PONTIFÍCIA UNIVERSIDADE CATÓLICA DO RIO GRANDE DO SUL
FACULDADE DE BIOCIÊNCIAS
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA CELULAR E MOLECULAR

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INVESTIGAÇÃO DO EFEITO PROLIFERATIVO E MIGRATÓRIO DO PEPTÍDEO LIBERADOR DE GASTRINA (GRP) SOBRE UMA LINHAGEM DE ADENOCARCINOMA PULMONAR

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Dissertação de mestrado apresentado ao Programa de Pós-Graduação em Biologia Celular e Molecular da Pontifícia Universidade Católica do Rio Grande do Sul como requisito para obtenção do título de mestre.

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LISTA DE ABREVIATURAS E SIGLAS

AKT – *protein kinase B*

AREG – *amphiregulin*

BB4-R – *bombesin receptor subtype 4*

BRS-3 – *bombesin receptor subtype 3*

c-Src – *proto-oncogene tyrosine-protein kinase*

EGFR – *epidermal growth factor receptor*

ERK – *extracellular signal-regulated kinase*

FAK – *focal adhesion kinase*

GPCR – *G-protein coupled receptor*

GRP – *gastrin-releasing peptide*

GRPR – *gastrin-releasing peptide receptor*

HB-EGF – *heparin-binding EGF-like growth factor*

MAPK – *mitogen activated protein kinase*

MMP – *matrix metalloproteinase*

mTOR – *mammalian target of rapamycin*

NMB – *neuromedin B*

NMBR – *neuromedin receptor*

NSCLC – *non-small cell lung cancer*

PBMC – *peripheral blood mononuclear cells*

PDK-1 – *phosphoinositide-dependent kinase-1*

PI3-K – *phosphoinositide 3-kinase*

PTEN – *phosphatase and tensin homolog*

SCLC – *small cell lung cancer*

TACE – *tumor necrosis factor-alpha converting enzyme*

TAM – *tumor associated macrophage*

TAN – *tumor associated neutrophil*

TGF-α – *transforming growth factor alpha*

VEGF – *vascular endothelial growth fator*

RESUMO

O câncer de pulmão é o tipo de câncer que mais comumente diagnosticado e o que mais mata no mundo levando a quase 1 milhão de mortes por ano. Entre todos os tipos histológicos, o adenocarcinoma é o mais frequente (75-80%). O peptídeo liberador de gastrina (GRP) é considerado um agente mitogênico, capaz de induzir a proliferação celular, uma vez que está envolvido no desenvolvimento fetal dos pulmões. Este peptídeo teve sua ação sobre o crescimento tumoral primeiramente identificada em células humanas de câncer de pulmão de pequenas células, atuando como fator autócrino de crescimento de tecidos e tumores através da ligação ao seu receptor GRPR. Este receptor foi encontrado em diversos tipos de tumores como próstata, mama, estômago, pâncreas e cólon. Além disso, este peptídeo atua como um morfógeno, na angiogênese e, está relacionado a processos inflamatórios e na regulação de células do sistema imune. E, fumantes assintomáticos possuem altos níveis de GRP no lavado broncoalveolar e na urina.

No entanto, pouco se conhece sobre os seus efeitos na tumorigênese e metástase e, quais os mecanismos moleculares e as vias de sinalização que são responsáveis pelos efeitos encontrados. Nosso grupo demonstrou, recentemente, que o GRP pode atuar como uma molécula quimiotática para neutrófilos. Desta forma, hipotetizamos que o GRP poderia constituir num estimulo quimiotático também para as células tumorais que expressão o GRPR. Neste trabalho, testamos essa hipótese, analisando o efeito do GRP sobre a proliferação, sobrevivência e migração de células da linhagem de adenocarcinoma A549, buscando identificar mecanismos de ação desse peptídeo. Esta linhagem expressa altos níveis de GRPR. O tratamento com GRP leva a ativação de quinases como a AKT e ERK1/2 que estão envolvidas nestes processos celulares. Nossos resultados sugerem que o GRP é principalmente um estímulo migratório para estas células, sem evidências de efeito significativo sobre a sua proliferação ou sobrevivência ao tratamento com a droga quimioterápica cisplatina, mas tornam-se mais sensíveis quando a droga é combinada com um antagonista do GRPR. Dessa forma, acreditamos que estudos futuros devam considerar um possível papel para o GRP na metástase.

ABSTRACT

Lung cancer is the most commonly diagnosed type of cancer and the leading cause of cancer related mortality in the world, causing nearly one million deaths per year. Among all histological types, adenocarcinoma is the most frequent one (75-80%). Gastrin-releasing peptide (GRP) is considered to be a mitogen, capable of inducing cell proliferation, since it is involved in fetal lung development. This neuropeptide had its effect on tumor growth first identified in human cells of small cell lung cancer, acting as an autocrine growth factor for tumor tissues by binding to its receptor GRPR. The receptor has been found in many tumor types such as prostate, breast, stomach, pancreas and colon. Moreover, this peptide acts as a morphogen, in angiogenesis and is related to inflammatory processes and in the regulation of cells of the immune system. Furthermore, asymptomatic smokers have high levels of GRP in bronchoalveolar lavage and urine. However, little is known about its effects in tumorigenesis and metastasis, and which molecular mechanisms and signaling pathways are responsible for the effects found. Our group demonstrated recently that GRP could act as a chemotactic molecule for neutrophils. Thus, we hypothesized that GRP could be also a chemotactic stimulus to tumor cells expressing the GRPR. In this study, we tested this hypothesis by examining the effect of GRP on proliferation, survival and migration of cells from the adenocarcinoma cell line A549, seeking to identify the mechanisms of action of this peptide. These cells express high levels of GRPR and treatment with GRP leads to activation of kinases such as AKT and ERK1/2 that are involved in the cellular processes mentioned. Our results suggest that GRP is a migratory stimulus to these cells without evidence of significant effect on their proliferation or survival to treatment with the chemotherapy drug cisplatin (CDDP). Nonetheless, they become more sensitive to CDDP when the drug is combined with a GRPR antagonist. Thus, we believe that future studies should consider a possible role for GRP in metastasis of NSCLC.

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1. CAPÍTULO 1

1.1 INTRODUÇÃO

1.1.1 O Peptídeo Liberador de Gastrina (GRP)

Erspamer e colaboradores (1971) isolaram e caracterizaram, primeiramente, o tetrapeptídeo bombesina a partir da pele dos anfíbios *Bombina bombina*. Além disso, os autores demonstraram que este peptídeo de 14 aminoácidos possui diversos efeitos biológicos quando administrado em mamíferos (ERSPAMER; ERPAMER; INSELVINI, 1970).

Quase uma década após este isolamento, foram caracterizados peptídeos análogos da bombesina em mamíferos. McDonald e colaboradores (1979) foram os primeiros a isolar o peptídeo liberador de gastrina (*Gastrin-releasing peptide:* GRP) e, logo depois, Minamino e colaboradores (1983) identificaram a neuromedina (NMB) na medula espinhal de porcos. Todos os três peptídeos estão relacionados em sequência de aminoácidos (Figura 1) e compartilham um grupamento metilamida em sua porção carboxi-terminal (PATEL; SHULKES; BALDWIN, 2006).

Família dos peptídeos da Bombesina

Bombesina	
	pGlu-Gln-Arg-Leu-Gly-Asn-Gln-***Trp***-***Ala***-Val-***Gly***-His-Leu-***Met***-$\mathbf{NH_2}$
GRP	
	Gly-Asn-His-***Trp***-***Ala***-Val-***Gly***-His-Leu-***Met***-$\mathbf{NH_2}$
Neuromedina B	
	Gly-Asn-Leu-***Trp***-***Ala***-Thr-***Gly***-His-Phe-***Met***-$\mathbf{NH_2}$

Figura 1 – Alinhamento da sequência dos aminoácidos dos peptídeos da família da Bombesina.
Fonte: Adaptado (PATEL et al., 2006).

Assim como outros neuropeptídeos com este grupamento, o GRP é formado a partir do processamento pós-traducional de um único produto inicial de tradução (preproGRP). Este peptídeo sofre reações de clivagem que geram um peptídeo de 27 aminoácidos chamado de $GRP_{1\text{-}27}$ e, este pode sofrer nova clivagem formando o $GRP_{1\text{-}17}$ e o $GRP_{18\text{-}27}$, mantendo sua atividade biológica (Figura 2) (PATEL; SHULKES; BALDWIN, 2006).

