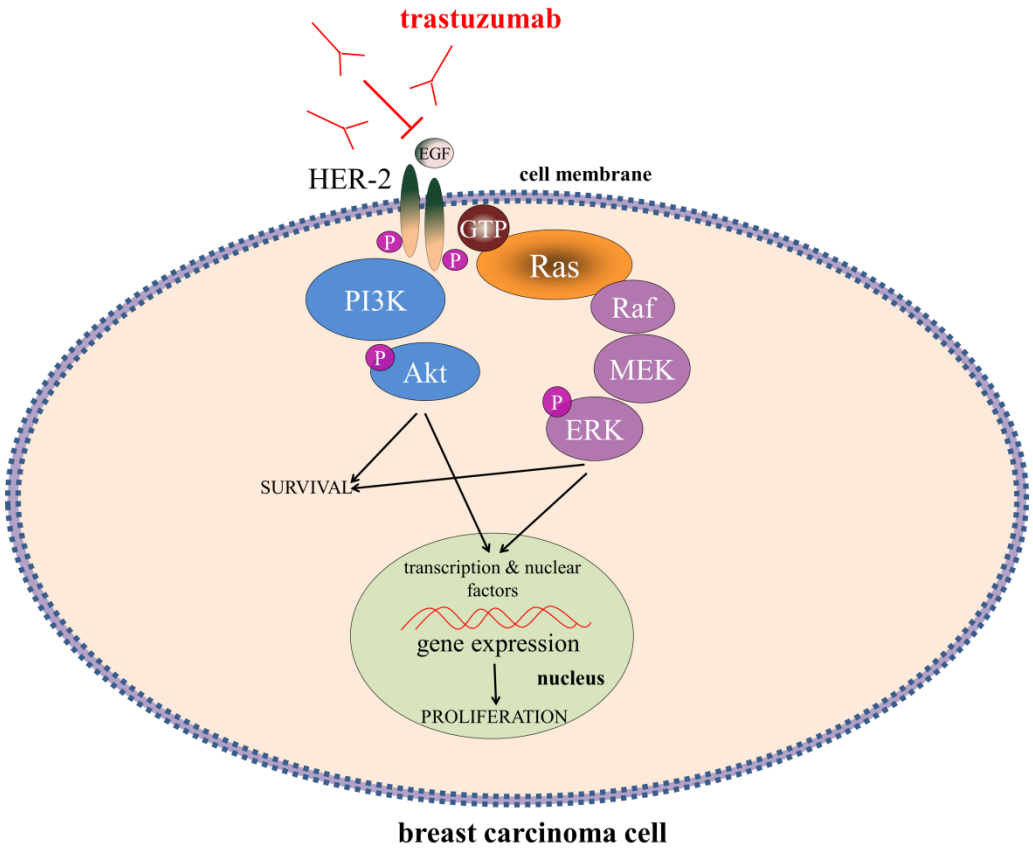


Figure 7. Representation of HER-2 signaling pathways and the action of Herceptin

Activation of HER-2 occurs through homo- or heterodimerization with one of the other members of the HER family. Following activation of HER-2, signal transduction cascades are initiated involving the MAPK and PI3K pathways resulting in cell proliferation and enhanced anti-apoptotic signaling. Herceptin binds to the extracellular segment of the HER-2 receptor to suppress therefore leading to disruption of receptor dimerization and HER-2 receptor signaling.

Major clinical problem: resistance

Despite the positive clinical results many ER/PR and HER-2 positive tumors are *de novo* resistant to Tamoxifen and Herceptin without any prior exposure, furthermore many of the tumors that initially respond to treatment can aquire resistance during and after therapy which presents a major clinical problem [Clarke et al., 2001; Ali and Coombes, 2002; Jensen and Jordan, 2003; Musgove and Sutherland, 2009].. From experimental studies, many different mechanisms have been suggested to explain Tamoxifen and Herceptin resistance.

Potential genetic or epigenetic changes within the tumor that might contribute to **Tamoxifen resistance** include: (1) altered ER expression; (2) an increased crosstalk between ER and HER-2 receptor, (3) ligand independent phosphomodification of ER which has been reported in response to EGF or IGF-I by stimulating the phosphorilation of Ser 118 on the ER and (4) loss of ER expression [Johnston et al., 1995].

A potential mechanism by which **Herceptin resistance** may develop is via disruption of the interaction between the therapeutic agent and the target protein: (1) the membrane-associated glycoprotein Mucin 4 (MUC4) masks the Herceptin binding epitopes of HER-2 resulting in steric hindrance of the interaction [Price-Schiavi et al., 2002; Nagy et al., 2005;] (2) aminoterminal-truncated p95-HER-2 lacks the Herceptin binding site, enabling activated signaling in the presence of Herceptin. Apart from changes in the HER-2 receptor structure alterations in the pathways it activates may also mediate Herceptin resistance: (1) increased expression of insulin-like growth factor-1 receptor (IGF-IR). It is showed that IGF-IR physically interacts with and phosphorylates HER-2 in Herceptin-resistant cells. Resistant cells exhibited more rapid IGF-I stimulation of downstream PI3K and MAPK pathways relative to parental cells [Lu et al., 2001 and 2004; Nahta et al., 2005] (2) mutations in PI3K pathway, e.g. PTEN-deficiency [Chan et al., 2005; Yakes et al., 2002; Nagata et al., 2004] (3) overexpression of HER-3 or EGFR and generation of high levels of ligand-stimulated Herceptin resistant HER-2/HER-3 or HER-2/EGFR heterodimers [Motoyama et al., 2002; Diermerier et al., 2005].

Molecular subtypes

The disease is heterogeneous in its histopathology, therapeutic response, metastatic patterns and outcome. Current treatment decisons are guided by histopathological grading, tumour size, lymph node-, hormone receptor-, HER-2- and proliferation status (Ki67

staining). More recently, gene expression profiling using microarray analysis has proved to be a powerful tool in the identification of profiles of specific tumor subtypes providing a deeper understanding of the complexity of breast cancer [Perou et al., 2000; Jacquemier et al., 2005; Badve and Nakshatri, 2009]. Studies have shown that breast tumors can be divided into at least five major molecular subtypes: (1) luminal A, (2) luminal B, (3) luminal B with enriched HER-2 expression, (4) HER-2 and (5) triple-negative or basal-like. These subtypes have been suggested to originate from different precursor cells and follow different progression pathways and they also differ in their response to treatment and outcome. Researchers are studying how molecular subtypes may become useful in planning treatment and developing new therapies. The St. Gallen Consensus Discussion in 2011 opened for molecular subtyping of breast cancer using ER, PR, HER-2 and Ki67/grade, all factors are already in clinical use. The complex profile of each subtype is shown in **Table 1**.

Table 1. Major molecular subtypes of breast cancer determined by gene expression profiling

	Invasive Ductal Adenocarcinoma				
Groups	Luminal A	Luminal B	Luminal B/ HER-2	HER-2	Triple negative
Receptor expression	ER+ and PR+ HER2 -	ER+ and PR+ HER2 +	ER+ and PR+ HER2 +++	ER- and PR- HER2 +++	ER- and PR- HER2 -
Proliferation rate	Ki67<10%	10%<Ki67<20%	Ki67>20%		
Tumor grade	moderate	poor			

ER+ = estrogen receptor-positive, **ER-** = estrogen receptor-negative, **PR+** = progesterone receptor-positive, **PR-** = progesterone receptor-negative, **HER-2+** = HER-2 receptor-positive, **HER-2+++** = HER-2 receptor is overexpressed, **HER-2-** = HER-2 receptor-negative.

Most breast cancers are luminal tumors. Luminal cancers start in the inner (luminal) cells lining the mammary ducts. Luminal A tumors tend to be estrogen receptor-positive (ER+), progesterone receptor-positive (PR+) and HER-2-negative (HER-2-) with moderate tumor grade. Luminal B tumors tend to be ER+, PR+; highly positive for Ki67 and

HER-2-positive (HER-2+). Luminal B/HER-2 tumors –often designated as a luminal B subgroup- tend to be ER+, PR+; highly positive for Ki67 and HER-2+. Compared to luminal A tumors, luminal B and luminal B/HER-2 tumors tend to have factors that lead to a poorer prognosis including poorer tumor grade, larger tumor size and lymph node-positivity. HER-2 type tumors are named for their high HER-2+ status. They tend to be estrogen and progesterone receptor-negative (ER-/PR-) with low tumor grade. Triple negative/basal-like breast cancers are ER-, PR- and HER-2-negative (HER-2-). HER-2 and triple-negativ type tumors have high Ki67 positivity, a fairly poor prognosis and are prone to early metastases.

Comparison of the variation among ER, PR and HER-2 receptor expression in breast cancers has been proven to be a useful strategy to predict the pathological feature and clinical outcome. Patients with ER+/PR+/HER-2− tumors always have the best survival advantage under chemotherapy.

n**PKC**δ **AS A SIGNIFICANT PLAYER IN SKELETAL MUSCLE PATHOLOGY**

It has been previously shown [Czifra et al., 2006] that nPKCδ plays a pivotal and exclusive role in mediating the *in vitro* growth-promoting effect of IGF-I both in human skeletal muscle cultures and in the mouse C2C12 skeletal muscle myoblast cell line. Our recent publication [Czifra et al., 2014] introduced this isoform as a key player in promoting cellular growth and inducing malignant transformation. Overexpression of the constitutively active nPKCδ stimulated whereas the kinase inactive DN-nPKCδ mutant inhibited *in vitro* growth of C2C12 myoblasts. Most importantly, 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).

RHABDOMYOSARCOMA -histology, classification and pathogenesis

Pediatric soft tissue sarcomas are a heterogeneous group of tumors that are presumed to arise from a primitive mesenchymal cell. Rhabdomyosaroma (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. Although RMS can arise anywhere in the body, distinct patterns link primary site, histology, and age at diagnosis: The most common site in which RMS occurs is in the head and neck structures (~40%), genito-urinary tract (~25%), and extremities (~20%).

The International Classification of Rhabdomyosarcoma was derived from detailed pathologic study of patients who were enrolled in the first four IRSG therapeutic trials (IRS I-IV). Histologically, pediatric RMS tumors are classified into 3 different histologic subtypes: (1) embryonic rhabdomyosarcoma (ERMS) being the most prevalent; (2) alveolar rhabdomyosarcoma (ARMS), the most aggressive; and (3) pleomorphic rhabdomyosarcoma (PRMS), a rare adult variant [Paulino and Okcu, 2008; Parham and Ellison, 2006; Ognjanovic et al., 2009; Wang , 2012; De Giovanni et al., 2009; Breneman et al., 2003]. The forms of RMS are thought to be derived from myogenic progenitors as the consequence of impaired differentiation due to genetic lesions [Charytonowicz E et al., 2009; Nitzki F et al., 2011].

The etiology and pathogenesis of RMS are still poorly understood. The prognosis the patient is dependent on the localization of the primary lesion, stage of disease, age at diagnosis and histological subtype [Pappo et al., 1995]. Chemotherapy is one of the three most common treatment modalities in RMS, but often, the resistance of cancer cells to drugs limits its efficacy [Melguizo et al., 2001; Cocker et al., 2001]. More than half of RMS patients present with unresectable or metastatic disease, which tends to be the main cause of death [Arndt and Crist, 1999]. 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.

The molecular pathogenesis of RMS is not clear. The majority of ARMS are characterized by specific translocations between the DNA binding encoding domain of either the PAX3 or PAX7 genes and the transactivation encoding domain of FOXO1 [Galili et al., 1993; Sharpio et al., 1993; Davis et al., 1994]. Other genetic events are associated with these tumors including MYCN amplification and overexpression [Driman et al., 1994; Williamson et al., 2005; Xia et al., 2002]. ERMS are not characterized by specific fusion genes but are aneuploid with frequent gain of chromosome 8 and have activating mutations of *ras* gene [Kratz et al., 2007; Langenau et al., 2007]. Another frequent genetic alteration present in RMS is loss of heterozygosity at the 11p15.5 locus.

In RMS receptor tyrosine kinases (RTKs) have been identified to be high-value target candidates based on clinical survival data [Blanford et al., 2006]. IGF-I is represented as an important growth factor. Overexpression of a receptor IGF-IR, is frequently found in RMS, occasionally associated with genomic amplification events [Bridge et al., 2002]. Evidence supports IGF-IR signaling in the genesis, growth, proliferation and metastatic behavior of RMS [Yun K, 1992].

Although several biomarkers, such as the PAX3-FOXO1 or PAX7-FOXO1 fusion gene have been reported to be of diagnostic and prognostic values in RMS, the poor clinical outcomes give motivation for a better understanding of the tumorigenic mechanisms so that new therapeutic targets can be identified [Breneman et al., 2003; Wachtel and Schafer, 2010].

PKC isozymes have been implicated in the pathogenesis of many human cancers including breast, colon, lung, prostate, pancreatic, liver and hematopoietic cancers [Fields and Murray, 2008]. 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 [Fields, 2007]. Although few reports are available on 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 [Ehrhardt and Morgan, 2005; Amstutz et al., 2008; Kikuchi et al., 2013].

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 potential function of **RasGRP3** in one of the most malignant cancer type, the breast-derived ductal adenocarcinoma. Within this broad theme, the research had a number of specific objectives, we intented:

(1) to characterize the expressions of RasGRP3 and phosphoRasGRP3 in human breast-derived invasive ductal adenocarcinoma samples and cell lines at mRNA and protein levels using molecular biological approaches

(2) to establish RasGRP3-silenced ductal adenocarcinoma-derived cell lines by using retroviral transfection for further *in vitro* and *in vivo* investigations

(3) to define the role of RasGRP3 in the regulation of proliferation and survival of RasGRP3 modified ductal adenocarcinoma cells by using quantitative molecular biological assays

(4) to analyze the role of RasGRP3 in the regulation of tumorigenic mechanisms by using SCID mouse model injected with the RasGRP3 modified ductal adenocarcinoma cells

(5) to estimate the difference in the sensitivity of RasGRP3 silenced cells against endocrine- and chemotherapeutic drugs Tamoxifen and Herceptin

(6) to investigate the role of RasGRP3 in growth factor coupled Ras-related downstream signaling

Since **nPKCδ** has been reported to play a pivotal role in mediating *in vitro* and *in vivo* cell growth as well as the malignant transformation of C2C12 skeletal muscle myoblasts, as a continuation of our previous studies, an attempt was made to further dissect the role of nPKCδ in the regulation of *in vitro* and, or further importance, *in vivo* growth of human rhabdomyosarcoma cells. In our experiments, the following experimental goals have been defined:

(1) to establish nPKCδ and dominant-negative nPKCδ overexpressor rhabdomyosarcoma cells for further *in vitro* and *in vivo* investigations

(2) to investigate the role of nPKCδ in the regulation of cell proliferation of nPKCδ modified rhabdomyosarcoma cells by using quantitative molecular biological assay

(3) to analyze the role of nPKCδ in the regulation of tumorigenic mechanisms by using SCID mouse model injected with the nPKCδ modified rhabdomyosarcoma cells

(4) to investigate the role of nPKCδ in growth factor coupled Ras-related downstream signaling

MATERIALS AND METHODS

HUMAN STUDY- human tissues and sample preparation

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. All patients provided written informed consent for participation in the study and publication of the results. The samples were obtained as part of routine diagnosis from surgical specimen. Neither the control patients nor the diseased patients had history of previous or contemporary breast malignancies. The tumor samples were divided into two parts. One part was processed to obtain formalin-fixed, paraffin-embedded sections which were subjected to routine haematoxylin-eosin staining-based grading, the second part was frozen in liquid nitrogen and processed for Q-PCR and Western blot analyses (see page 39 and 41).