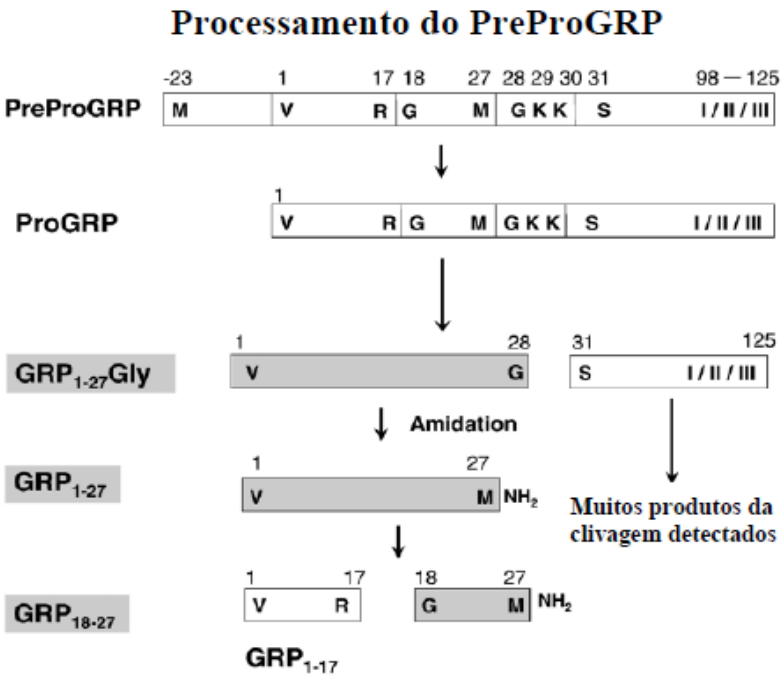

Figura 2 – Processamento do PreProGRP até o peptídeo funcional GRP_{18-27}.
Fonte: Adaptado (PATEL; SHULKES; BALDWIN, 2006).

Um estudo realizado por Rozengurt e colaboradores (1983) demonstrou um efeito mitogênico em culturas da linhagem murina de fibroblastos Swiss 3T3 expostas à bombesina, sem a presença de qualquer outro fator de crescimento no meio de cultivo. Logo após, outros estudos verificaram que a bombesina e seus análogos induzem a proliferação e crescimento de diversos tipos celulares como células da mucosa gastrointestinal (PUCCIO; LEHY, 1989) e pulmão (WILLEY; LECHNER; HARRIS, 1984), através de ensaios de incorporação de timidina e clonogênico, estimando esse aumento da divisão através da avaliação do ciclo celular e do aumento da capacidade de gerar um clone, respectivamente.

Em condições fisiológicas o GRP possui um papel importante na regulação da contração da musculatura lisa, no trânsito intestinal, na liberação de hormônios no trato gastrointestinal, na secreção de enzimas pancreáticas e como um neurotransmissor no sistema nervoso central (CORNELIO; ROESLER; SCHWARTSMANN, 2007). A bombesina e o GRP também estão relacionados ao controle da saciedade pela supressão da ingestão de alimentos (GIBBS et al., 1979; STEIN; WOODS, 1982; WASHINGTON; AGLAN; SAYEGH, 2014). Ainda, o GRP está relacionado com a patogênese da doença de Alzheimer, de Parkinson e da esquizofrenia além de ter sido caracterizado como mediador da ansiedade e da reposta ao estresse (ROESLER et al., 2006). Adicionalmente, o GRP participa da regulação o ritmo circadiano, ansiedade e a resposta ao medo, assim como da termorregulação (MAJUMDAR; WEBER, 2011).

1.1.2 GRP e o Sistema Imune

Del Rio e colaboradores (1994) verificaram que a bombesina, o GRP e a Neuromedina C estimularam a proliferação de linfócitos e induziram a produção de IL-1β em macrófagos peritoneais. Atualmente, há estudos relacionando o aumento na produção de GRP à regulação de células do sistema imune e, por conseguinte, ao envolvimento em processos inflamatórios, como asma (ZHOU et al., 2011) e artrite (GREEN, 2005; GRIMSHOLM; RANTAPAA-DAHLQVIST; FORSGREN, 2005; OLIVEIRA et al., 2011). Dal-Pizzol e colaboradores (2006) mostraram que um antagonista do receptor para o GRP, o RC-3095, reduziu a liberação de citocinas pró-inflamatórias por macrófagos ativados *in vitro* e em um modelo animal de sepse, resultando em melhora da sobrevida.

Nosso grupo recentemente relatou que o GRP pode ser um mediador inflamatório endógeno, agindo como um agente quimiotático de neutrófilos através do GRPR. Além disso, ele ativa vias de sinalização específicas que promovem a migração de neutrófilos (CZEPIELEWSKI et al., 2012). Como sabemos, o sistema imune regula de maneira multicelular e complexa o desenvolvimento tumoral, assim como por vezes os tumores podem induzir respostas imunes favoráveis ao tumor, como a inflamatória, para este progredir (HANAHAN; WEINBERG, 2011; SWANN et al., 2008). Os neutrófilos são conhecidos tradicionalmente por suas funções na resposta imune inata, no entanto, está cada vez mais claro que neutrófilos associados a tumores (TANs), assim como os macrófagos associados a tumores (TAMs), desempenham um papel importante na biologia tumoral (MANTOVANI, 2009). TAMs e TANs podem controlar o crescimento do câncer, podendo desenvolver um fenótipo antitumoral (M1 para TAMs ou N1 para TANs) ou pró-tumoral (M2 ou N2) (Figura 3) (FRIDLENDER; ALBELDA, 2012; PICCARD; MUSCHEL; OPDENAKKER, 2012).

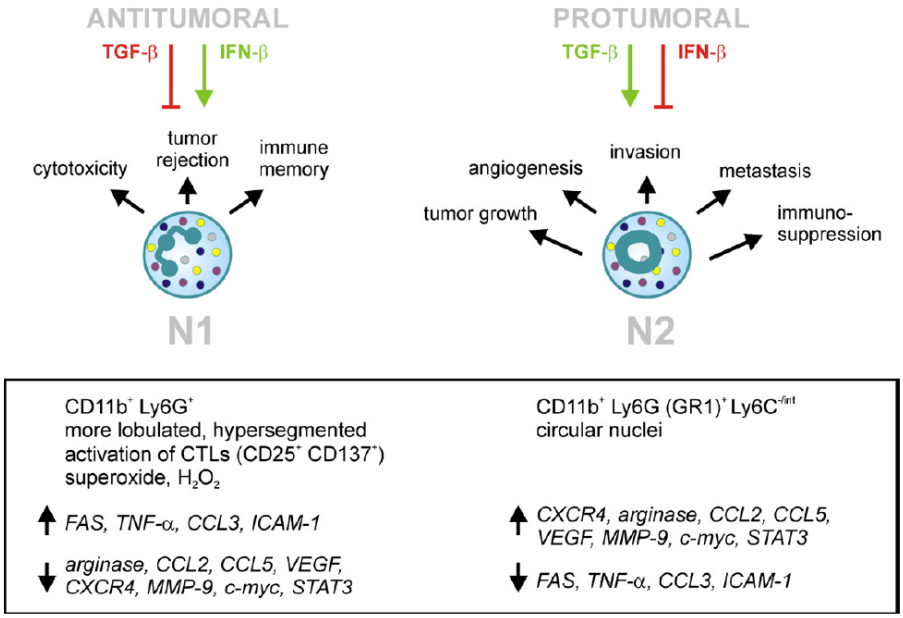

Figura 3 – Esquema simplificado de polarização de neutrófilos associados a tumores (TAN). Fonte: Adaptado (PICCARD et al, 2012),

Devido ao conjunto destas descobertas, podemos hipotetizar que o GRP apresenta um efeito modulador do sistema imune, possivelmente pela ativação e quimioatração de neutrófilos para o microambiente tumoral que, por sua vez, promoveriam o seu crescimento ou por uma ação direta do GRP nas células tumorais. Sendo assim, essa dissertação vem investigar paralelamente as ações diretas do GRP na célula tumoral, fazendo parte de um estudo mais amplo dentro da hipótese acima.

1.1.3 O Receptor do GRP (GRPR)

Até o momento, quatro subtipos de receptores para *bombesin-like peptides* já foram descritos: GRPR ou BB_2 (BATTEY; WADA, 1991; SPINDEL et al., 1990a), receptores para neuromedina B (NMBR) ou BB_1 e receptores para bombesina (BRS-3 ou BB_3) e BB4-R (GONZALEZ et al., 2008). Contudo, o BB4-R possui alta afinidade para a bombesina de anfíbios, portanto, não é encontrado em mamíferos (NAGALLA et al., 1995). Estes receptores são acoplados à proteína G (GPCR) via o seu domínio intracelular (XIAO et al., 2001). Dois grupos distintos isolaram e clonaram o GRPR de células 3T3 derivadas de camundongos Swiss, as quais expressam altos níveis deste receptor (BATTEY et al., 1991; SPINDEL et al., 1990b). O GRPR humano possui 384 aminoácidos e possui uma alta homologia (90%) com o

GRPR murino (CORJAY et al., 1991). Atualmente, sabe-se que o gene que codifica o GRPR está localizado no cromossomo X, tanto em humanos, como em murinos (XIAO et al., 2001).

Sano e colaboradores (2004)) verificaram a distribuição de mRNA deste receptor em primatas e, o mesmo foi identificado em grandes quantidades, no estômago, próstata, músculo esquelético e sistema nervoso central. Outros estudos verificaram que o mRNA do GRPR está expresso em fases embrionárias iniciais de vários sistemas, como o nervoso, urogenital, respiratório e gastrointestinal (BATTEY; WADA; WRAY, 1994).

Outros trabalhos observaram que o GRP pode ser secretado por terminações nervosas, tendo um efeito neuroendócrino em alguns tecidos com receptores específicos para este peptídeo (XIAO; QU; WEBER, 2003). A via do GRPR parece ter interações funcionais com outros neurotransmissores e sistemas de receptores como GABA, dopamina e receptores para glicocorticóides. Estes dados suportam o possível envolvimento do GRPR em doenças como Parkinson, Alzheimer, esquizofrenia e autismo (CORNELIO; ROESLER; SCHWARTSMANN, 2007; JENSEN et al., 2008; ROESLER et al., 2006).

Além disso, Sun & Chen (2007) descreveram primeiramente que o GRPR presente na medula espinhal medeia o comportamento de coceira em camundongos. Desde então, outros pesquisadores tem avaliado como o GRP e células que expressam GRPR atuam na via geradora de coceira, uma vez que esta via pode ser um novo alvo terapêutico contra prurido (AKIYAMA et al., 2014; MACIEL et al., 2014; MISHRA; HOON, 2013; SUKHTANKAR; KO, 2013). Outro estudo mostrou que terminações de neurônios sensoriais primários secretam GRP na pele e, desta forma, induzem uma resposta de coceira através da ativação de receptores para GRP presentes nos mastócitos, promovendo a liberação de histamina e triptase (ANDOH et al., 2011).

1.1.4 GRP e sua função no tecido pulmonar normal

O GRP é o peptídeo análogo da bombesina mais presente no pulmão de mamíferos. A bombesina e o GRP são capazes de induzir a proliferação de células epiteliais normais dos brônquios, de fibroblastos pulmonares e embrionários, sendo assim relacionado ao desenvolvimento pulmonar fetal (DEGAN et al., 2008).

Foi observado que macrófagos alveolares de humanos e de porcos da índia produzem bombesina em diferentes condições de cultura (WIEDERMANN et al., 1986). Alguns anos depois, foi verificado que a bombesina, GRP e NMB eram capazes de estimular a função

fagocítica de monócitos, macrófagos e leucócitos polimorfonucleares de camundongo *in vitro* (JIN et al., 1990). Além disso, Meloni e colaboradores (1995) verificaram que a bombesina é capaz de aumentar a atividade pró-coagulante de macrófagos alveolares e que indivíduos com bronquite crônica tem um aumento na produção de análogos da bombesina por monócitos, macrófagos alveolares e PBMCs (MELONI et al., 1991). Outros ensaios mostraram que o GRP e a bombesina são mediadores de broncoconstrição 10 vezes mais potentes que a substância P e 100 vezes mais que a histamina (IMPICCIATORE; BERTACCINI, 1973; LACH; HADDAD; GIES, 1993).

Sunday e colaboradores avaliaram o papel do GRP no desenvolvimento pulmonar e em patologias pediátricas no pulmão, como a asma (ZHOU et al., 2011) e a displasia broncopulmonar (SUNDAY et al., 1998). Recentemente, a autora descreveu sua hipótese, baseada nos resultados encontrados, de que espécies reativas de oxigênio geradas pela exposição a hipóxia, ozônio e radiação ionizante podem induzir a degranulação de células neuroendócrinas pulmonares, que são sensíveis ao oxigênio, levando a secreção de GRP. O GRP produzido atua sobre células de músculo liso, células endoteliais e fibroblastos, assim como macrófagos, células T CD4 e neutrófilos, resultando em lesão pulmonar com inflamação aguda e crônica, podendo levar à fibrose pulmonar (Figura 4) (SUNDAY, 2014).

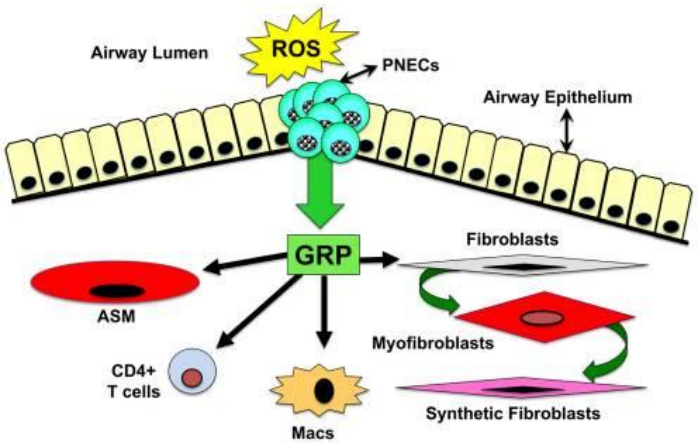

Figura 4: Desenho esquemático da hipótese geral : mecanismos pelos quais GRP medeia lesão pulmonar e fibrose.
Fonte: (SUNDAY, 2014)

Estes estudos demonstram que o GRP e o GRPR possuem um papel importante no desenvolvimento pulmonar, mas podendo também estar envolvido na patogênese de algumas doenças através da ativação do sistema imune. Além disso, fumantes assintomáticos possuem altos níveis de GRP no lavado broncoalveolar (AGUAYO et al., 1989) e na urina (AGUAYO et al., 1992), uma vez que o fumo leva a hiperplasia das células neuroendócrinas pulmonares.

Outro estudo mostrou que a expressão de GRPR está associada ao hábito de fumo (SIEGFRIED et al., 1997). Fumantes possuem um aumento no risco de desenvolver bronquite crônica, enfisema e câncer de pulmão, todos estes apresentam proliferação anormal do epitélio, indicando um possível papel do GRP no desenvolvimento destas patologias (AGUAYO et al., 1989), além de contribuir para a inflamação e fibrose do pulmão (AGUAYO et al., 1990).

1.1.5 GRP, GRPR e Câncer

A hipótese de que o GRP atua como fator de crescimento autócrino em tumores foi proposta no estudo de Cuttitta e colaboradores no qual foi verificado que anticorpos monoclonais para GRP tinham efeito anti-proliferativo em células de câncer de pulmão de pequenas células (SCLC) (CUTTITTA et al., 1985). Estas células produzem e secretam este peptídeo, e possuem receptores de alta afinidade (GRPR) para tal (CARNEY et al., 1987a; MOODY et al., 1985). Desde esta descoberta, outros pesquisadores constataram a presença de GRPR e o efeito mitogênico em tumores provenientes de vários tecidos como próstata , mama, estômago, pâncreas e cólon (revisado em PATEL; SHULKES; BALDWIN, 2006).

Recentemente, foi verificado que células-tronco tumorais (CD133+) de SCLC apresentam alta expressão de GRPR e que apesar de serem resistentes à quimioterapia, são altamente sensíveis a um antagonista de neuropeptídeo de amplo espectro (Peptide-1). Estes dados indicam que a combinação destes medicamentos seria mais eficaz no tratamento de SCLC do que a quimioterapia sozinha (SARVI et al., 2014). Outro grupo identificou uma nova rede molecular agindo no SCLC a qual liga a bombesina autócrina à via da Sonic hedgehog (Shh) e, com isso, sugerem os inibidores da Shh como novas estratégias terapêuticas contra este tipo de câncer agressivo (CASTELLONE et al., 2014).

O GRP também tem habilidade de causar a secreção de vários outros hormônios e proteínas, muitos dos quais possuem potencial efeito mitogênico. Portanto, é possível que o GRP proporcione um estímulo mitogênico indiretamente, *in vivo*, através da ação de outros hormônios peptídicos (PRESTON; MILLER; PRIMROSE, 1996) como, por exemplo, a liberação de TGF-α, anfiregulina (AREG) e HB-EGF, visto em estudos com linhagens de tumores de pulmão de não pequenas células (NSCLC) e carcinoma de células escamosas de cabeça e pescoço (LIU et al., 2007; LUI et al., 2003; SIEGFRIED et al., 1994; ZHANG et al., 2004).

Além disso, foi visto que no câncer de tireoide, o efeito mitogênico do GRP é parácrino, uma vez que o aumento da expressão de GRP ocorre em células C adjacentes aos adenomas foliculares e carcinomas papilares, com o potencial de atuar como fator trófico, ectópico e parácrino nas células tumorais (Figura 5) (MATSUBAYASHI et al., 1984; SUNDAY et al., 1988).