CELL CULTURING

Culturing of human breast derived tumor cell lines

During the RasGRP3 study, we employed a human primary breast derived ductal adenocarcinoma cell line called BT-474, five different metastatic ductal adenocarcinoma cell lines (MDA-MB-453, MCF7, SK-BR-3, JIMT-1, T-47D) and a prostate derived carcinoma cell line (PC-3) as positive control [Yang et al., 2010]. PC-3, BT-474 and T-47D cells were maintained in RPMI-1640 Medium (Sigma-Aldrich; St. Louis, MO), MCF7 cells were maintained in Eagle's Minimum Essential Medium (EMEM) (Sigma-Aldrich), JIMT-1 cells were maintained in 1:1 Dulbecco’s Modified Eagle Medium (DMEM)/Ham’s F12 Medium (Sigma-Aldrich), 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 (Invitrogen; Paisley, UK). All medium were supplemented with 10% (v/v) fetal bovine serum (FBS,

Invitrogen), 2 mM Glutamine (Sigma-Aldrich), 50 U/ml penicillin and 50 μg/ml streptomycin (both from PAA Laboratories GmbH, Austria). In addition, medium for T47-D cells was supplemented with 0.2 U/ml bovine insulin (Sigma-Aldrich), 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.

Culturing of rhabdomyosarcoma cells

Human rhabdomyosarcoma (RD) cells (obtained from the American Type Culture Collection, ATCC No. CCL-136) were maintained in DMEM (Sigma-Aldrich) supplemented with 10% (v/v) FBS (Invitrogen), 2 mM Glutamine (Sigma-Aldrich), 50 U/ml penicillin and 50 μg/ml streptomycin (both from PAA Laboratories). 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

Immunohistochemistry of human breast carcinoma samples

The expression of RasGRP3 and phosphoRasGRP3 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; Sigma-Aldrich) 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 (all from Sigma-Aldrich) for 30 min and probed with the appropriate primary antibodies for 2 hours at 37 °C. Sections were then stained for 30 minutes with the appropriate horseradish peroxidase-

labeled polymer conjugated secondary antibodies. Immunoreactions were visualized using DAB substrate (EnVision kit, DAKO; Glostrup, Denmark) for 3-5 minutes and the sections were counterstained by Mayer's hematoxylin (Sigma-Aldrich) 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.

Immunohistochemistry of xenograft tumors

The histological parameters of the xenograft isolated tumors were determined on formalin-fixed, paraffin-embedded, and hematoxylin-eosin (HE)-stained sections of the developed tumors. In addition, to assess the number of proliferating cells, formalin-fixed, paraffin-embedded sections were immunostained against the nuclear marker Ki67 using a streptavidine-biotin-complex three-step immunohistochemical technique (DAKO). First, the inhibition of endogenous peroxidase activity was performed using 3 % H_2O_2 in 100 % methanol (both from Sigma-Aldrich). Then, non-specific binding was blocked by 1 % bovine serum albumin (Sigma-Aldrich) in PBS buffer (pH 7.5). After testing various concentrations of the anti-Ki-67 monoclonal mouse primary antibody (DAKO), an optimal 1:50 dilution was employed. Sections were then incubated for 30 minutes with the appropriate horseradish peroxidase-labeled polymer conjugated secondary antibody. Immunoreactions were visualized using DAB substrate (EnVision kit, DAKO) and the sections were counterstained by Mayer's hematoxylin (Sigma-Aldrich) and cover-slipped for microscopic examination.

MICROSCOPY AND IMAGE ANALYSIS

Immunohistochemical images were captured and digitalized using an RT Spot Colour CCD camera (Diagnostic Instruments Inc.) integrated on a Nikon Eclipse 600 fluorescence and light microscope (Nikon Tokyo, Japan). 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

Antibodies for Western blotting and immunohistochemistry

Throughout the experiments several primary and secondary antibodies were used. For clarity, antibodies are summarized in **Table 2** and **3**.

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; all from Sigma-Aldrich) and the protein content of samples was measured by the BCA protein assay kit (Pierce, Rockford, IL). Total cell lysates were mixed with 2x SDS-PAGE sample buffer (Sigma-Aldrich) and boiled for 10 mins at 100 ^{o}C. The samples containing 20-60 μg total protein were separated by electrophoresis on 7.5 % or 10 % SDS-polyacrylamide gels (Invitrogen) and transferred onto BioBond nitrocellulose membranes (Whatman, Maidstone, UK). After the membranes were blocked with 5% dry milk in PBS and labeled with the appropriate primary and secondary antibodies (see Table 2 and 3), the immunoreactive bands were visualized by SuperSignal West Femto Chemiluminescent Substrate-enhanced chemiluminescence (Pierce) using a Gel Logic 1500 Imaging System (Kodak, Tokyo, Japan). 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.

Table 2. Primary antibodies used throughout the experiments

ANTIBODY (application)	SPECIFICITY	DILUTION	HOST SPECIES	MANUFACTURER	EPITOPE
anti-RasGRP3 (WB)	Hu, Ms	1:200	Rb	Cell Signaling Technology	carboxy terminus of human RasGRP3
anti-RasGRP3 (IHC)	Hu	1:50	Ms	Abcam	full length of human RasGRP3
anti-phospho-RasGRP3 (WB and IHC)	Ms, Hu	1:200 (WB) 1:50 (IHC)	Rb	Abcam	phospho-specific peptide surrounding Threonine 133 of human RasGRP3
anti-ERK ½ (WB)	Hu, Rat, Ms	1:1000	Rb	Sigma-Aldrich	peptide sequence 317-339 of human ERK ½
anti-phospho-ERK ½ (WB)	Hu, Rat, Bov, Ms, Hms	1:1000	Ms	Sigma-Aldrich	phosphorylated form of the ERK-activation loop
anti-Akt (WB)	Hu, Ms, Rat, Hms	1:1000	Ms	Cell Signaling Technology	peptide sequence 140-480 of human Akt1
anti-phospho-Akt (WB)	Hu, Ms, Rat, Hms	1:1000	Ms	Cell Signaling Technology	phospho-specific peptide surrounding Serine 473 of mouse Akt1
anti-ERα (WB)	Hu, Ms	1:1000	Rb	Sigma-Aldrich	full length of human Estrogen Receptor-α
anti-phospho-ERα (WB)	Hu, Rat, Ms	1:1000	Rb	Sigma-Aldrich	phospho-specific peptide surrounding Serine 118 of human Estrogen Receptor-α
anti-HER2/neu (WB)	Hu, Rat, Ms	1:200	Rb	Sigma-Aldrich	peptide sequence 1246-1250 of protein HER2/neu
anti-IGF-IR (WB)	Hu	1:200	Ms	Santa-Cruz Biotechnology	carboxy teminus of human IGF-IR
anti-EGFR (WB)	Hu	1:200	Ms	Santa-Cruz Biotechnology	peptide sequence 985-996 of human EGFR
anti-PKCδ (WB)	Hu, Ms, Rat	1:1000	Rb	Sigma-Aldrich	full length of human PKCδ
anti-actin β (WB)	Ms, Hu, Ck, Rat	1:1000	Rb	Sigma-Aldrich	amine terminus of human actin β
anti-GAPDH (WB)	Hu, Ms, Rat, Nhp	1:1000	Rb	Novus Biologicals	peptide sequence 150-200 of human GAPDH

WB = Western blot; **IHC**= immunohistochemistry; bolded antibodies are monoclonal, the others are polyclonal.

Cell Signaling Technology (Beverly, MA), Abcam (Cambridge, UK), Santa Cruz Biotechnology (Santa Cruz BioTech, CA), Novus Biologicals (Cambridge, UK).

Table 3. Secondary antibodies used throughout the Western blot experiments

ANTIBODY	SPECIFICITY	DILUTION	HOST SPECIES	MANUFACTURER
Peroxidase-conjugated IgG	Rb	1:1000	Gt	Bio-Rad Laboratories
Peroxidase-conjugated IgG	Ms	1:1000	Gt	Bio-Rad Laboratories

Bio-Rad Laboratories (Hercules, CA)

Quantitative real-time PCR (Q-PCR)

Quantitative real-time PCR was performed on an ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA) by using the 5′ nuclease assay, as we have previously described [Bodó et al., 2005]. Total RNA was extracted with TRIzol reagent (Invitrogen) according to manufacturer's protocol. One microgram of total RNA were then reverse transcribed into cDNA by using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions. PCR amplification was performed by using the TaqMan primers and probes (Assay ID: Hs 00964396_m1 for human RasGRP3) using the TaqMan Universal PCR Master Mix Protocol (Applied Biosystems). The threshold cycle (Ct) of RasGRP3 was determined and normalized to that of human GAPDH (Assay ID: Hs 03929097_g1) to obtain a ΔCt value (Ct_{GAPDH}-$Ct_{RasGRP3}$) 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, Piscataway, NJ) containing a RasGRP3-specific hairpin RNA (shRasGRP3; sequence listed in **Table 4**). Scrambled control plasmid consisted of a sequence (shSCR; sequence listed in **Table 4**) which has no specific targets in mammalian cells [Yang et al., 2010, Yang et al., 2011].

Table 4. shRNA sequences used throughout the experiments

NAME	shRNA SEQUENCES
shRasGRP3	Mlu I ACGCGTCGGAATTACCGCAAGGCCTTTCTTGATATCCGGAAAGGCCTTGCGGTAATTGCTTTTTTTCCAACTCGAG SENSE STRAND LOOP ANTISENSE STRAND TERMINATION SIGNAL Xho I
shSCR	Mlu I ACGCGTCGCTGATAGCATTCGACGTCTATTGATATCCGTAGACGTCGAATGCTATCAGCTTTTTTTCCAACTCGAG SENSE STRAND LOOP ANTISENSE STRAND TERMINATION SIGNAL Xho I

The designed single-stranded oligonucleotides were synthesized, annealed, inserted into the pRNA-H1.1/Retro vector by GenScript. All constructs were verified by DNA sequencing. The production of the retroviral particles and the infection of the target cells were conducted by following the manufacturer's instructions (Clontech, CA). Briefly, RetroPack PT67 cell line was transfected with the shRasGRP3 or shSCR constructs and selected in Hygromycin B (400 µg/ml; Invitrogen) for 2 weeks. The supernatants containing retroviral particles of the selected packaging cells were collected and the virus titers were determined using NIH/3T3 cells. The two host cell lines, MCF7 and T-47D were infected with each retroviral supernatant (MOI=5) and subjected to Hygromycin B (400 µg/ml) selection for 3 weeks. Studies were carried out on the cloned, antibiotic-resistant cells. Efficiency of the suppression of RasGRP3 expression was determined using Western blotting.

Generation of PKC constructs

PKC constructs were engineered as described previously [Papp et al., 2004; Griger et al., 2007; Ács et al., 1997; Li et al., 1999]. Briefly, the cDNA sequences of nPKCδ, and the kinase (dominant)-negative (DN-nPKCδ) were subcloned into a metallothionein promoter-driven eukaryotic expression vector (pεMTH) [Bodó et al., 2005].

RD cells were transfected by either the empty pεMTH vector or by the vectors encoding the cDNA sequences of nPKCδ, or DN-nPKCδ [Papp et al., 2004; Griger et al., 2007; Ács et al., 1997]. Transfections were performed using a Lipofectamine anionic detergent (Invitrogen) in serum-free DMEM solution using 4 μg cDNA according to the protocol suggested by the manufacturer. Cells were selected in DMEM containing 750 μg/ml Geneticin (Invitrogen) for 3 weeks. Studies were carried out on the cloned, antibiotic-resistant cells. The efficacy of recombinant overexpression was monitored by Western blotting.

Determination of cell proliferation

Determination of cell proliferation of breast carcinoma cells

The degree of cellular growth was determined by measuring the DNA content of cells using CyQUANT GR Cell Proliferation Assay Kit (Invitrogen) according to the protocol of the manufacturer. MCF7 and T-47D cells (10^4 cells/ well) were cultured in 96-well black-well/clear-bottom plates (Greiner Bio-One, Baden-Württemberg, Germany) in quadruplicates and were growth for 5 days. The assay was performed each day. Supernatants were removed, and the plates were frozen at −70 °C. The plates were then thawed at room temperature, and 200 μl of CyQUANT GR dye/cell lysis buffer mixture was added to each well. After 5 minutes of incubation, fluorescence signals were quantitated on a Fluorescence Imaging Plate Reader FlexStation^{III} (FLIPR, Molecular Devices, CA) at an excitation wavelength of 485 nm and an emission wavelength of 530 nm [Ramot et al.,2010].

Determination of cell proliferation of rhabdomyosarcoma cells

Proliferation of RD cells was determined by MTT assay (Sigma-Aldrich) according to the protocol of the manufacturer. Cells were plated in 96-well multi-titer plates (10^3 cells/ well) in quadruplicates and were cultured for 4 days. Cells were washed, then incubated for 3 hours at 37 °C with 0.5 mg/ml MTT reagent [3-(4,5-dimethilthiasol-2-il)-2,5-

diphenyltetrasolium bromide; Alfa Aesar GmbH, Karlsruhe, Germany]. 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; all from Sigma-Aldrich) for 30 mins 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 [Bodó et al., 2005].

Detection of apoptotic and necrotic cells

Cells were harvested, washed once in ice-cold Dulbecco's phosphate buffered saline (DPBS), and re-suspended in 0.5 ml DPBS. Annexin-V (Invitrogen) was added to a final concentration of 1 μM and the cells were incubated for 20 min at 4 °C in the dark. Propidium iodide (PI) (Invitrogen) was then added at a 5 μg/ml final concentration 10 minutes before analysis by flow cytometry using the FACScan system (BD Biosciences, Oxford, UK). Data were analyzed with FSC Express software (De Novo Softwer, CA).

Mitochondrial membrane potential was also assessed by MitoProbe DilC_1(5) Assay Kit (Invitrogen). T-47D and MCF7 derived cells (5 x 10^3 cells/ well) were cultured in 96-well black-well/clear-bottom plates in quadruplicate. After removal of supernatants, cells were incubated with DilC_1(5) working solution –prepared according to the manufacturer's instructions- 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}.