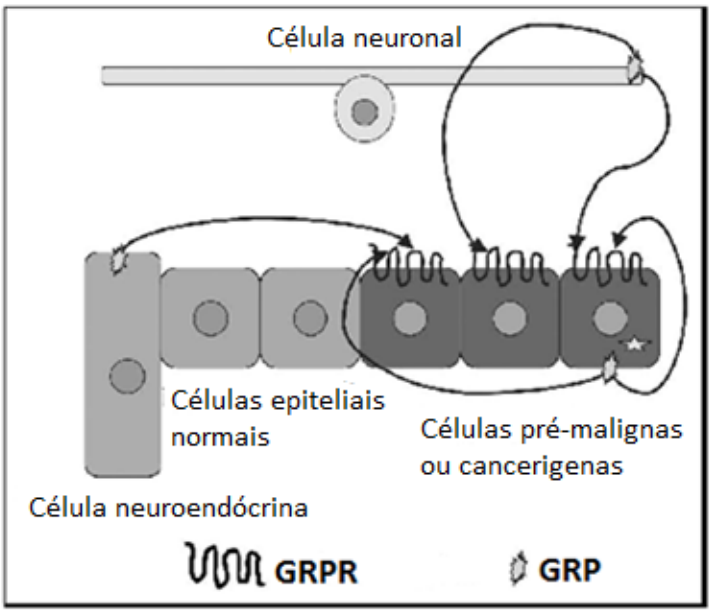

Figura 5 – Interação autócrina, neuroendócrina e parácrina do GRP com o GRPR de células tumorais.
Fonte: Adaptado (QU et al., 2003).

A ação mitogênica do GRP em tumores é a mais descrita na literatura para diversos tipos de tumores (PATEL; SHULKES; BALDWIN, 2006); no entanto, pouco se conhece sobre os seus efeitos na tumorigênese e metástase. Além disso, há estudos mostrando o GRP e seu receptor (GRPR) medeiam a angiogênese através do aumento da migração de células endoteliais (MARTÍNEZ et al., 2005) e também por estimular a liberação de VEGF (ISHOLA et al., 2007; SCHLEGEL et al., 2012) e IL-8 (QIAO et al., 2007).

O GRP também atua como um morfógeno, uma vez que regula a morfologia do tumor, alterando o citoesqueleto de actina da célula e, consequentemente, a sua forma (GLOVER et al., 2004; LINE et al., 1999). O processo regulatório do desenvolvimento chamado de transição epitélio-mesenquimal (TEM) é utilizado pelos tumores para adquirirem a capacidade de invadir, resistir à apoptose e difundir células epiteliais transformadas. Este processo, que pode ser ativado transitoriamente ou estavelmente, recapitula diversos passos envolvidos na morfogênese embrionária e da cicatrização, assim as células neoplásicas adquirem múltiplos atributos que permitem a invasão e metástase (HANAHAN;

WEINBERG, 2011). Portanto, a capacidade morfogênica, ou morfogênese, é um passo decisivo para a mobilidade celular durante o desenvolvimento da natureza invasiva de diversos tumores (LEE et al., 2012).

Tumores com células diferenciadas expressam concentrações altas de GRPR quando comparado a tumores pobremente diferenciados, como em câncer de próstata (MARKWALDER; REUBI, 1999). Ainda, tumores com células diferenciadas co-expressam GRPR e GRP (JENSEN; CARROLL; BENYA, 2001).

Além disso, o GRP aumenta a adesão celular, através da ativação de FAK (GLOVER et al., 2005; TAGLIA et al., 2007), proteína relacionada à proliferação, sobrevivência e migração celular (MCLEAN et al., 2005). Lee e colaboradores (2012) observaram que a alta expressão de GRPR correlaciona com o aumento na expressão de FAK em neuroblastomas. Estimulando estas células com GRP, houve um aumento de FAK fosforilada (pFAK), resultando em um aumento na proliferação e migração celular *in vitro*. Neste mesmo estudo, *knockdown* para GRPR nesta linhagem inibiu o seu potencial metastático tanto *in vitro* como *in vivo*. Outro estudo verificou que a alta expressão de GRP no tumor primário pode ser considerado um fator de prognóstico de metástase no linfonodo e reduz a taxa de sobrevida de pacientes com câncer de mama, possivelmente promovendo habilidades invasivas de células tumorais (NI et al., 2012). E recentemente, Patel *et al* descobriram um mecanismo chave para a migração de células de câncer de cólon reguladas pelo GRPR através da via $G\alpha_{13}$-PRG-RhoA-ROCK (PATEL et al., 2014).

1.1.6 Inibidores de GRP e antagonistas de GRPR

Uma vez que o potencial mitogênico do GRP em tumores ficou estabelecido, pesquisadores procuraram desenvolver inibidores de GRP e antagonistas para receptores de GRP que poderiam ser eficazes no tratamento de várias malignidades. Muitas opções surgiram, entre eles o anticorpo monoclonal 2A11 contra GRP, o qual, após sucesso em experimentos com camundongos, foi aprovado para ensaios clínicos de fase I e II. Contudo, apesar de não apresentar efeitos tóxicos, os resultados não foram muito promissores; uma vez que apenas 01 de 12 pacientes apresentou resolução completa do tumor. Porém, este teve uma recidiva alguns meses após o tratamento com o anticorpo (KELLEY, 1997).

Outras opções que surgiram foram os antagonistas seletivos para receptores de GRP como, por exemplo: RC-3094 II, RC-3095 e RC-3940-II (CORNELIO et al., 2007). Um

destes antagonistas, RC-3095 ou [D-Tpi^{6}, Leu$^{13}\Psi$ (CH_2NH)-Leu^{14}] bombesin (6-14) sintetizado por Pinski e colaboradores foi testado *in vitro,* e em camundongos *nude* (PINSKI et al., 1992). Estes testes mostraram que o RC-3095 foi capaz de produzir uma regressão significativa em vários tipos de células tumorais, incluindo o glioblastoma (KIARIS et al., 1999), carcinoma de pulmão de células pequenas (KOPPAN et al., 1998), pâncreas (QIN et al., 1994) e ovário (CHATZISTAMOU et al., 2001).

Devido a estes resultados promissores, o primeiro teste clínico de fase I foi realizado em pacientes com tumores sólidos. Este estudo utilizou doses que variavam entre 8-96 μg/kg administradas de uma a duas vezes ao dia, subcutaneamente, em pacientes com diferentes tipos de tumores. No entanto, não foi possível verificar uma regressão significativa dos tumores (SCHWARTSMANN et al., 2006).

Em conclusão, apesar dos resultados do tratamento com RC-3095 de camundongos portando tumores demonstrarem uma diminuição do crescimento tumoral *in vivo* e *in vitro*, os mecanismos envolvidos no efeito desse antagonista do GRPR ainda são pobremente compreendidos. Já o efeito da expressão de GRPR em tumores parece ser melhor compreendido.

1.1.7 Sinalização Intracelular Via GRP/GRPR

Como dito anteriormente, GRP e seu receptor GRPR, estão envolvidos no crescimento de diversos tipos de tumores. No entanto, os mecanismos moleculares e as vias de sinalização que são responsáveis pelos efeitos induzidos por GRP/GRPR permanecem obscuros para a maioria dos tipos de câncer.

O GRPR interage com subfamílias de proteínas G, como Gαq e Gα12/13. A ativação de Gαq leva à estimulação de fosfolipase C-β (PLC-β), resultando num aumento da concentração de Ca^{++} citoplasmático e na geração de inositol trifosfato (IP_3) e diacilglicerol (DAG) (ROZENGURT, 1998). Na revisão feita por Flores e colaboradores (2009), os autores descrevem diversas vias de sinalização ativadas pela ligação de GRP e/ou bombesina ao GRPR em diferentes tipos de câncer (Figura 6). No entanto, isto não significa que em todos os tipos de câncer o estímulo com GRP e/ou bombesina irá desencadear a ativação de todas estas vias de sinalização em conjunto.