Determination of chemotherapeutic sensitivity

The CyQUANT assay was employed to assess the chemotherapeutic sensitivity of the cells. For this, MCF7 and T-47D cells were plated in 96-well black-well/clear-bottom plates (Greiner Bio-One) in quadruplicates at a density of 10^4 cells/ well. After overnight culturing,

various concentrations of chemotherapeutics (see page 68), i.e. Herceptin (F.Hoffmann-LaRoche Ltd., Switzerland) and Tamoxifen (Sigma-Aldrich) were administered for 72 h. After the indicated time, CyQuant GR cell proliferation assay was performed as discussed before.

XENOGRAFT EXPERIMENTS

Severe combined immunodeficiency (SCID) mice were bred and maintained in the animal facility of the Department of Dermatology (University of Debrecen, Medical and Health Science Center Faculty of Medicine) in accordance with the animal-welfare ordinance. The studies were performed under the current regulations and standards of the Institutional Research Ethics Committee of the University of Debrecen, Debrecen, Hungary. Cells were harvested by trypsinization and washed twice with DPBS. Cell pellets [4×10^6 viable cells (T-47D, MCF7 and RD cells) and $2x10^6$ viable cells (C2C12 cells)] were re-suspended in 0.1 ml appropriate medium and 0.1 ml Matrigel (BD Biosciences) and injected in a single subcutaneously site on the right flank of the mice (0.2 ml/injection). Animals were sacrificed 12 week after injection in case of T-47D and MCF7 breast carcinoma cells while RD cells derived tumors were observed over a period of 30 days. After the animals were finally euthanized the tumors were excised, and the averaged three-dimensional size, the weight 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 EXPRESSION IN HUMAN BREAST CANCER

RasGRP3 is highly expressed in human breast derived ductal adenocarcinoma samples

Using Q-PCR and Western blot, we assessed the expression of the RasGRP3 protein and presumably its active form, phosphoRasGRP3, in human breast derived ductal adenocarcinoma samples as well as in normal human breast tissue samples.

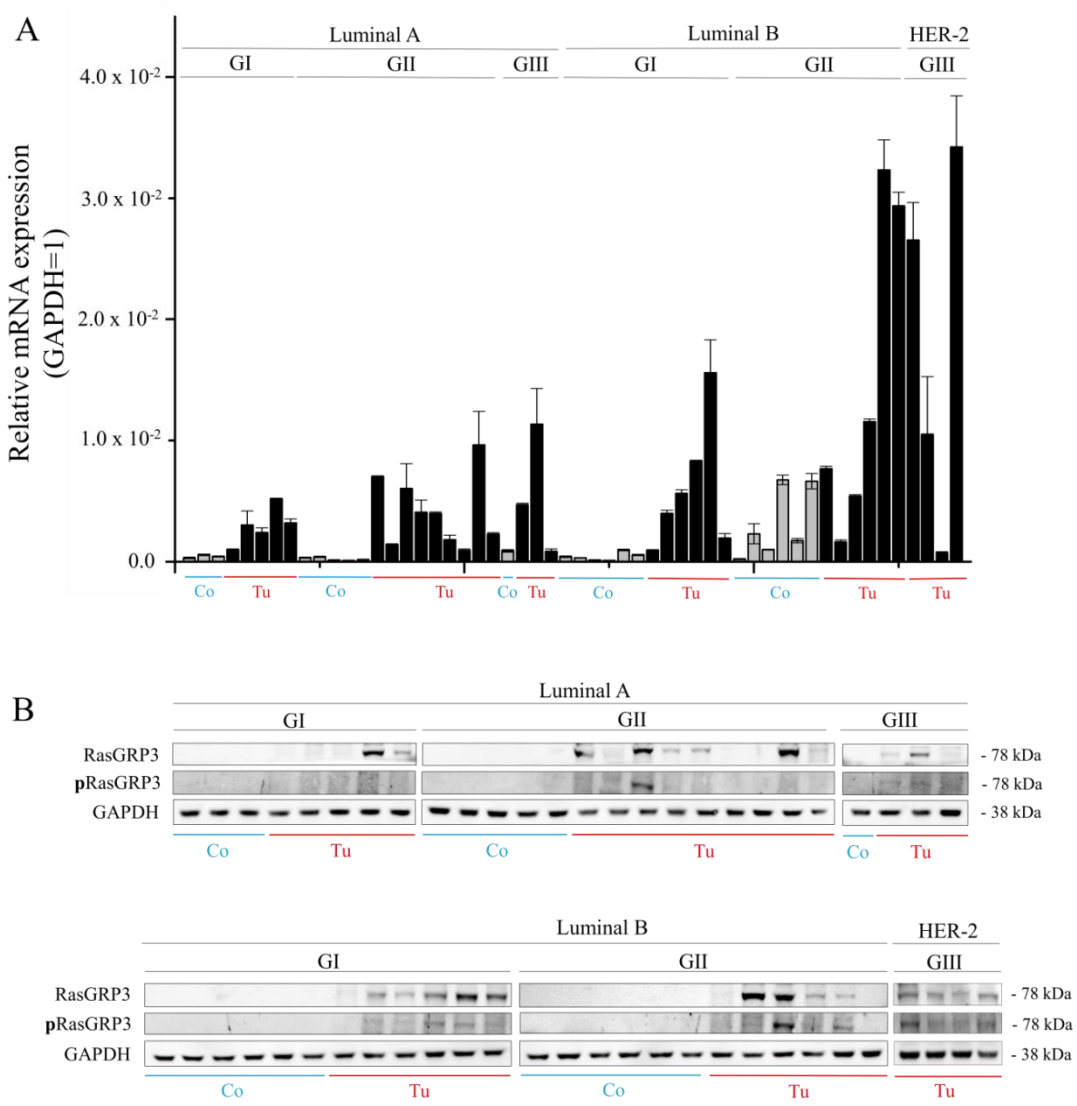


Figure 8. RasGRP3 expression in breast derived ductal adenocarcinoma samples

(A) mRNA (Q-PCR) and **(B)** protein (Western blot) analyses of RasGRP3 and phosphoRasGRP3 expressions in human normal breast (Co; n=21) and breast derived ductal adenocarcinoma (Tu; n=33) with various grades (GI-GIII) presented according to groups Luminal A, Luminal B and HER-2 of human breast derived ductal adenocarcinoma (Luminal A; Luminal B and HER-2). GAPDH was used as an internal control. The results are representative of three independent experiments for Q-PCR and two independent experiments for Western blot. Values represent the mean ± SEM.

Results are presented according to the molecular classification of breast derived ductal adenocarcinoma (groups Luminal A, Luminal B and HER-2). We found that the levels of RasGRP3 and phosphoRasGRP3, albeit exhibiting marked inter-individual variations, were significantly higher in the tumor samples compared to the controls (**Figure 8**). The elevated RasGRP3 and phosphoRasGRP3 expressions in the tumor tissues might correlate with the degree of malignancy and progression; indeed, the expression of the proteins in groups Luminal B and HER-2 were higher compared to group Luminal A. Althought further investigations and increased sample number are needed to fully prove this hypothesis.

In addition, we also determined the cellular localizations of RasGRP3 and phosphoRasGRP3 by immunohistochemistry in sections prepared from normal or diseased breast tissues. RasGRP3 was localized in the cytoplasm of the cells whereas the active form exhibited mostly nuclear immunoreactivity **(Figure 9)**. Human kidney derived samples [Yamashita et al., 2000] served as positive controls in immunohistochemical experiments.

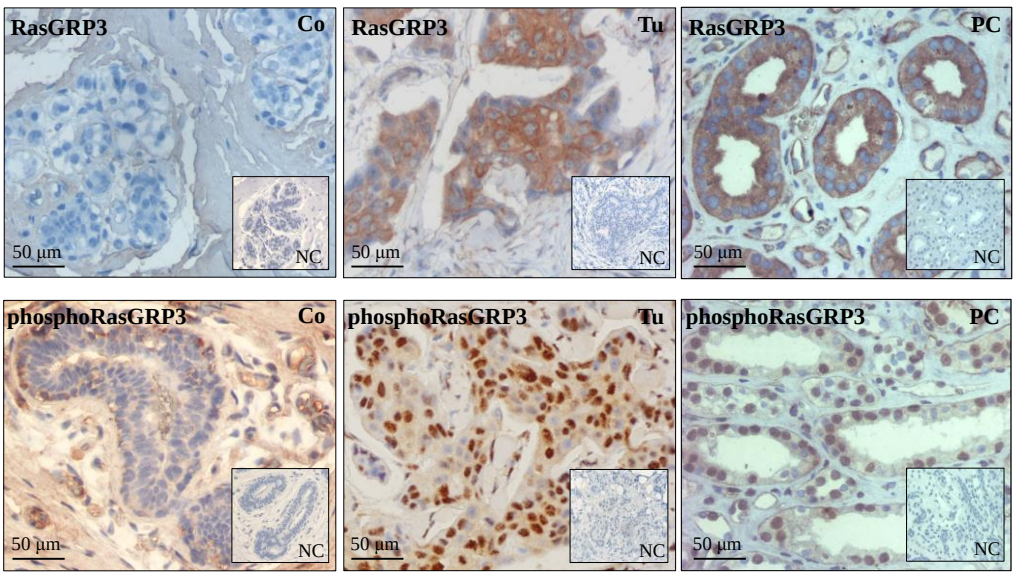


Figure 9. RasGRP3 localization in breast derived ductal adenocarcinoma samples

Representative images of RasGRP3- and phosphoRasGRP3-specific immunreactivity with diaminobenzidine as a chromogen (brown staining) on human normal (Co; n=4) and breast derived ductal adenocarcinoma (Tu; n=4) sections. Human kidney samples were included as a positive control (PC). Nuclei were co-stained by Mayer's Hematoxylin (blue staining). Preabsorption negative control (NC) was used. The results are representative of four independent experiments.

RasGRP3 is expressed in human breast derived ductal adenocarcinoma cell lines

Starting the *in vitro* experiments, we determined 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. The characteristics of the different cell lines regarding their origin, estrogen- and HER-2 receptor status and therapeutic sensitivity are detailed in **Table 5**.

Table 5. Characteristics of the breast derived ductal adenocarcinoma cell lines

CELL LINE	primary	metastatic (from)	estrogen receptor	HER-2 receptor	tamoxifen resistant	trastuzumab resistant
BT-474	yes		expressed	expressed	no	no
JIMT-1		yes (pleura)	not expressed	overexpressed	yes	yes
MCF7		yes (pleura)	expressed	not expressed	yes	yes
SK-BR-3		yes (pleura)	not expressed	expressed	no	no
MDA-MB-453		yes (pericardium)	not expressed	expressed	no	no
T-47D		yes (pleura)	expressed	expressed	no	no

Q-PCR and Western blot analyses also confirmed the expression of RasGRP3 in all cell lines with barely detectable levels in the primary BT-474 cells and in one of the metastatic cell lines, MDA-MB-453 (**Figure 10**). For comparison, the relatively high expression of RasGRP3 in the PC-3 prostate adenocarcinoma derived cell line [Yang et al., 2010] served as a positive control in both assays.

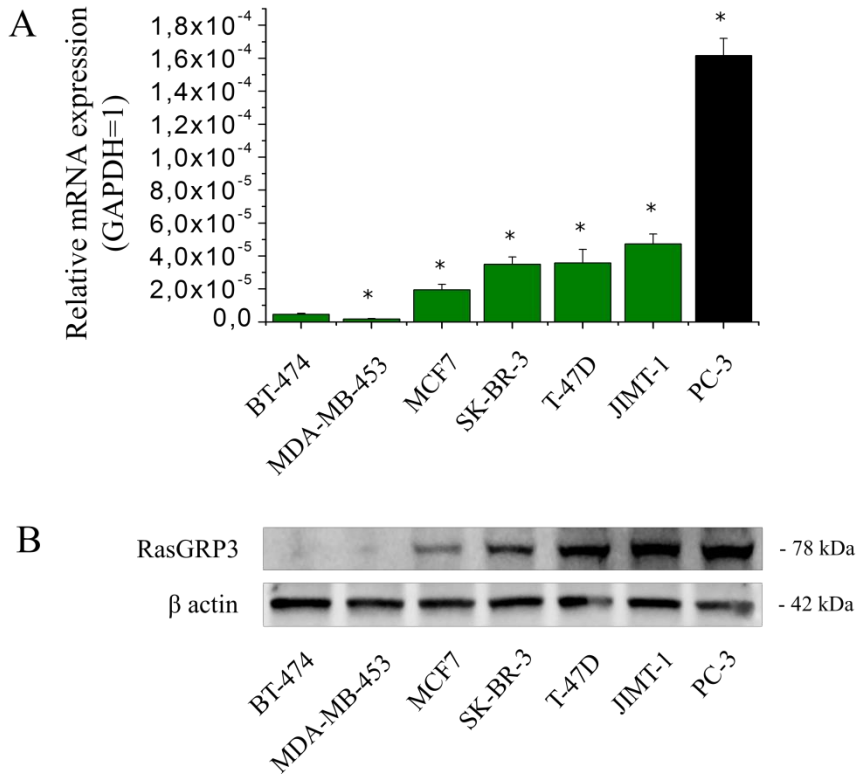


Figure 10. RasGRP3 expression in breast derived ductal adenocarcinoma cell lines

(A) mRNA (Q-PCR) and **(B)** protein (Western blot) analyses of RasGRP3 expression in multiple breast derived ductal adenocarcinoma cell lines. PC-3 cells were included as a positive control. GAPDH gene expression was used as an internal control. The results are representative of three independent experiments. Values represent the mean ± SEM. * indicates significant difference ($p < 0.05$) compared to primary BT-474 cells.

GENERATION OF "RASGRP3-SILENCED" BREAST CANCER CELLS AND nPKCδ OVEREXPRESSOR RHABDOMYOSARCOMA CELLS

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

To explore the functionality of RasGRP3, namely the role of the protein in the regulation of proliferation, viability, chemotherapeutic resistance and tumorigenesis 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) or against a non-targeting scrambled control (shSCR) to achieve a long term

stable suppression (sequences of the shRNA probes are shown in **Table 4**). As determined by Western blot analysis, levels of RasGRP3 could be effectively reduced in both MCF7 and T-47D cancer cells whereas in cells expressing shSCR significant modulation of RasGRP3 expression was not observed **(Figure 11)**.

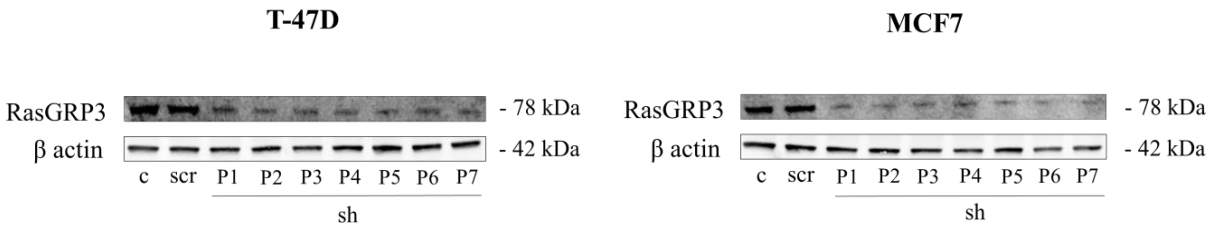


Figure 11. Confirmation of the extent of RasGRP3 expression in T-47D and MCF7 cells

Expression of RasGRP3 was inhibited using a specific shRNA (shRasGRP3). A non-targeting scrambled shRNA (shSCR) was used as a control. Confirmation of the extent of suppression of RasGRP3 expression of cells investigated was determined by Western blotting. β actin was used as an internal control. P indicates the number of subculturing of the RasGRP3 silenced cells. Results are representative of 2 independent experiments.

Overexpression of nPKCδ in human rhabdomyosarcoma cells

Our previous data argued for the key role of nPKCδ in promoting proliferation and inducing malignant transformation of myoblasts. 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. As determined by Western blot analysis (**Figure 12**), levels of the overexpressed nPKCδ were several-fold higher compared to the control (empty MTH vector expressing) cells.

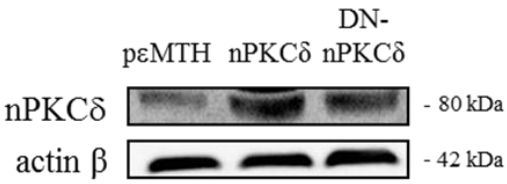


Figure 12. Confirmation of the overexpression of nPKCδ in human rhabdomyosarcoma cells

Confirmation of the stable transfectants of RD cells expressing the empty MTH (pεMTH) vector or vectors encoding nPKCδ or the dominant-negative (DN-nPKCδ) mutant was determined by Western blotting. β actin was used as an internal control. Results are representative of 2 independent experiments.

ROLES OF RASGRP3 AND nPKCδ IN THE REGULATION OF *IN VITRO* PROLIFERATION

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

We then assessed the possible alterations in cellular functions of the "RasGRP3-silenced" cells. As revealed by growth curve analysis using fluorimetric CyQUANT GR cellular proliferation assays from days 0-5 of culturing **(Figure 13)**, 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.

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

We investigated the effect of overexpression of the nPKCδ on the proliferation of RD cells. As revealed by growth curve analysis using MTT assays from days 0-4 of culturing (**Figure 14**) 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.

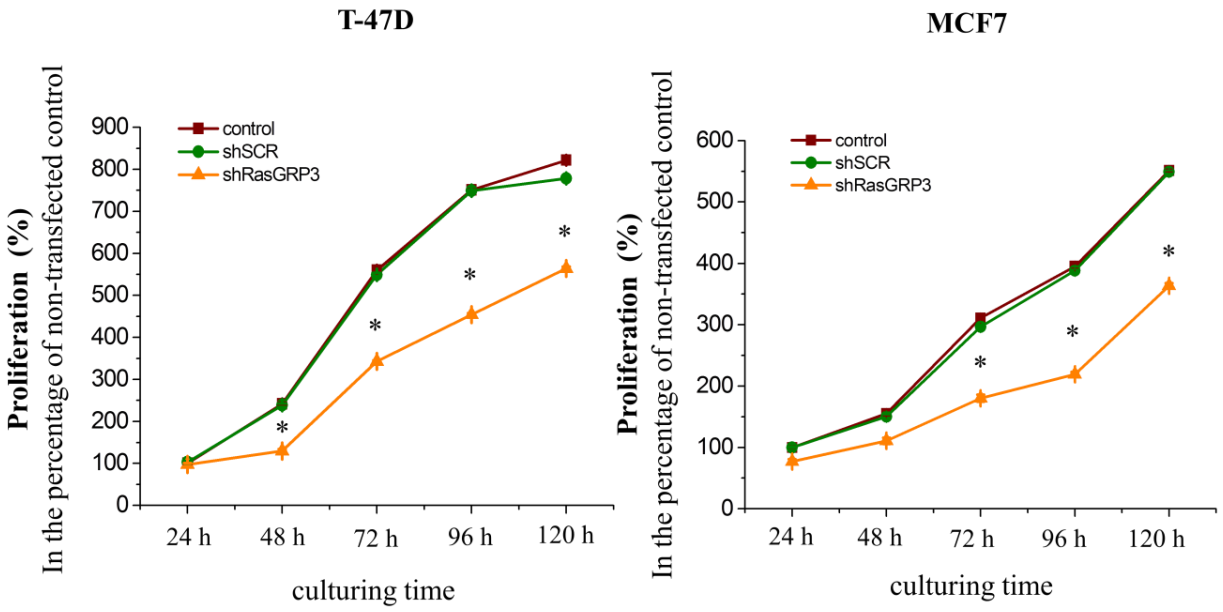


Figure 13. Down-regulation of RasGRP3 expression represses cell proliferation of both MCF7 and T-47D derived cells

The proliferation of T-47D and MCF7 derived cells was determined using the CyQuant GR cell proliferation assay, with values normalized to the levels of non-transfected control cells. CyQuant assay was conducted every 24 hours. Values represent the mean ± SEM for 2 independent experiments. * indicates significant difference ($p < 0.05$) compared to scrambled control cells.

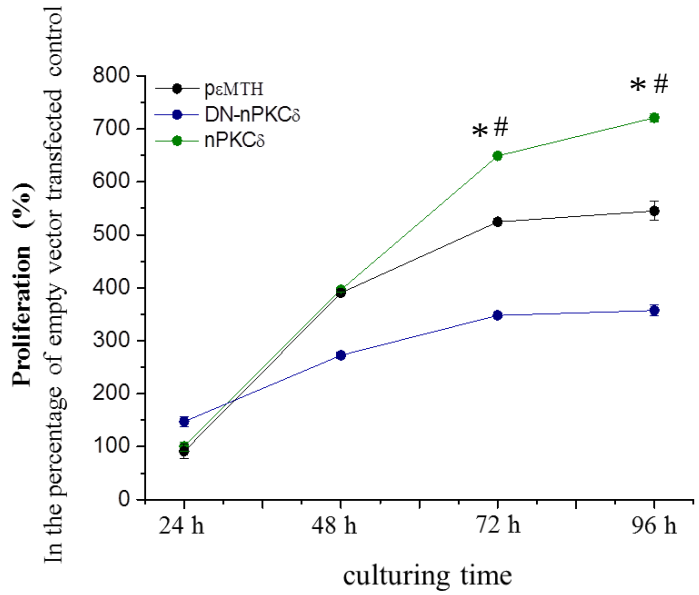


Figure 14. Overexpression of nPKCδ promotes cell proliferation of RD cells

The proliferation of RD cells was determined using the MTT assay. Values were normalized to the levels of empty MTH vector-transfected control cells. MTT assay was conducted every 24 hours. Values represent the mean ± SEM for 2 independent experiments. * indicates significant difference ($p < 0.05$) compared to control (empty MTH) vector-transfected cells. # indicates significant difference ($p < 0.05$) compared to DN-nPKCδ vector-transfected cells.

ROLE OF RASGRP3 IN THE REGULATION OF SURVIVAL

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 **(Figure 15).**

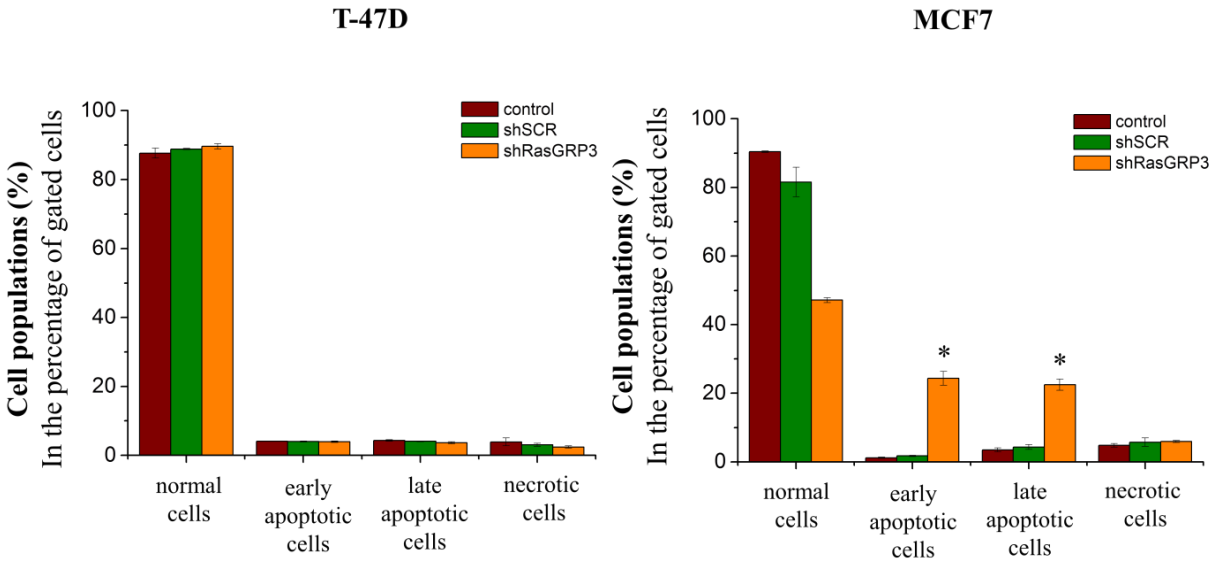


Figure 15. Down-regulation of RasGRP3 expression induces apoptosis in MCF7 derived cells I.

The survival of MCF7 and T-47D derived cells were determined staining with Annexin-V and PI analyzed by flow cytometry. Data were evaluated with FSC Express softwer and presented in the percentage of gated cells. Values represent the mean ± SEM for three independent experiments. * indicates significant difference ($p < 0.05$) compared to scrambled control cells.

To further support the role of RasGRP3 in the regulation of apoptosis a quantitative fluorimetric MitoProbe^{TM} $DilC_1$ (5) assay was performed. A reduction in the mitochondrial transmembrane potential is one of the earliest markers of apoptosis [Green and Reed, 1998; Zamzami et al., 1995]. 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. We

found that RasGRP3 silencing significantly decreased mitochondrial membrane potential **(Figure 16)**, in MCF7 cells, while no significant change was observed in T-47D cells.

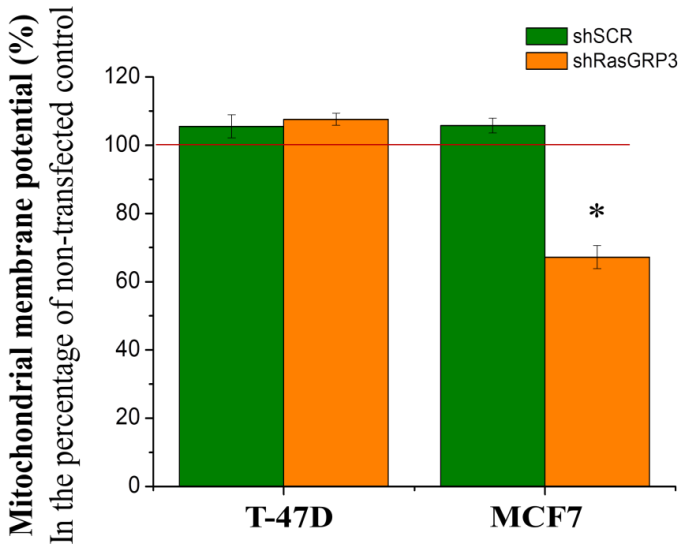


Figure 16. Down-regulation of RasGRP3 expression induces apoptosis in MCF7 derived cells II.

Assessment of mitochondrial membrane potentials of MCF7 and T-47D derived cells by fluorimetric DilC1 (5) assay with values normalized to the levels of non-transfected control cells. Values represent the mean ± SEM for four independent experiments. * indicates significant difference ($p < 0.05$) compared to scrambled control cells.

ROLE OF RASGRP3 IN THE REGULATION OF CHEMOTHERAPEUTIC SENSITIVITY

RasGRP3 expression contributes to sensitivity to Tamoxifen and Herceptin in T-47D cells

We also investigated the sensitivities of "RasGRP3-silenced" MCF7 and T-47D cells to Tamoxifen used in endocrine therapy and to the chemotherapeutic drug Herceptin [Clark et al., 2002; Wang et al., 2005]. 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 **(Figure 17)**. Suppression of RasGRP3 levels did not affect the sensitivity of the MCF7 cells to the actions of the drugs **(Figure 17)**.

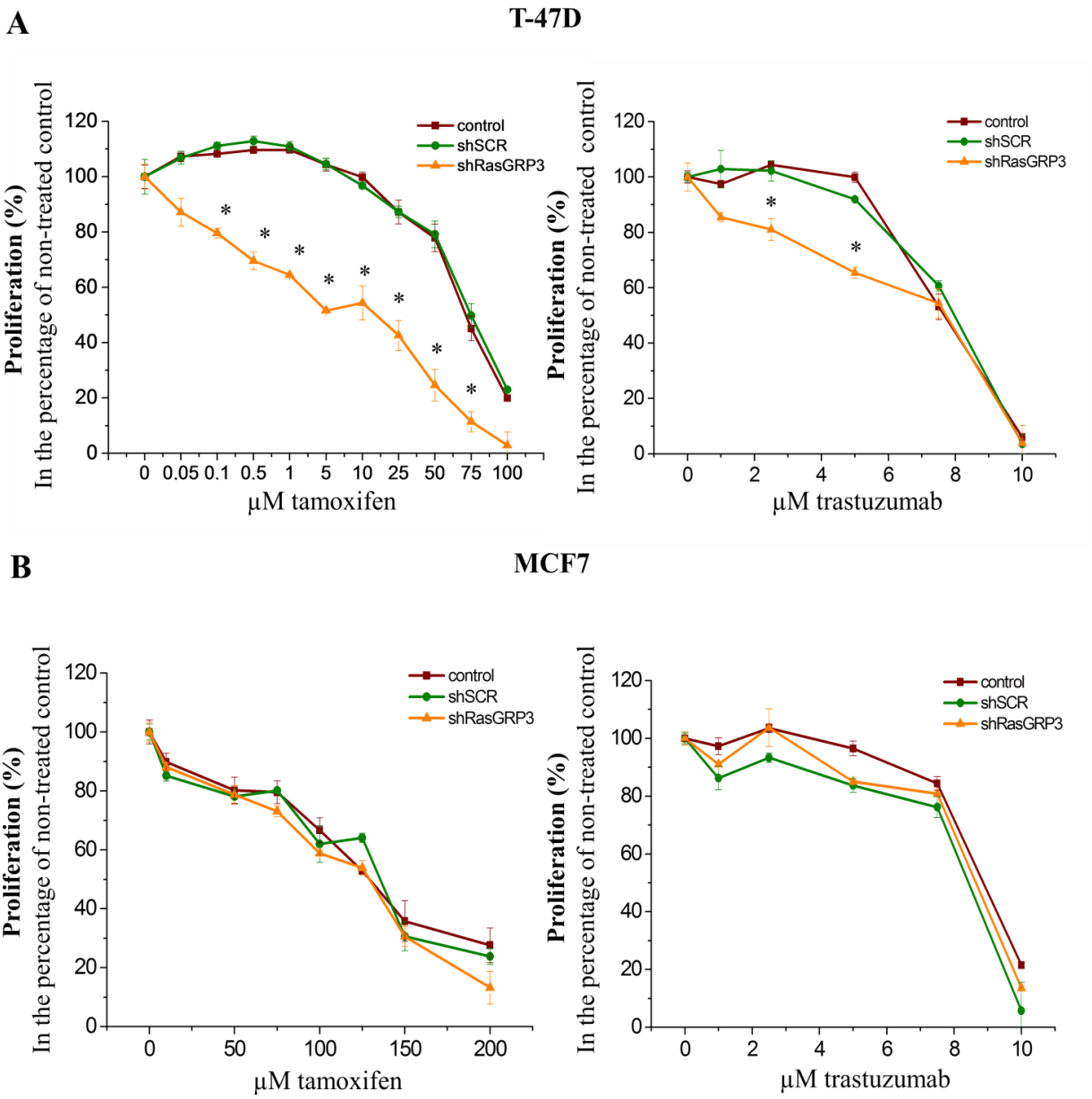


Figure 17. Down-regulation of RasGRP3 increases the sensitivity to Tamoxifen and Herceptin of the T-47D cells

The indicated T-47D (A) and MCF7 (B) derived cell lines were seeded at a density of 1 x 10^4 cells/ well and treated with Tamoxifen and Herceptin as indicated (Tamoxifen: MCF7 cells: 0-200 µM; T-47D cells: 0-100 µM; Herceptin: both MCF7 and T-47D cells: 0-10 µM). After 72 hours of incubation cell proliferation was determined using the CyQuant GR cell proliferation assay. The results were normalized to the levels of untreated cells. Values represent the mean ± SEM of 2 independent experiments. * indicates significant difference ($p < 0.05$) compared to scrambled control cells.