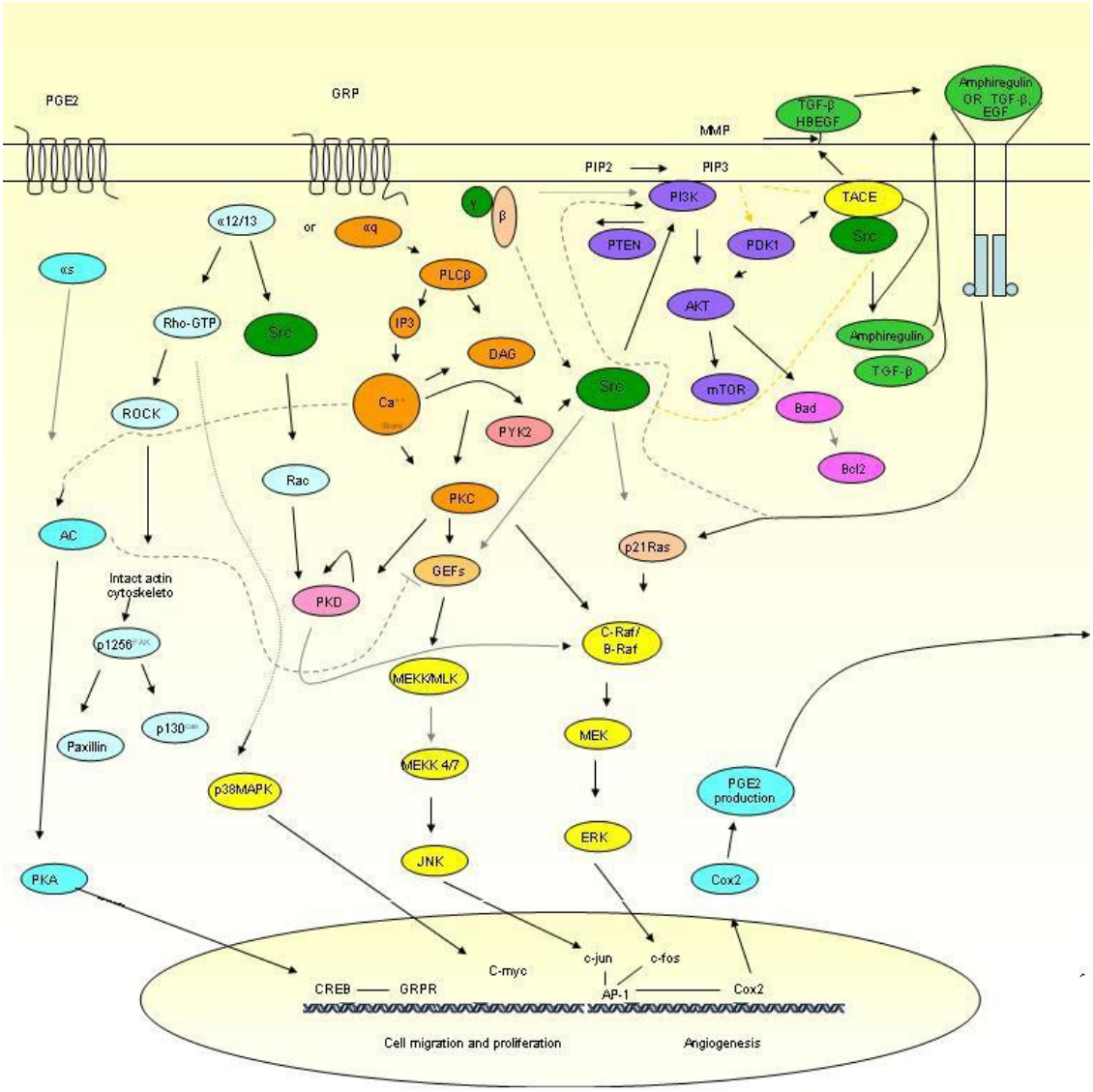

Figura 6 – Vias de sinalização associadas ao receptor do peptídeo liberador de gastrina (GRPR) em câncer. Linhas cinzas indicam possíveis interações funcionais. As linhas pretas indicam já estabelecidas vias ativadas com a estimulação de GRPR.
Fonte: Adaptado (FLORES et al., 2009)

Um grupo que trabalha com linhagens de carcinoma de células escamosas de cabeça e pescoço verificou que após o estimulo com GRP (400 nM), a quinase e, proto-oncogene, c-Src é ativada (ZHANG et al., 2004) que, por sua vez, ativa PI3-K resultando na fosforilação da metaloproteinase TACE (também chamada de ADAM17) por intermédio da quinase PDK-1 (ZHANG et al., 2006). TACE fosforilada acoplada a c-Src, se desloca para a membrana celular para promover a liberação de ligantes do EGFR, como o TGF-α e AREG. Estes ativam o EGFR levando à proliferação celular através da ativação da via MEK/MAPK e AKT (LUI et al., 2003; THOMAS et al., 2005; ZHANG et al., 2006). Este mesmo grupo verificou que em linhagens provenientes de tumores de NSCLC possuem uma discrepância quanto a

quantidade de GRP produzido por estas células. Enquanto que a linhagem 201T produz 4.2 nM de GRP, a linhagem A549 não teve níveis detectáveis, sendo que ambas são classificadas como adenocarcinomas (SIEGFRIED et al., 1999). Além disso, os autores observaram que quando cultivada sem soro por dois meses, a linhagem A549 produz 2,4 ng/10^5 células de GRP (SIEGFRIED et al., 1994).

Outro grupo que trabalha com linhagens humanas de neuroblastomas verificou um sistema de sinalização mais complexo após o estímulo com GRP (Figura 7). Os autores observaram que além de promover a proliferação celular (KIM et al., 2002), o GRP, através da ligação ao seu receptor GRPR, aumenta o potencial invasivo e metastático de neuroblastomas através da ativação da quinase de adesão focal (FAK) (LEE et al., 2012) e AKT (QIAO et al., 2013a). Além disso, através da ativação da quinase alvo da rapamicina humana (mTOR), o GRP é capaz de inibir a autofagia (WOON KIM et al., 2013). Verificaram também o aumento de proteínas relacionadas com o ciclo celular, como p21 (QIAO et al., 2013b) e a inibição da proteína supressora de tumores PTEN (QIAO et al., 2005). Os experimentos foram realizados em grande parte *in vitro* e em camundongos *nude*. Outros resultados encontrados pelo grupo foram incluídos na Figura 7 a fim de ilustrar todo o processo de sinalização mediado pelo GRP nas linhagens de neuroblastomas (ISHOLA et al., 2007; QIAO et al., 2007, 2008; SCHLEGEL et al., 2012).

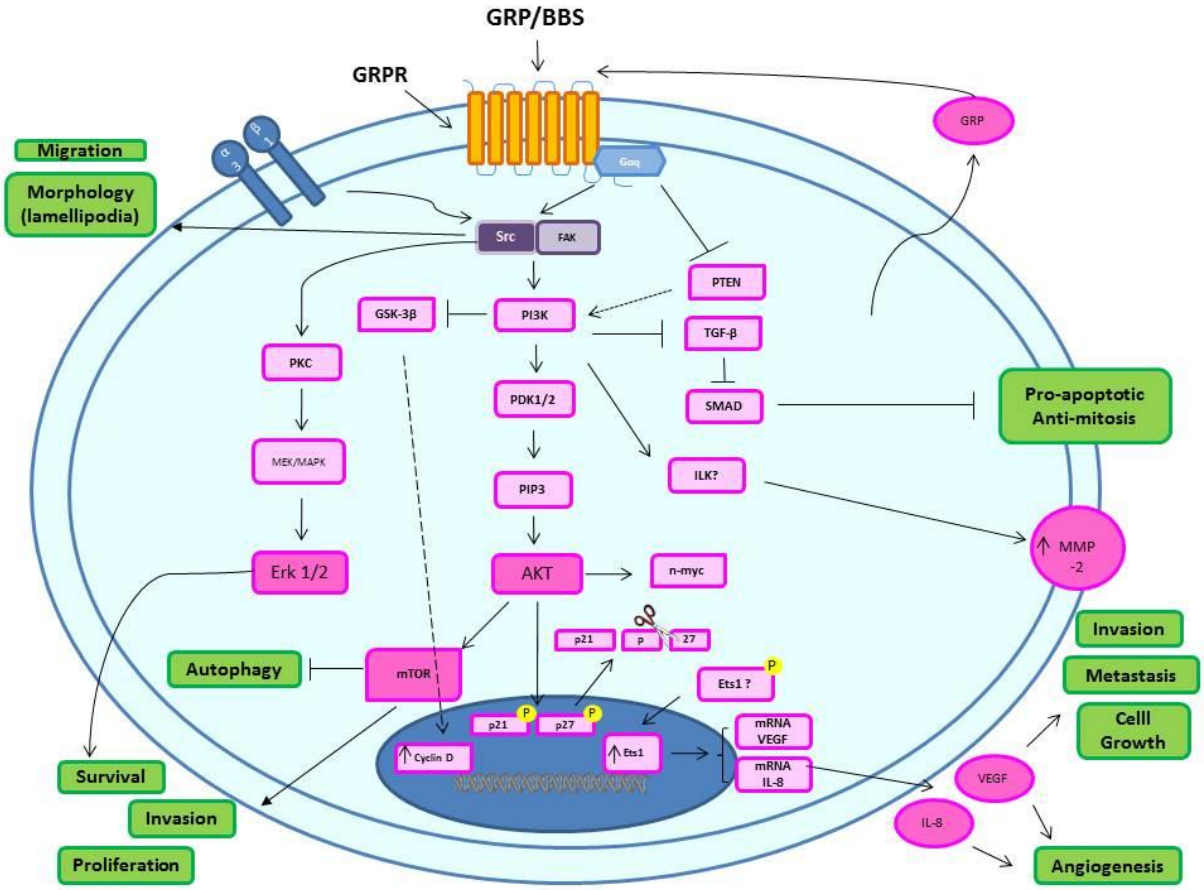

Figura 7 - Sinalização mediada por GRP/GRPR identificadas em linhagens de neuroblastomas. Fonte: Ilustração original baseada em revisão bibliográfica.