ROLES OF RASGRP3 AND nPKCδ IN THE REGULATION OF TUMORIGENESIS

Down regulation of RasGRP3 suppressed xenograft tumor formation

To assess furthermore 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 **(Figure 18A)** as measured by weight of excised tumors in comparison of those induced by the shSCR-expressing cells. As revealed by the Haematoxylin-eosin staining based immunohistochemical analysis **(Figure 18B)** of the xenograft tumors T-47D cells derived tumors showed „ Pushing-type" of growth reflected on 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. In these tumors no large necrotic areas were present.

These differential features of the various cells on tumorigenesis were also proven by immunohistochemical analysis of the expression of the proliferation marker Ki67 (**Figure 19**). Digitalized images of Ki67-specific immunreactivity obtained on sections prepared from tumors developed by MCF7 and T-47D derived cells (**Figure 19B**) were analysed using Image J image analysis softwer (**Figure 19A**). In each section the number of cells were counted at 5 randomly placed, equal areas of interest (AOI) and the average of Ki67 imunnopositive cells of the 5 AOI was defined. Ratios representing total cell number/Ki67 positive cells: in T-47D shSCR expressing cells n=1213/1098; in T-47D shRasGRP3 expressing cells n=1216/1096; in MCF7 shSCR expressing cells n=1268/757; in MCF7 shRasGRP3 expressing cells n=1112/553. According to the analysis RasGRP3 downregulation decreased the number of proliferating cells in MCF7 derived xenograft tumors.

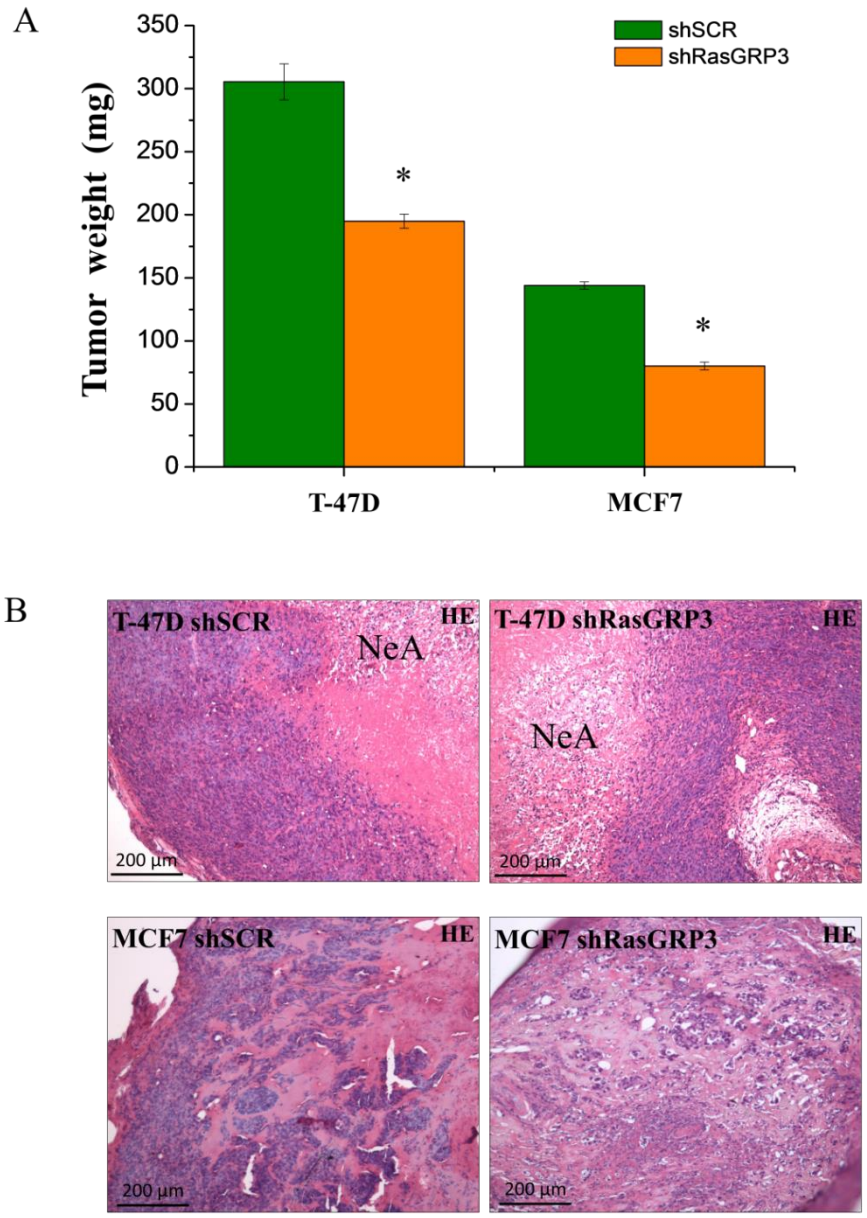


Figure 18. Suppression of RasGRP3 expression inhibits xenograft tumor growth of both MCF7 and T-47D cells

SCID mice were injected with cells of the indicated MCF7 and T-47D derived cell lines. The animals were sacrificed 12 weeks after injection, and the tumors were excised and weighed **(A)**. Values represent the mean ± SEM of four independent experiments **(B)** Haematoxylin-eosin stained representative images of the developed shSCR or shRasGRP3 xenograft tumors. T-47D cells derived tumors shows „Pushing-type" of tumor growth destroying the sorrounded tissues. (Necrotic areas (NeA). MCF7 derived tumors show less infiltrative nature. * indicates significant difference ($p < 0.05$) compared to scrambled control cells.

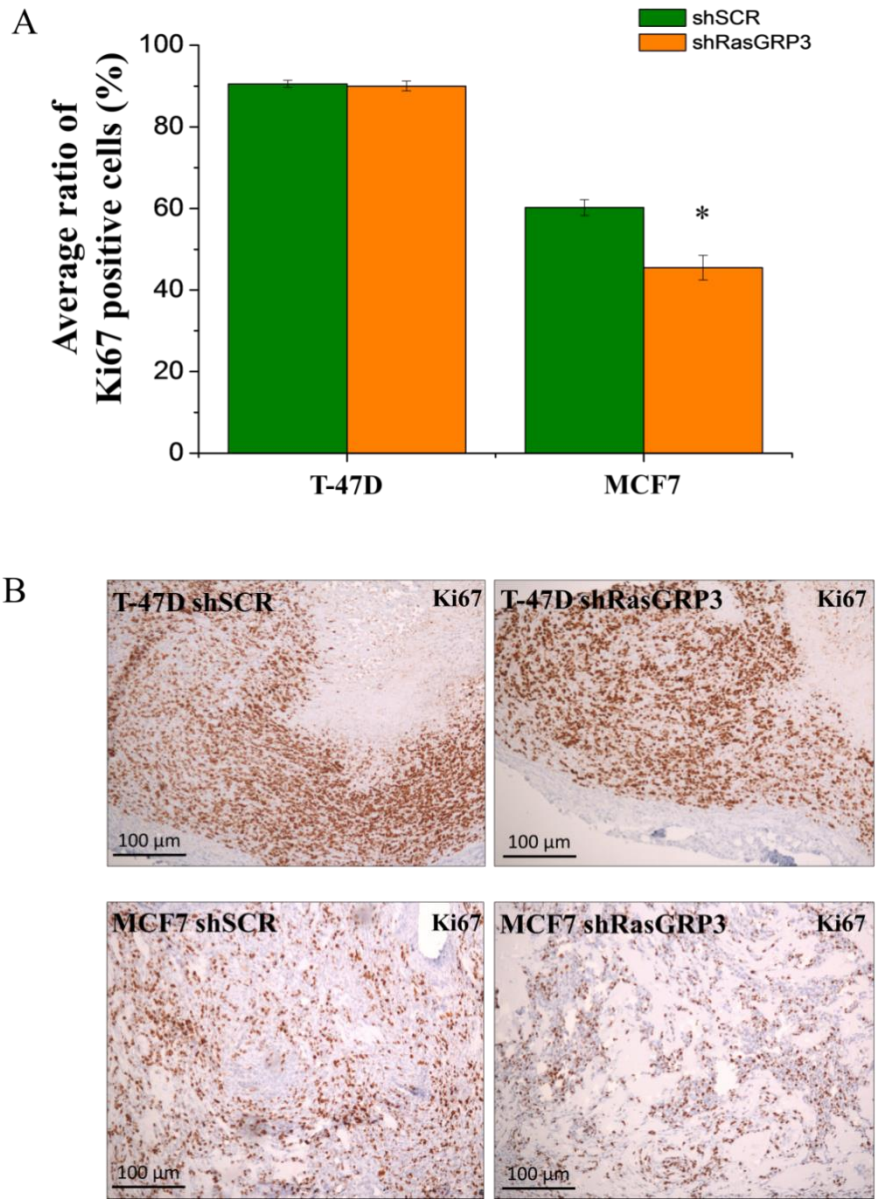


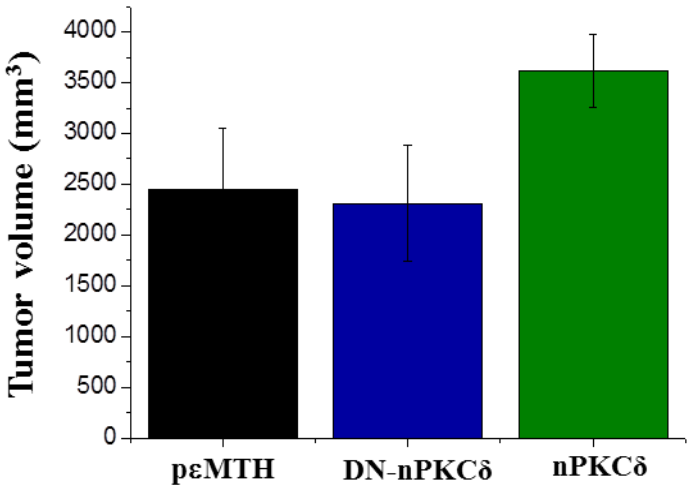
Figure 19. Suppression of RasGRP3 expression decreases the proliferation rate of MCF7 derived xenograft tumors

(A) Results of Image J image analysis of Ki67-specific immunreactivity obtained on sections prepared from tumors developed by MCF7 and T-47D derived cells. In tumors induced by RasGRP3-silenced MCF7 cells the number of Ki67 positive cell was significantly less than in the control shSCR-expressing cells. Statistical analysis did not reveal differences in the Ki67 positivity in case of the T-47D cells. Values represent the mean ± SEM. **(B)** Representative images of Ki67-specific immunoreactivity with diaminobenzidine as a chromogen (brown staining) on sections prepared from tumors developed by shSCR or shRasGRP3 derived cells. Nuclei were co-stained by Mayer's Hematoxylin (blue staining). * indicates significant difference ($p < 0.05$) compared to scrambled control cells.

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.

A



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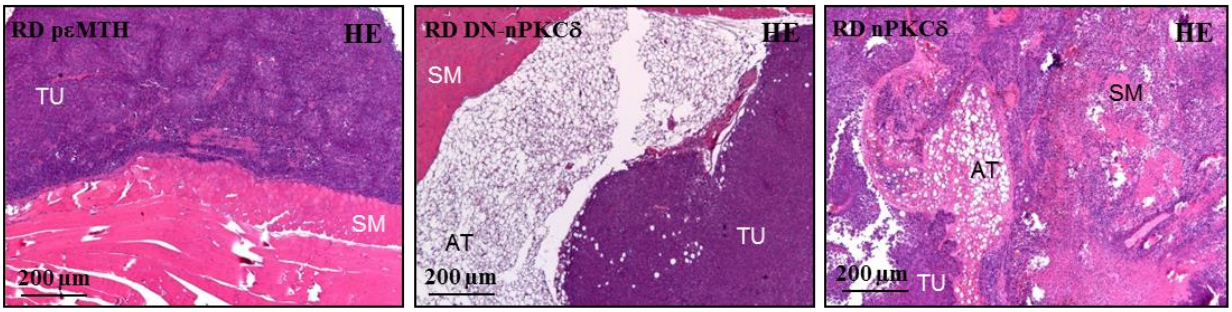


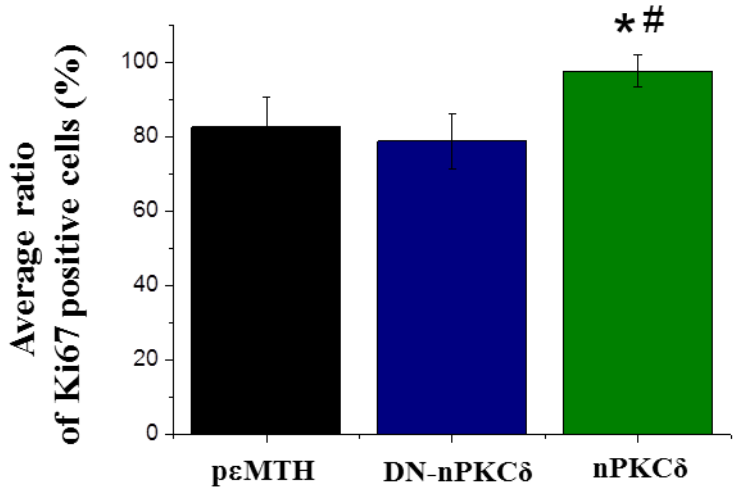
Figure 20. Overexpression of nPKCδ mutants induces xenograft tumor growth of RD cells I.

SCID mice were injected with cells of the indicated RD cells. The animals were sacrificed 30 days after injection and the three-dimensional size of the tumors were determined **(A)**. Values represent the mean ± SEM of five independent experiments**. (B)** Haematoxylin-eosin stained representative images of the developed xenograft tumors. Note that the tumor (TU) induced by the nPKCδ overexpresser cells infiltrated and destroyed the subcutaneous adipose tissue (AT) and skeletal muscle (SM) layer compared to the tumors induced by the control MTH empty vector or DN-nPKCδ overexpresser RD cells.

As expected, injection of all RD cell types resulted in tumor development in immunodeficient mice (**Figure 20**), among them, unless the difference is not significant tumors induced by nPKCδ overexpressers 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 **(Figure 20)**.

Immunohistochemical analyses of the expression of the proliferation marker Ki67 revealed that in tumors induced by nPKCδ overexpressers were characterized by the highest percentage of Ki67 positive (i.e. proliferating) cells within the sarcomas **(Figure 21)**, the latter value was significantly different from those measured in tumors induced by control (pεMTH) or DN-nPKCδ overexpressing cells. Digitalized images of Ki67-specific immunreactivity obtained on sections prepared from tumors developed by nPKCδ overexpressors, control (pεMTH) or DN-nPKCδ overexpressing cells (**Figure 21**) were analysed using Image J image analysis softwer (**Figure 21**). In every each section the number of cells were counted at 5 randomly placed, equal areas of interest (AOI) and the average of Ki67 imunnopositive cells of the 5 AOI was defined. Ratios representing total cell number/Ki67 positive cells: in control (pεMTH) overexpressing cells n=2003/1655; in DN-nPKCδ overexpressing cells n=1590/1253; in nPKCδ overexpressing cells n=1493/1457. As revealed by the analysis nPKCδ overexpressor cells induced tumors had the highest cell proliferation rates **(Figure 21)**.

A



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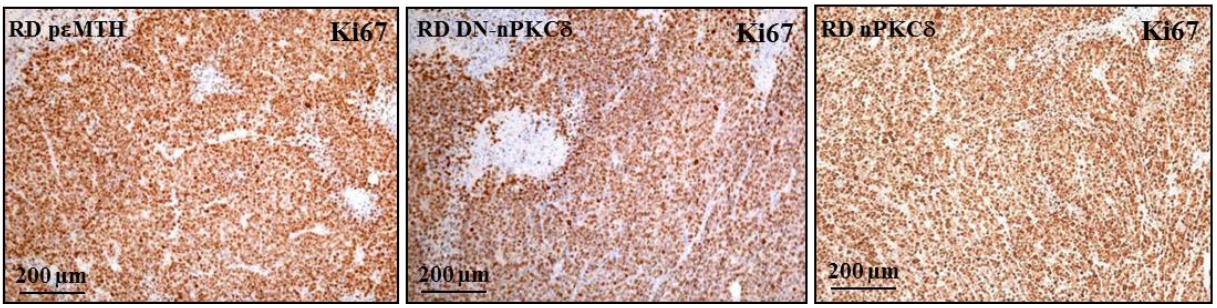

Figure 21. Overexpression of PKCδ mutants induces xenograft tumor growth of RD cells II.

(A) Results of Image J image analysis of Ki67-specific immunreactivity obtained on sections prepared from tumors developed by the indicated RD cells. In tumors induced by nPKCδ overexpressers RD cells the number of Ki67 positive cell was significantly higher than in tumors induced by control (pεMTH) or DN-nPKCδ overexpressing cells. Values represent the mean ± SEM. **(B)** Representative images of Ki67-specific immunoreactivity with diaminobenzidine as a chromogen (brown staining) on sections prepared from the developed tumors. Nuclei were co-stained by Mayer's Hematoxylin (blue staining). * indicates significant difference ($p < 0.05$) compared to control (empty MTH) vector-transfected cells, while # indicates significant difference ($p < 0.05$) compared to DN-nPKCδ overexpressing cells.

ROLES OF RASGRP3 AND nPKCδ IN THE REGULATION OF GROWTH FACTOR MEDIATED RAS SIGNALING

RasGRP3 is involved in growth factor-induced Akt, ERK 1/2 and estrogen receptor activation

Growth factors such as IGF-I and EGF represent important signaling molecules in breast cancer [Nahta et al., 2005; Zielinski et al., 2009]. In the final stage of our experiments, 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 **(Figure 22 and 23)** as indicated, and the activation of the possibly most important downstream molecules related to Ras, 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 to the basal level **(Figure 22 and 23)**. 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 **(Figure 22 and 23)**.

According to our results MCF7 cells were more sensitive to the growth factors than T-47D cells and the onset of phosphorylation events was different, with MCF7 derived "RasGRP3-silenced" cells appearing to respond to growth factors induced Akt and ERK 1/2 down-regulation earlier. In case of IGF-I application **(Figure 22)** in MCF7 cells, pAkt was affected in 5-30 min, while pAkt is affected only at 30-40 min in T-47D cells. A marked reduction in ERK 1/2 activation can be observed in both cells at 5 and 10 min, which is still present at 20 and 30 mins in T-47D cells. A decrease in ERα phosphorylation can be noticed in MCF7 cells earlier, but the down-regulation is most affected in T-47D cells. In case of EGF treatment **(Figure 23)** the RasGRP3 mediated down-regulation of Akt (5-40 min) and ERK 1/2 (5-20 min) activation was more effective in MCF7 cells compared to T-47D, in which pAkt is affected only at 30 min and there is no significant change in ERK 1/2 activation.

However T-47D cells show a significant decrease in ERα activation (20-30 min) compared to MCF7 cells, in which pERα is affected only at 20 min. Importantly, down regulation of RasGRP3 had no measurable effect on basal Akt, ERK 1/2 and ERα phosphorylation and on the total Akt, ERK 1/2 and ERα expression levels.

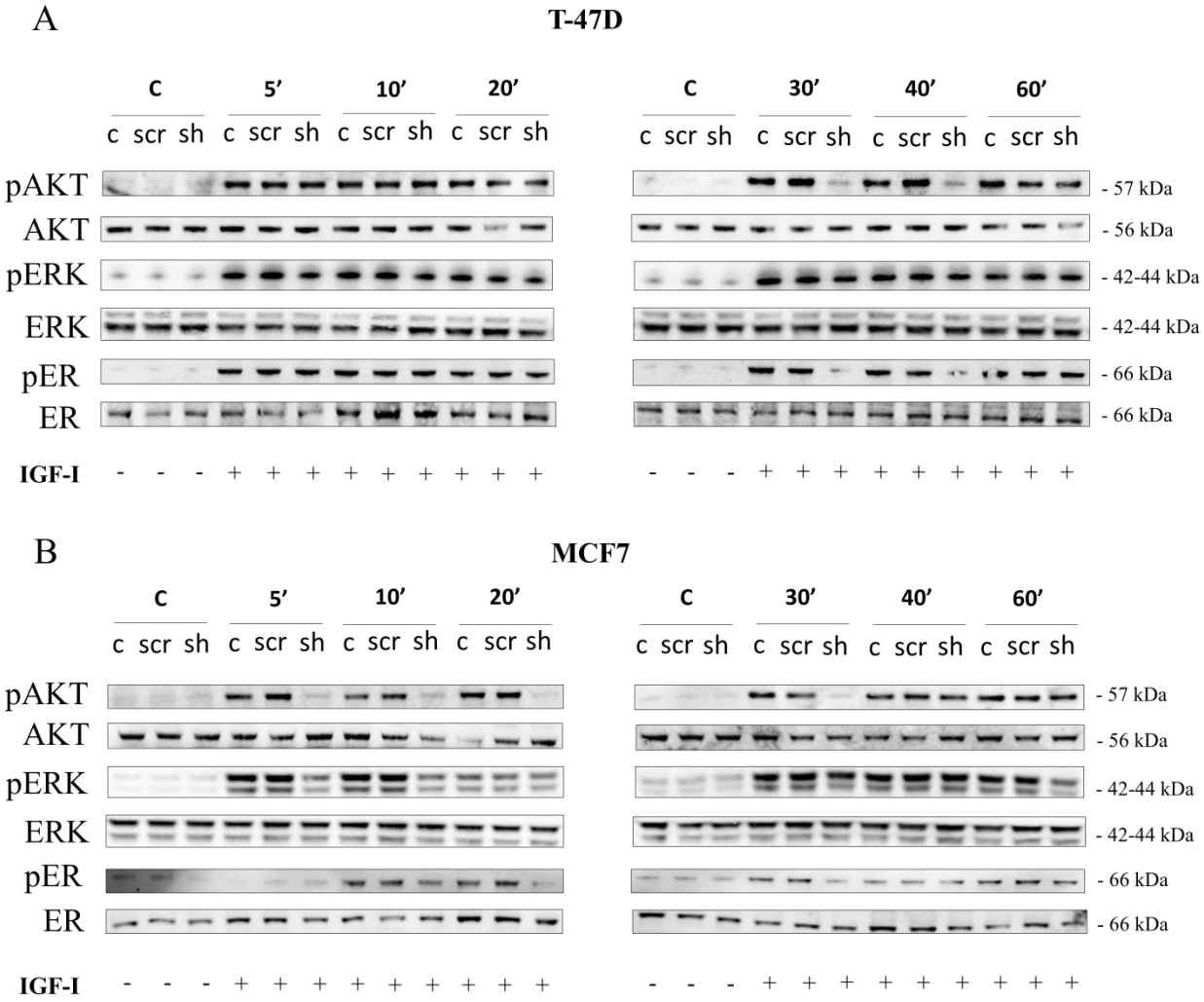

Figure 22. Effects of down-regulation of RasGRP3 on the IGF-I related Ras signaling pathway

RasGRP3 is involved in IGF-I dependent Akt, ERK 1/2 and ERα activation. RasGRP3 knockdown cell lines created from T-47D **(A)** and MCF7 **(B)** cells were treated with or without IGF-I (100 pg/ml) as indicated. Akt and phosphorylated Akt, ERK 1/2 and phosphorylated ERK 1/2, ERα and phosphorylated ERα were detected by immunoblotting of cell lysates. Levels of total Akt, ERK 1/2 and ERα were used as control. All results were representative of 2 independent experiments.

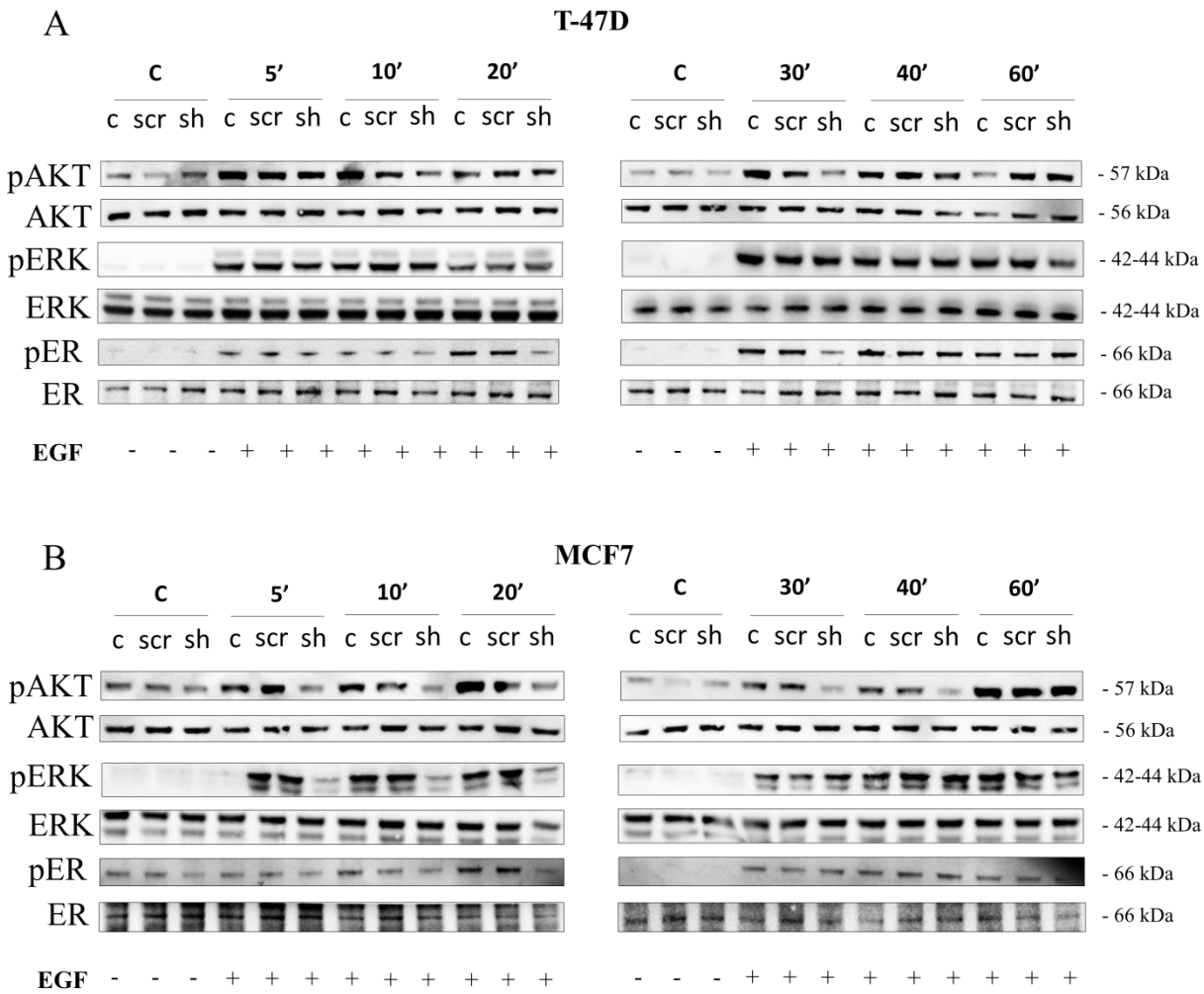

Figure 23. Effects of down-regulation of RasGRP3 on the EGF related Ras signaling pathway

RasGRP3 is involved in EGF dependent Akt, ERK 1/2 and ERα activation. RasGRP3 knockdown cell lines created from T-47D **(A)** and MCF7 **(B)** cells were treated with or without EGF (100 pg/ml) as indicated. Akt and phosphorylated Akt, ERK 1/2 and phosphorylated ERK 1/2, ERα and phosphorylated ERα were detected by immunoblotting of cell lysates. Levels of total Akt, ERK 1/2 and ERα were used as control. All results were representative of 2 independent experiments

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 [Lin et al., 2004], namely progesterone receptor (PGR), cathepsin D (CTSD), cytochrome C (CYCS) and loricrin (LOR) 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 **(Figure 24)**. In case of T-47D cells a significant decrease was observed in shSCR cells in their cytochrome C expression compared to the non-treated cells, but in shRasGRP3 cells this decrease was more expressive.