Todas estas descobertas indicam que o GRP, através da ligação ao seu receptor, é capaz de aumentar o potencial invasivo e migratório de células tumorais pela ativação de vias de sinalização intracelular responsáveis por estes processos. Nosso grupo descobriu recentemente que o GRP pode atuar como uma molécula quimiotática para neutrófilos (CZEPIELEWSKI et al., 2012) e, desta forma, hipotetizamos que o GRP tenha um efeito migratório sobre a linhagem de adenocarcinoma de pulmão A549.

1.1.8 Epidemiologia do câncer de Pulmão

Entre todos os tumores malignos, o câncer de pulmão é o mais comum no mundo e continua sendo a principal causa de mortalidade relacionada ao câncer (COLLISSON et al., 2014; JEMAL; BRAY; FERLAY, 2011; SIEGEL et al., 2014). Ele foi responsável por 22.424

mortes em 2011 somente no Brasil e o Instituto Nacional de Câncer estima 27.330 novos casos em 2014, sendo 16.400 homens e 10.930, mulheres. Devido à agressividade da doença, a difícil detecção e tratamento, o câncer de pulmão é considerado altamente letal, com sobrevida média total cumulativa em cinco anos de 13 a 21% em países desenvolvidos e de 7 a 10% nos países em desenvolvimento (INCA, 2014).

Os dois principais tipos de câncer de pulmão são SCLC e NSCLC. Sendo o primeiro relacionado a 15-20% dos casos de câncer de pulmão e o segundo, a 80-85% dos casos (HERBST; HEYMACH; LIPPMAN, 2008). O NSCLC pode ser classificado de acordo com a sua histologia em carcinoma escamoso, carcinoma de grandes células e adenocarcinoma (STINCHCOMBE, 2014), sendo o último o mais comum entre todos os tipos de câncer de pulmão (COLLISSON et al., 2014)

A linhagem celular A549 é proveniente de um adenocarcinoma de NSCLC. No entanto, pouco se sabe sobre a biologia desta doença. Este tipo de tumor faz metástase principalmente para o fígado, assim como nos ossos, glândulas supra-renais e cérebro (INCA, 2002). Recentemente, Mattei *et al* demonstraram a expressão de GRPR em uma grande população de pacientes com câncer de pulmão. Embora a expressão de receptores de GRP tenha sido semelhante em SCLC e NSCLC, a expressão foi mais pronunciada em NSCLC avançado e, particularmente nos casos de adenocarcinoma, mostrando que a via do GRP/GRPR pode representar um potencial alvo para o desenvolvimento de novas abordagens de tratamento nesta população (MATTEI et al., 2014).

Portanto, estudos que avaliem o efeito do GRP sobre a migração de células tumorais e os mecanismos envolvidos neste efeito, são de grande relevância para uma melhor compreensão do seu papel na biologia tumoral.

1.2 OBJETIVOS

1.2.1 Objetivo geral

Avaliar o efeito proliferativo e migratório do peptídeo liberador de gastrina sobre a linhagem tumoral A549.

1.2.2 Objetivos específicos

1) Quantificar a expressão de GRPR, por *Real-Time* PCR, na linhagem A549.
2) Analisar o ciclo celular da linhagem A549 após tratamento com GRP.
3) Avaliar o efeito do GRP sobre a proliferação da linhagem A549 através de ensaios de proliferação.
4) Avaliar o efeito migratório *in vitro* do GRP sobre a linhagem A549.
5) Avaliar o efeito do GRP na sobrevivência da linhagem A549 tratada com agente quimioterápico.
6) Investigar o envolvimento do GRPR no processo de migração da linhagem A549 utilizando um antagonista seletivo do GRPR (RC-3095).
7) Investigar as vias de sinalização envolvidas na proliferação, sobrevivência e/ou migração induzidas pelo GRP e RC-3095 na linhagem A549.

2 CAPÍTULO 2

2.1 Artigo Científico

Artigo será enviado para: **Peptides**

Manuscript

Full Title: Gastrin-releasing peptide (GRP) induces migration but not proliferation in a NSCLC cell line
Running Title: GRP induces NSCLC migration
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Abstract

Lung cancer is the most commonly diagnosed type of cancer and the leading cause of cancer related mortality in the world, causing nearly one million deaths per year. Among all histological types, adenocarcinoma is the most frequent one (75-80%). Gastrin-releasing peptide (GRP) is considered to be a mitogen, capable of inducing cell proliferation, since it is involved in fetal lung development. This neuropeptide had its effect on tumor growth first identified in human cells of small cell lung cancer, acting as an autocrine growth factor for tumor tissues by binding to its receptor GRPR. The receptor has been found in many tumor types such as prostate, breast, stomach, pancreas and colon. Moreover, this peptide acts as a morphogen, in angiogenesis and is related to inflammatory processes and in the regulation of cells of the immune system. Furthermore, asymptomatic smokers have high levels of GRP in bronchoalveolar lavage and urine. However, little is known about its effects in tumorigenesis and metastasis, and which molecular mechanisms and signaling pathways are responsible for the effects found. Our group demonstrated recently that GRP could act as a chemotactic molecule for neutrophils. Thus, we hypothesized that GRP could be also a chemotactic stimulus to tumor cells expressing the GRPR. In this study, we tested this hypothesis by examining the effect of GRP on proliferation, survival and migration of cells from the adenocarcinoma cell line A549, seeking to identify the mechanisms of action of this peptide. These cells express high levels of GRPR and treatment with GRP leads to activation of kinases such as AKT and ERK1/2 that are involved in the cellular processes mentioned. Our results suggest that GRP is a migratory stimulus to these cells without evidence of significant effect on their proliferation or survival to treatment with the chemotherapy drug cisplatin (CDDP). Nonetheless, they become more sensitive to CDDP when the drug is combined with a GRPR antagonist. Thus, we believe that future studies should consider a possible role for GRP in metastasis of NSCLC.

Introduction

Among all malignant tumors, lung cancer is the most common in the world and remains the leading cause of cancer related to mortality [1,2], causing over a million deaths each year [3]. NSCLC is related to 75-80% of lung cancer cases and can be classified according to their histology in squamous cell carcinoma, large cell carcinoma or adenocarcinoma, which is the most common type of lung cancer [3].

Gastrin-releasing peptide (GRP) is a mammalian bombesin-like peptide which was firstly isolated by McDonald and his colleagues [4] a decade after the isolation of bombesin (BBS) from the skin of the amphibian *Bombina bombina* [5]. GRP is a mitogenic agent capable of inducing proliferation and cell growth [6,7] since it is involved in the development of fetal lungs [8,9] and acts as an autocrine and paracrine growth factor for human tissues and tumors [10–12]. Moreover, it has important physiological functions like in the regulation of smooth muscle contraction in the bowel, the release of hormones in the gastrointestinal tract, pancreatic enzyme secretion and as a neurotransmitter in the central nervous system [11].

Currently, there are studies linking an increased production of GRP to the regulation of immune cell and, therefore, to the involvement in inflammatory processes such as asthma [13], bronchopulmonary dysplasia [14], arthritis [15] and sepsis [16]. Furthermore, asymptomatic smokers have high levels of GRP in bronchoalveolar lavage [17] and urine [18] since smoking leads to hyperplasia of pulmonary neuroendocrine cells, which secrete GRP. Another study showed that the expression of GRPR is associated with long-term smoking [19].

GRP is known to act by interacting with its preferential receptor, GRPR (Gastrin-releasing peptide receptor) [20] and it has been found in lung, prostate, breast, stomach, pancreas, and colorectal carcinoid tumors [reviewed in 12]. It is usually viewed as a mitogenic factor in cancer, however, little is known about its effects on tumorigenesis, metastasis and the pathways involved in mediating this process. There are studies showing that GRP and its receptor (GRPR) mediate angiogenesis [21]. GRP has been shown to also act as a morphogen when its receptor is aberrantly upregulated [22]. It can regulate tumor morphology by altering the actin cytoskeleton and consequently cell shape [23] as well as increasing cellular adhesion through activation of FAK (Focal adhesion kinase) [22,24]. And the knockdown of GRPR inhibited the metastatic potential of neuroblastoma cells, both *in vitro* and *in vivo* [25]. Another study found that GRP was an important prognostic factor in breast cancer metastasis, possibly promoting the invasive abilities of tumor cells [26].

Since the mitogenic potential of GRP in tumors was established, researchers began to develop selective antagonists for GRPR, which could be effective in the treatment of various malignancies. One of the antagonists, RC-3095 [D-Tpi6, Leu13Ψ (CH2 NH) Leu14] bombesin synthesized by Schally et al [27] was tested *in vitro* and with *in vivo* xenographs. These tests showed that RC-3095 was able to produce a significant regression in several types of tumor cells, including glioblastoma [28,29], small cell lung carcinoma [30], pancreas [31] and ovarian [32]. Due to these promising results, the first phase I clinical trial was performed in patients with solid tumors. However, no significant tumor regression was observed [33].

Because GRP is overexpressed in tumors and metastasis, and we have recently found that it can act as a chemotactic molecule for neutrophils [34], we questioned whether GRP could act as a chemoattractant for cancer cells as well. Thus, to investigate the proliferative, survival and migratory effect of GRP we used a NSCLC cell line A549. It is the type of lung cancer with the highest mortality related to malignant diseases in the world. This tumor metastasizes primarily to the liver as well as in bone, adrenal glands and brain. However, little is known about the biology of this disease, therefore studies that evaluate the effect of GRP on tumor cell migration are of great relevance to a better understanding of its role in tumor biology.

Material and Methods

Reagents

Dulbecco's Modified Eagles Medium (DMEM) and Trizol were obtained from Gibco Invitrogen. Fetal bovine serum was from Cultilab. Primers were synthetized by Taqman. Gentamicin was purchased from Novafarma. Qubit, Superscript III kit was obtained from Invitrogen. Human gastrin-releasing peptide (GRP_{1-27}), MTT powder, trypan blue, RNase were purchased from Sigma-Aldrich. Cisplatin was purchased form Libs. DAPI was from Life Technologies. RC-3095, which was originally synthesized in the Schally laboratory by solid-phase methods, was supplied by Cristália. All antibodies, propidium iodide, cytofix and PermIII buffers were obtained from BD Bioscience except for the anti-Ki67 and fixable viability dye, which were from eBioscience.

Cell Culture

Human alveolar epithelial adenocarcinoma (A549) was grown and maintained in DMEM supplemented with 10% FBS and 0.1 mg/mL of gentamicin antibiotic. All cells were incubated at 37°C, 5% CO^2 in a humidified incubator with medium being replaced every 48 hours.

GRPR expression by Real-time quantitative PCR

Total mRNA was extracted from A549 cells using Trizol®. Quantification of mRNA was measured using the protocol from Qubit™ assay. cDNA was synthesized by Superscript III kit. Sequences of primers for human GRPR: 5'-CCAACCGCAAGACCTTCATT-3' (forward) and 5'-GGAAGATCCACGTTGTAAGC-3' (reverse). Quantification of GRPR expression was performed using an Applied Biosystems StepOne™ Real-Time PCR instrument.

Trypan Blue Exclusion Assay

Cell proliferation was determined by counting cells that excluded trypan blue with a hemocytometer, and treated under the same conditions described for Ki67 assay for 24 and 48 hours.

MTT Assay

A549 cells ($1x10^4$) were plated on 96-well culture plates until reached 50% confluence and later starved (0.5% FBS) for 24 hours. For the proliferation assay, cells were treated with GRP (10 nM, 100 nM and 1 μM) with DMEM 0.5% FBS, or medium alone for 24 hours in 37°C and 5% CO_2. For the viability assay, cells were pre-treated with GRP (10, 50, 100 and 500 nM) for 15 min in the incubator for 24 and 48 hours. Cisplatin (CDDP) 20 μg/mL was then added to each well. Cells were also treated with RC-3095 (100 nM, 500 nM, 1 μM and 10 μM) alone and with CDDP (20 and 40 μg/mL) for 24 and 48 hours. Cell proliferation and viability was determined by the 3-(4,5-dimethylthi- azol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay in each treatment group described above. Briefly, MTT (5 mg/mL) solution was added to each well. Cells were incubated at 37°C for 2 hours in a humidified incubator with 5% CO_2. The formed formazan crystals were solubilized by the addition of 120 μL of DMSO and the content was transferred to a new plate. The optical density was measured at a wavelength of 570 nm with an ELISA plate reader (Antros Zenyth 340r).

Ki67 expression

A549 cells ($5x10^4$) were plated on 24-well culture plates until reached 50% confluence. Cells were starved (0.5% FBS) for 24 hours then treated with GRP (50 and 100 nM) with 0.5% or 10% FBS, or medium alone for 24 hours in 37°C and 5% CO_2. Cells were detached from the plate using trypsin, centrifuged and resuspended in DMEM. Cells were then stained for Ki67 (anti-Ki67 eFluor 710) and viability (Fixable Viability Dye eFluor 780) following eBioscience protocol for Staining Intracellular nuclear proteins and Viability staining Protocol C. Data were obtained using FACSCantoII (Beckton Dickinson) and BD FACSDiva software, and analyzed using Flowjo v10.

Propidium Iodide (PI) Cell Cycle analysis

A549 cells ($5x10^4$) were plated on 24-well culture plates until reached 50% confluence. Cells were starved (0.5% FBS) for 24 hours then treated with GRP (50 and 100 nM) with 0.5% or 10% FBS, or medium alone for 24 hours in 37°C and 5% CO_2. Cells were detached from the plate using trypsin, centrifuged, resuspended and $5x10^5$ cells/mL were placed in flow cytometry tubes. Cells are fixed with 70% ethanol for 1 hour on ice. Then, cells are washed and centrifuged twice and 50 uL of RNase solution (10mg/mL) is added to each tube for 15 minutes at 37°C. After incubation, DNA is stained with propidium iodide (10µg) for 30 min at room temperature. Data were obtained using FACSCantoII (Beckton Dickinson) and BD FACSDiva software, and analyzed using Flowjo software v.10.

BrdU Assay

A549 cells ($5x10^4$) were plated on 24-well culture plates until reached 50% confluence. Cells were starved (0.5% FBS) for 24 hours then treated with GRP (50 and 100 nM) with 0.5% or 10% FBS, or medium alone for 24 hours in 37°C and 5% CO_2. Cell cycle analysis was performed using the BD Pharmogen BrdU Flow Kit (anti-BrdU FITC) and protocol was performed according to manufactures instruction manual. Data were obtained using FACSCantoII (Beckton Dickinson) and BD FACSDiva software, and analyzed using Flowjo v10.

Clonogenic assay

This assay was performed according to Franken *et al* [35]. Briefly, 100 cells (A549) were plated on 6-well plated and let adhere for approximately 4 hour. Cells were washed then treated with GRP (50 and 100 nM), or medium alone (10% FBS) for 15 days in 37°C and 5% CO_2. On the 15^{th} day, cells were washed 3x with cold PBS 1x and fixed with freezer-cold methanol for 10 min. Colonies were stained with 1 mL of crystal violet for 10 min and the excess washed with running water. Only colonies with 50 cells or more were counted.

Expression of phospho-AKT and phospho-ERK1/2

A549 cells ($5x10^4$) were plated on 24-well culture plates until reached 50% confluence. Cells were starved (0.5% FBS) for 24 hours then treated with GRP (50 and 100 nM) with 0.5%, or medium alone for 5 and 15 minutes in 37°C and 5% CO_2. The expression of phospho-AKT, and phospho-ERK1/2 was measured by flow cytometry using BD Phosflow (BD Bioscience) protocol for adherent cells using trypsin to detach cells. Briefly, cells were fixed in Phosflow Buffer I for 10 min at 37 °C. After washing, permeabilization was performed with Phosflow Perm Buffer II for 30 min on ice. Then, cells were washed twice and stained with PE anti-phospho-AKT, and FITC anti–phospho-ERK 1/2 Ab for 30 min on ice. Data were obtained using FACSCantoII (Beckton Dickinson) and BD FACSDiva software, and analyzed using Flowjo v10.

Wound healing assay

A549 cells ($2x10^6$) were seeded on 6-well plates DMEM 10% FBS for 24 hours or until reached 90% confluence at 37°C under 5% de CO_2. Subsequently, cells were starved (0.5% FBS) for 24 hours. Then, using a p200 pipette tip a straight line was scraped into the well to mimic an injury. Cells were washed three times with PBS to remove the debris/serum and treated with GRP 50 nM in DMEM 0.5% or 10% FBS, or medium alone and allowed to migrate for 48 hours. Cultures were photographed at day zero, 24 hours and 48 hours via an inverted optical microscope with a capturing digital image system (Olympus IX51), always in the same field. Photographs were further analyzed by using Adobe® Photoshop® CS5.

Transwell Chemotaxis Assay

We have followed the protocol described by Shi *et al* with minor modifications [36] for the Transwell system (Corning). Briefly, A549 cells ($2x10^6$) were seeded on a 6-well plate and starved (0.5% FBS) for 24 hours at 37°C under 5% de CO_2. Cells were then suspended in DMEM 0.5% FBS (2×10^5 cells/200 µL) and added to top wells of 8 µm-transwell inserts (Corning). GRP was added to the bottom wells at 50 nM containing 10% FBS, or medium alone. Cells were incubated for 8, 12 and 24 hours at 37°C under 5% de CO_2. To evaluate the involvement of ERK and GRPR on GRP-induced cell migration, cells were pretreated with

selective inhibitors (PD98059 and RC-3095, respectively) at 37°C under 5% de CO_2 for 1 hour. At last, inserts were removed from the plate; cells on the bottom were fixed in the membrane and stained with DAPI (1:50) for 5 minutes. Using a scalpel, membranes were removed and placed on a glass slide, mounted with Vectashield® and covered with a coverslip. Slides were examined at 200x magnification using an optical microscope Olympus BX41 and six random fields were captured and further analyzed using ImageJ software (NIH) to perform cell count.