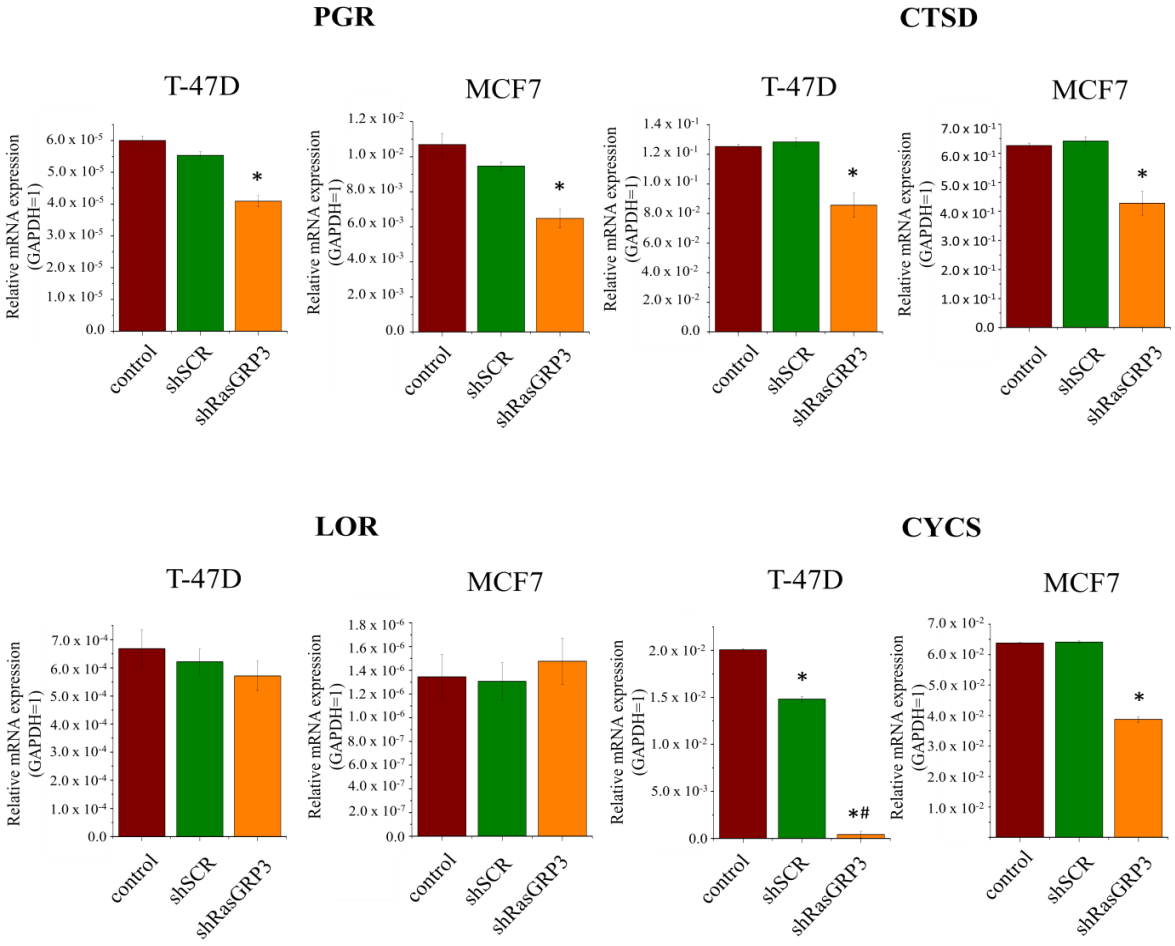

Figure 24. Effects of down-regulation of RasGRP3 on the Ras signaling pathway

Q-PCR analyses of ERα-regulated genes: PGR, CTSD, CYCS and LOR. GAPDH was used as an internal control. The results are representative of three independent experiments. Values represent the mean ± SEM. In case of loricrin expression of T-47D cells * indicates significant difference ($p < 0.05$) compared to non-transfected control cells, while $^{\#}$ indicates significant difference ($p < 0.05$) compared to scrambled control 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 **(Figure 25).** Suppression of RasGRP3 levels decreased the expression of both IGF-I and EGF receptors in MCF7 cells (**Figure 25B**), whereas it had no effect on the receptor expression of T-47D cells **(Figure 25A)**. RasGRP3 down-regulation did not modify the expression of HER-2 receptor of the cells.

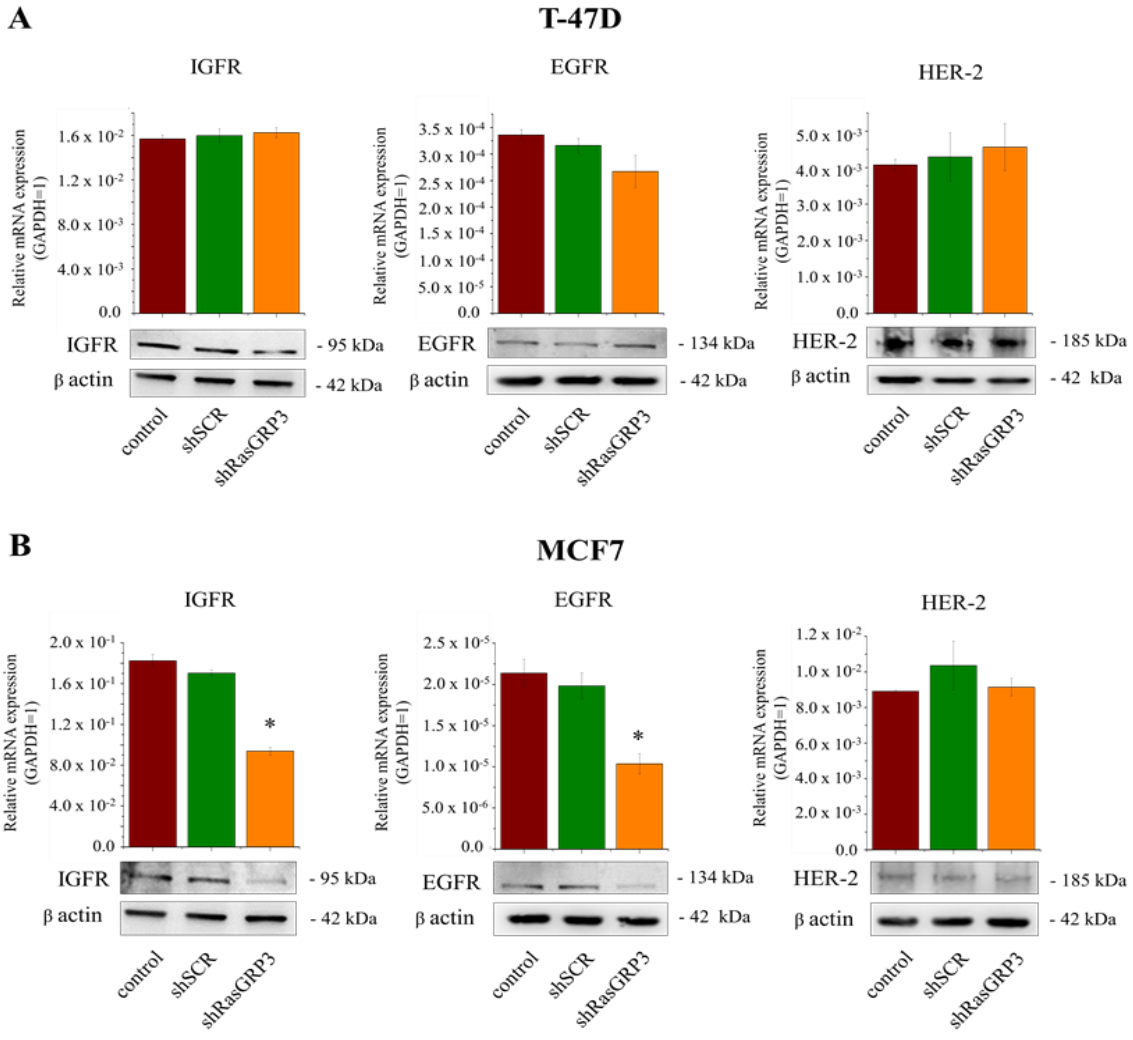

Figure 25. Effects of down-regulation of RasGRP3 on the Ras signaling pathway IV

IGF1R, EGFR and HER-2 expressions were detected by Q-PCR and immunoblotting of RasGRP3 knockdown cell lines created from T-47D (**A**) and MCF7 (**B**) cells. GAPDH (Q-PCR) and β actin (Western Blot) were used as internal controls. The results are representative of three (Q-PCR) or two (Western blot) independent experiments. Values represent the mean ± SEM. * indicates significant difference ($p < 0.05$) compared to scrambled control cells.

nPKCδ is involved in IGF-I-induced ERK 1/2 **activation**

The insulin-like growth factor pathway are involved in sarcomas, including RMS. Therefore, in the next stage of our experiments, we investigated the role of nPKCδ in modulating the IGF-I induced activation of the Ras-related ERK 1/2 signaling pathway in RD cells. Cells were treated with IGF-I **(Figure 26)** as indicated, 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 **(Figure 26)** compared to the control (empty MTH) vector transfected or DN-nPKCδ mutant cells. Overexpreesion of nPKCδ had no measurable effect on the total ERK 1/2 expression level.

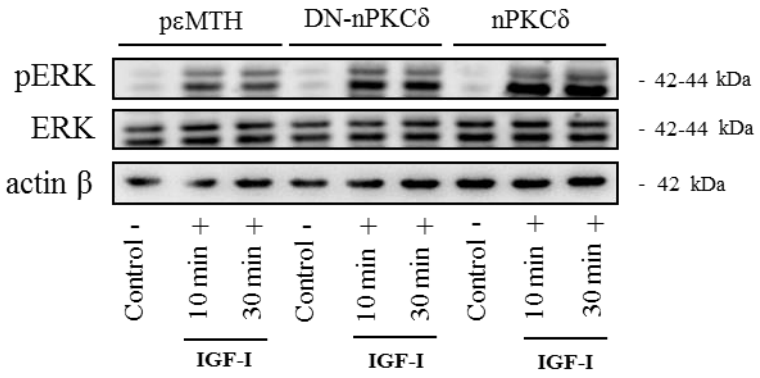

Figure 26. Effects of overexpression of nPKCδ on the IGF-I related ERK 1/2 activation in RD cells

RD cells transfected either with empty MTH (pεMTH) vector or with vectors encoding the active (nPKCδ) or dominant-negative (DN-nPKCδ) nPKCδ isoforms were treated with or without IGF-I (100 ng/ml) as indicated. ERK 1/2 and phosphorylated ERK 1/2 were detected by immunoblotting of cell lysates. β actin was used as an internal control. All results were representative of 2 independent experiments.

DISCUSSION

Overactivation of Ras signaling pathways -by various upstream and downstream signaling elements- has been recognized to be important for tumorigenesis [Bos, 1989; Rajagopalan et al., 2002; Kennecke et al., 2010; Vetter and Wittinghofer, 2001]. Here we investigated the potential oncogenic roles of two Ras regulator proteins: (1) RasGRP3 and (2) PKCδ in the formation of human cancer.

RASGRP3 EXPRESSION IN HUMAN BREAST CANCER

Publications have demonstrated RasGRP3 expression in different types of human cancers such as e.g. Burkitt's lymphoma, pre-B-cell leukemia, natural killer-like T-cell leukemia, and revealed informations regarding its possible oncogenic functions in metastatic prostate cancer and melanoma [Teixeria et al., 2003; Yang et al., 2010; Yang et al., 2011], In this study we described the expression of RasGRP3 on human breast cancer cells using Q-PCR, Western blot and immunohistochemical analyses. We showed that the expression of RasGRP3 is elevated in tumor-derived samples compared to non-diseased breast samples. We also reported the subcellular localization of RasGRP3 and its presumably active form, the phosphoRasGRP3. We observed RasGRP3 expression in the cytoplasm whereas phosphoRasGRP3 showed nuclear/perinuclear immunreactivity in the breast cancer cells. The function of RasGRP3 in the nucleus has not been investigated before. Another potential target of the protein over H-Ras and R-Ras, is the small GTPase Rap1. Rap1 has been found in the nuclei of liver tumor cells and squamous cell carcinoma lines [Wurzer et al., 2001; Mitra et al., 2003]. Our result raises the possibility that RasGRP3 prefer Rap1 activation over Ras in breast cancer cells. Another possible explanation for the nuclear localization is the interaction of the protein with specific intracellular proteins like dynein light chain 1 (DLC1) – a novel RasGRP3-interacting protein [Okamura et al., 2006]- that mediate the nuclear transport of many certain proteins, namely p53 and brain-enriched protein PMES-2 [Lo et al., 2005;

Ninomiya et al., 2005]. DLC1 also participates in the transactivation functions of ERα [Rayala et al., 2005]. As DLC1 is often overexpressed in breast cancer [Vadlamudi et al., 2004] it is interesting to note that DLC1–RasGRP3 interaction may act as a chaperone for nuclear translocation of RasGRP3 in breast cancer cells suggesting a role for RasGRP3 in amplifying Ras initiated cellular responses in breast cancer cells.

ROLES OF RASGRP3 AND nPKCδ IN THE REGULATION OF *IN VITRO* AND *IN VIVO* PROLIFERATION

Both *in vitro* and *in vivo* studies were carried out to define the exact functional role of RasGRP3 and nPKCδ in the regulation of proliferation.

Similar to those described in prostate carcinoma and melanoma [Yang et al., 2010; Yang et al., 2011], suppression of RasGRP3 inhibited cell proliferation *in vitro*. Of further importance, down-regulation of RasGRP3 expression reduced T-47D and MCF7 cells derived tumor formation in SCID mice. According to the "*in vivo"* proliferation analysis RasGRP3 down-regulation decreased Ki67 positivity –a prognostic marker used in grading method[Perou et al., 2000; Jacquemier et al., 2005; Badve and Nakshatri, 2009]- in MCF7 cells derived tumors.

nPKCδ isoform is implicated in the regulation of proliferation of numerous cell types [Mischak et al., 1993; Gutcher et al., 2003]. In human keratinocytes [Li et al., 1999; Papp et al., 2004] and fibroblast [Ács et al., 1997] the isoform was suggested to inhibit proliferation; however, nPKCδ was shown to stimulate proliferation and acting as a prosurvival factor in breast cancer cell lines [McCracken et al., 2003]. According to our previous results the overexpression of the constitutively active nPKCδ stimulated *in vitro* growth of C2C12 myoblasts [Czifra et al., 2014]. Moreover, nPKCδ overexpresser C2C12 cells, when injected to immunodeficient mice, initiated the development of malignantly transformed rhabdomyosarcomas [Czifra et al., 2014]. Similar to those described in case of C2C12 cells,

overexpression of nPKCδ enhanced cell proliferation of RD cells compared to control (pεMTH) cells or DN-nPKCδ mutant. Interestingly, the size of tumors induced by cells overexpressing nPKCδ did not differ from the control (pεMTH or DN-nPKCδ). These findings suggest that other factors than nPKCδ may also be involved in promoting the aggressive growth of RMS-derived cells. Although the overexpression of nPKCδ did not increase xenograft tumor growth, but according to Ki-67 proliferation analysis the proliferation rate of nPKCδ derived cells was the highest, these developed tumors showed the most infiltrative growth pattern destroying the surrounded tissues.

ROLE OF RASGRP3 IN THE REGULATION OF SURVIVAL

Again, similar to those described in prostate carcinoma and melanoma [Yang et al., 2010; Yang et al., 2011], down-regulation of RasGRP3 induced apoptosis in MCF7 cells. Why do MCF7 cells show signs of apoptosis in response to RasGRP3 silencing? The major signaling pathway implicated in supporting survival is the PI3K cascade. Studies indicate that the protective effect of PI3K is mediated primarily by Akt [Datta et al., 1997]. In addition, inhibition of Akt signaling can induce apoptosis in some human cancer cell lines [Yuan et al., 2000; Page et al., 2000]. Akt delivers antiapoptotic survival signals by phosphorilating Bad therefore inhibits apoptosis by maintaining Bcl-x_L function and preventing cytochrome c release from mitochondria. We found that MCF7 „RasGRP3-silenced" cells showed decreased Akt activation downstream from both IGF-I or EGF stimulation more intensively in MCF7 cells compared to T-47D cells. Since the decrease of mitochondrial membrane potencial is one of the first marker of mitochondria-induced apoptosis we investigated the mitochondrial membrane potential of T-47D and MCF7 derived cells using a MitoProbe^{TM} DilC_1(5) assay. RasGRP3 down-regulation caused a significant decrease in the mitochondrial membrane potencial of MCF7 cells but not in T-47D cells indicating that RasGRP3 possibly

through the modulation of Akt activation may lead to the activation of the intrinsic pathway of apoptosis in MCF7 cells.

ROLE OF RASGRP3 IN THE REGULATION OF CHEMOTHERAPEUTIC SENSITIVITY

The primary treatment of ER positive breast cancer is the non-steroidal anti-estrogen Tamoxifen, while HER-2 positive tumor treatment is focused on Herceptin.

Many ER positive tumors are *de novo* resistant to Tamoxifen. Ligand independent phosphomodification of ERα is one of the many different mechanisms have been suggested to explain Tamoxifen [Schiff et al., 2004; Shou et al., 2004; Arpino et al., 2008]. Ligand-independent activation of ERα has been reported in response to EGF or IGF-I through ERK 1/2, by stimulating the phosphorylation of Ser 118 on the ERα [Kato et al., 1995; Kok et al., 2009]. Clinical resistance to Tamoxifen can also be associated with EGFR and HER-2 upregulation [Wright et al., 1992; Pietras et al., 1995]. The Tamoxifen resistant MCF7 cell line demonstrates increased EGFR/HER-2/MAPK signaling activity; furthermore, it also shows high levels of phosphorylation of ERα at Ser 118 [Knowlden et al., 2003]. We demonstrated the role of RasGRP3 in decreasing the phosphorylation of ERα at Ser 118 through the down-regulation of Akt and ERK 1/2 pathways both in T-47D and MCF7 cells. In T-47D cells by decreasing ERα transactivation RasGRP3 down-regulation contributed to an increased Tamoxifen sensitivity. MCF7 cells are resistant to the growth inhibitory action of Tamoxifen, therefore decreasing pERα activation in MCF7 cells (otherwise more affected cells to EGF and IGF-I treatment) had no effect on Tamoxifen sensitivity. Furthermore we showed that RasGRP3 mediates the expressions of ERα-regulated genes; down-regulation of the protein decreased the expressions of PGR, CTSD and CYCS [Lin et al., 2004], although it had no effect on LOR expression.

We also investigated the role of RasGRP3 in regulation of Herceptin sensitivity. The potential mechanisms of resistance not only involves the structural mutation in HER2 protein, but the alternative elevations of other tyrosine kinase receptors, such as IGF-IR, or the intracellular alterations in growth factor downstream signaling like constitutive activity of PI3K pathway [Lu et al., 2001]. These findings imply that cancer cells, by utilizing other growth factor signaling, can compensate for the inhibition of HER-2 signaling mediated by Herceptin. A promising target whose aberrant expression may confer resistance to Herceptin is the IGF-IR [Jerome et al., 2001], the ectopic expression of IGF-IR drives Herceptin-sensitive SK-BR3 and MCF-7/HER-2-18 cells to resistant to the drug. Although the down regulation of RasGRP3 had no effect on HER-2, IGF-I and EGF receptor expression in T-47D cells, but caused the down-regulation of IGF-I and EGF induced downstream signaling suggesting that the protein can contribute to an increased sensitivity of the cells against Herceptin. MCF7 cells expresses low amount of HER-2 receptor, therefore show resistance to Herceptin. Decreasing IGF-IR expression in these cells did not effect Herceptin resistance.

ROLES OF RASGRP3 AND nPKCδ IN THE REGULATION OF GROWTH FACTOR MEDIATED RAS SIGNALING

Expressions of IGF-I receptor and EGF receptor (EGFR or HER-1) in breast cancer have been reported to be elevated [Papa et al., 1990; Papa et al., 1993]. IGF-I and EGF are potent mitogens for breast cancer cells inducing cellular proliferation and promoting the invasiveness and endocrine- or chemotherapeutic resistance of T-47D and MCF7 breast cancer cells [Karey and Sirbasku, 1988; Pekonen et al., 1988; Dufourny et al., 1997; Dunn et al., 1998; Gooch et al., 1999] via the activation of Akt and ERK 1/2 kinases [Downward, 2003; Roberts and Der, 2007]. We demonstrated that RasGRP3 contributed to signaling downstream of IGF-I and EGF in both cell lines by decreasing the level of phosphorylated Akt and ERK 1/2 induced by both IGF-I or EGF stimulations. Cells showed different

activation profile to the growth factors; compared to T-47D cells "RasGRP3-silenced" MCF7 cells appearing to respond earlier and more effectively to both IGF-I and EGF treatment (see also section 5.3.). RasGRP3 also decreased the expression of IGF-I and EGF receptors in MCF7 cells. The effects of RasGRP3 on Akt and ERK 1/2 phosphorylation in both T-47D and MCF7 cells are consistent with our demonstration that RasGRP3 is contributing to Ras activation and likewise support the effects we observed on cell proliferation, chemotherapeutic resistance and tumorigenesis. We think that the effects of upstream growth factors are mediated in part through RasGRP3 in breast cancer cells; we hope that these results help fill out the gaps of our current knowledge of the breast cancer signaling network **(Figure 27)**.

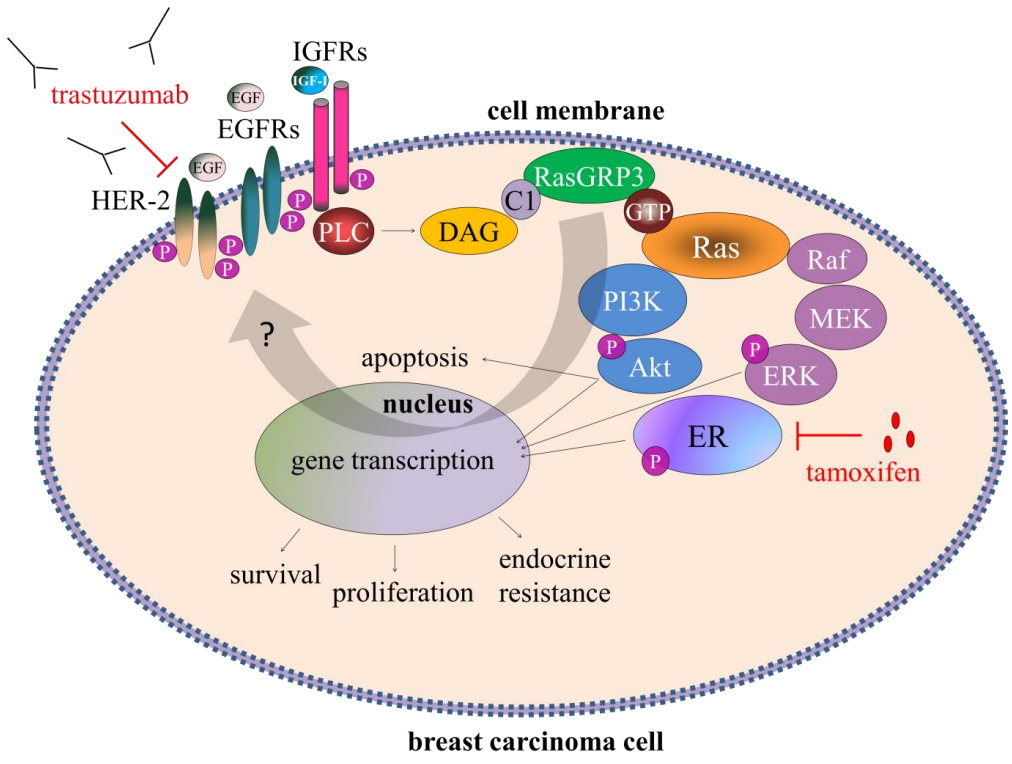

Figure 27. Schematic overview presenting the role of RasGRP3 in mediating the Ras signaling pathways in breast carcinoma cells

The signaling of growth factors (such as IGF-I and EGF) through downstream signaling molecules to ERα is mediated by RasGRP3. Inhibition of the Ras signaling pathway at the RasGRP3 level bears the potential to suppress downstream activation and ligand independent activation of ERα resulting in a decreased proliferation and chemotherapeutic resistance.

The significance of IGF-IR receptor has been reported to be elevated in RMS [Kalebic et al., 1998; Aslam et al., 2013]; IGF-I is one of the key autocrine and paracrine growth factors in skeletal muscle [Tidball, 2005] inducing cellular proliferation, invasion and angiogenesis enhancing ERK ½ activation. In one of our previous publications we presented that nPKCδ plays a central role in mediating the mitogenic effect of IGF-I both in human and C2C12 skeletal muscle cells [Czifra et al., 2006]. Although it was confirmed that RMS overexpresses IGF-IR, here we were the first to investigate the role of nPKCδ in the modulation of MAPK activation of RD cells. Overexpression of nPKCδ increased the level of ERK 1/2 phosphorilation induced by IGF-I compared to the pεMTH or DN-nPKCδ transfected cells. These data support the effects we observed on cell proliferation.

Collectively, these data describe RasGRP3 and nPKCδ as key players in promoting cellular growth and inducing malignant transformation of breast cancer and rhabdomyosarcoma cells, which findings identify these proteins as a novel, promising target molecule for pathway directed chemotherapy in the supportive treatment and diagnosis of breast cancer and rhabdomyosarcoma.

SHORT SUMMARY

The aim of our experiments was to investigate the potential function of RasGRP3 and nPKCδ in the formation and progression of human breast cancer and rhabdomyosarcoma.

First, the RasGRP3 expression was 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. According to our results RasGRP3 expression was elevated in human breast tumor tissue samples. To explore the biological function of the proteins, RasGRP3 knockdown and nPKCδ overexpressor cultures were established. 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, ERK 1/2 and estrogen receptor alpha phosphorylation downstream from insulin like growth factor-I (IGF-I) or epidermal growth factor (EGF) stimulation. Recombinant overexpression of 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.

Taken together, our results suggest that RasGRP3 and nPKCδ may have a role in the pathological behavior of breast cancer and rhabdomyosarcoma cells and may constitute a therapeutic target for these cancers.

ÖSSZEFOGLALÁS

A RasGRP3 és nPKCδ, a Ras-kapcsolt jelátviteli útvonalak aktivitását befolyásolni képes fehérjék. Mindkét fehérje potenciális onkogén hatású molekula; már több rosszindulatú daganattípusban is leírták a génjének amplifikációját és a fehérjetermék overexpresszióját. A fehérjék potenciális onkogén hatását figyelembe véve kísérleteink során egyrészt megvizsgáltuk a RasGRP3 kifejeződését és funkcionális szerepét az egyik legrosszindulatúbb daganattípusban, az emlő eredetű ductalis adenocarcinomában, továbbá az nPKCδ izoforma szerepét rhabdomyosarcoma sejtek *in vitro* és *in vivo* növekedésének szabályozásában.

Kísérleteink során sikerrel igazoltuk a RasGRP3 gén- és fehérjeszintű jelenlétét humán, emlő eredetű ductalis adenocarcinomából származó humán szöveti mintákon. Megállapítottuk, hogy míg a RasGRP3 jellemzően a sejtek citoplazmájában expresszálódik, addig az aktív foszforilált forma nukleáris/perinukleáris elhelyezkedést mutat. Ezt követően primer és metasztatikus emlő tumoros sejtvonalakon is kimutatnunk a fehérje expresszióját. A RasGRP3 és nPKCδ funkcionális szerepét tisztázandó RasGRP3 géncsendesített emlő tumoros és nPKCδ overexpresszált rhabdomyosarcoma tenyészeteket hoztunk létre. A RasGRP3 expresszió gátlásának segítségével megállapíthattuk, hogy a fehérje expressziójának gátlása a sejtek proliferációját *in vitro* és *in vivo* szignifikánsan csökkenti, valamint apoptotikus folyamatokat indukál az MCF7 sejvonalban. Továbbá kimutattuk, hogy a géncsendesítés következtében a T-47D sejtek rezisztenciája a kemoterápia során használt Tamoxifennel és Herceptinnel szemben csökken. Jelátviteli kísérleteink során megállapítottuk, hogy a fehérje gátlásával a Ras kapcsolt jelátviteli útvonalak aktivitása jelentős mértékben csökkenthető. Az nPKCδ overexpresszióját követően az RD sejtekben, MTT assay segítségével sikeresen kimutattuk, hogy ez az izoforma fokozza a sejtek *in vitro* és *in vivo* proliferációját SCID egerekbe injáktalva. Jelátviteli kísérleteink során igazoltuk, hogy a nPKCδ izoforma a MAPK útvonal „upstream" regulátoraként viselkedik rhabdomyosarcomában; a fehérje overexpressziójával az ERK ½ kináz aktivitása szignifikánsan fokozódik.

Eredményeink alapján feltételezhető, hogy mindkét fehérje potenciális onkogén szereppel bír az említett tumorok kialakításában és a sejtek növekedésének szabályozásában.

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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.
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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.
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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.
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RasGRP3; nPKCδ; breast cancer*;* rhabdomyosarcoma; chemotherapeutic resistance; Tamoxifen; Herceptin; proliferation; tumorigenesis; EGF; IGF-I; signaling pathways

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