Statistical Analysis

Data are presented as mean ± SEM. Results were analyzed using GraphPad Prism 6. Statistical differences among the experimental groups were evaluated by analysis of variance ANOVA with Tukey correction or with Student's t test. The level of significance was set at $P < 0.05$.

Results

A549 expresses high levels of GRPR mRNA

GRPR is the most commonly expressed GRP receptor in patients with lung cancer [37], which is of particular interest considering that adenocarcinoma expresses higher levels than all other histological types [38]. To further investigate GRPR presence in NSCLC *in vitro* models we examined expression of this receptor in A549 cell line. GRPR qPCR analysis showed high expression in A549 cell line when compared to 3T3 (Fig.1 A), which is also consistent with the results found by another study [39]. Thus, this result indicates that A549 would respond to GRP stimulus.

AKT and ERK1/2 are activated by GRP treatment

Previous studies investigating the effect of GRP on NSCLC cell lines have shown that GRP stimulates AKT [39]. Also, GRP induces rapid activation of p44/42 MAPK in lung cancer cells through EGFR [40]. In our study, we stimulated cells with GRP (50 nM) for 5 and 15 minutes and evaluated AKT and ERK1/2 phosphorylation by FACS. As shown by Liu *et al* study [39], our results also showed AKT activation but in 5 min only (Fig.1 B). Moreover, ERK1/2 was activated in 5 and 15 minutes after stimulation with GRP (Fig.1 C). Since these proteins are involved in several cell signaling that regulate like proliferation, survival and migration we sought to evaluate these parameters in A549.

GRP has no effect on cell proliferation

The role of GRP as a growth factor of normal cells and several tumor types is extensively described in the literature [reviewed in 12]. Therefore, we firstly used the hemocytometer count with trypan blue exclusion and MTT assay to verify the proliferative effect on A549 cells. To this end, we exposed cells to different concentrations of GRP (50 and 100 nM) for cell count and GRP (10 nM, 100 nM and 1μM) in the MTT assay. Interestingly, no difference was seen compared to medium alone in any of the concentrations used in cell count (Fig.1 D) and in MTT assay (Fig.1 E). Also, clonogenic assay showed no increase in the formation of colonies after treatment with GRP (50 and 100 nM) (Fig.1 F,G). Considering these

proliferation assays are not very comprehensive we wondered if more sensitive assays would dissect the proliferative effect of GRP (50 and 100 nM) on A549 cells. To better investigate, cells were also analyzed for the proliferation marker Ki67 (Fig.2 D,E), cell cycle accumulation (Fig.2 F) and for the ability to incorporate BrdU (Fig.2 G). However, similar results to the other assays were found showing that GRP had no significant effect on proliferation in this cell line even with low concentrations of medium. These results are consistent with other studies that have not found an increased proliferation of NSCLC cell lines after stimulation with bombesin and/or GRP [39,41].

GRPR antagonist (RC-3095) induced minor cytotoxicity

Gastrin-releasing peptide receptor synthetic antagonists, such as RC3095, have been developed as anticancer candidates, and have shown antitumor activity in both *in vivo* and *in vitro* murine and human tumor models, producing long-lasting tumor regression [28,30–32]. Therefore, we evaluated the cytotoxic effect of RC-3095 in A549 cell line. RC-3095 at 10 μM had no significant cytotoxic effect on cells treated for 24 and 48 hours with 10% FBS (Fig.3 A,B) but reduced significantly (22%) cell viability when treated with 0.5% FBS (Fig.3 C) for 24 hours. These data is in accordance to the results above since RC-3095 is not cytotoxic to A549, only when cell is under starving conditions.

Cell death-induced by cisplatin is not prevented by GRP treatment

To further investigate, we tested if pre-treatment with GRP could decrease cell death induced by cisplatin. However, no difference was seen in cell survival rate by MTT assay in any of the GRP concentrations tested either in 24 (Fig.4 A,E) or 48 hours (Fig.4 C,G). Notwithstanding, cells treated with medium supplemented with 0.5% FBS were less sensitive to cisplatin in 24 hours than with 10% (Fig.4 A,C). These findings show that although GRP activates proteins involved in survival signals it is does not prevent cells from cell death by CDDP.

Combination of RC-3095 and cisplatin increase cell death *in vitro*

Combined blockade of GRPR and EGFR pathways significantly inhibited head and neck squamous cell carcinoma (HNSCC) proliferation, invasion, and colony formation [42]. Thus, we questioned if RC-3095 alone could decrease cell viability and if could potentiate treatment

with cisplatin, since is still one of the main treatments used in NSCLC cases [43]. We found that only RC-3095 at 500 nM was able to reduce cell viability (57%) in 24 hrs with 10% FBS (Fig.4 B). Interestingly, when cells were exposure to cisplatin with 0.5% FBS they were less sensitive to its effect in 24 hours when compared to cells treated with cisplatin in 10% FBS (Fig.4 E,F). But when they were treated in combination with RC-3095 1 and 10 μM they became more sensitive having 34% and 27% cell viability reduction, respectively (Fig.4 F). No difference was seen in both culture conditions of FBS in 48 hours of treatment (Fig.4 C,D,G,H). These results suggest that combined therapy targeting GRPR and CDDP augments antitumor efficacy.

GRP induces cell migration

It is known that morphogenesis is an important step for cellular mobility during the development of invasive nature of many tumors. Recent data suggest that the GRP serves primarily as a morphogen and not as a mitogen as usually viewed [22,44]. In order to evaluate the migration potential of GRP on the A549 cell line we used two established migration assays. In the wound healing assay, cells were starved for 24 hours and then treated with GRP 50 nM with 0.5% or 10% FBS for 48 hrs. Although there was no difference when cell were treated with GRP 50 nM with 0.5% of serum, we found a significant difference when treating cells with GRP 50 nM at 10% of serum in 24 and 48 hours after the wound (Fig.5 A,B). However, some argue this assay does not evaluate migration alone and the effect seen could be due to cell proliferation, which was dismissed in our previous experiments (Fig. 1-2). To support our data, we also performed the transwell assay. There was no significant increase in the migration of cells exposed to 50 nM GRP with 10% of FBS for 12 (Fig.5 E,F) and 24 hours (Fig. 5 A,B) when compared to control. Nonetheless, we found a significant difference when cells were allowed to migrate for only 8 hours towards GRP 50nM with 10% of FBS (Fig.5 G,H). These results demonstrate that the rapid activation of AKT/MAPK pathway induced rather migration than solely proliferation or survival as the broad literature describes.

Discussion

The hypothesis that the GRP acts as an autocrine growth factor in tumors was proposed in Cuttitta *et al* study in which it was found that monoclonal antibodies to GRP had anti-proliferative effect on SCLC [10]. Lung cancer is the most commonly diagnosed type in the world [2] leading to over a million deaths per year [38]. Noting that adenocarcinoma, a NSCLC histological type, is the most frequent among all types of lung cancers and smoking is its major cause [3]. Asymptomatic smokers have high levels of GRP in bronchoalveolar lavage and urine [17,45] moreover, the expression of GRPR is associated with the smoking habit [19]. Smokers have an increased risk of developing chronic bronchitis, emphysema and lung cancer, all of these exhibit abnormal epithelial proliferation, indicating a possible role of GRP not only in the development of these disorders but also contributing to inflammation and fibrosis of the lung [46]. In this study, we characterize a new role of the interaction of GRP to its specific receptor, GRPR, in a lung adenocarcinoma cell line. Our results indicate that the neuropeptide GRP can induce migration of these cells, which express GRPR.

GRPR is the most commonly expressed GRP receptor in patients with lung cancer as it is detected in 85% of small and non-small cell lung cancer [37]. Recently, GRPR tissue expression was analyzed in a large population of patients with lung cancer. Both SCLC and NCLC cases had similar expression of GRPR however, the results showed higher expression in particularly adenocarcinoma cases than in SCLC cases [38]. Our initial investigation showed that A549 expressed higher levels of GRPR than 3T3 cell line (Fig.1 A), which has shown to proliferate when stimulated with bombesin, a GRP analogue. GRP is the mammalian counterpart of bombesin and its role as a growth factor of normal cells and several tumor types has been extensively described in the literature [reviewed in 11 and 12]. Therefore, we sought to investigate the proliferative effect of GRP in the A549 cell line. We first tested different doses of GRP (10–1000 nM) and in two different serum concentrations (05% and 10%) by Trypan exclusion, MTT and clonogenic assay. However, none of these assays showed difference in cell proliferation (Fig.1). Since theses assays are not very specific for this evaluation, we wondered if more elaborated assays would show proliferation induced by GRP. Ki67 is only expressed when cells are in an activate cell cycle phase thus being a well-established marker for tumor proliferation. Yet, no difference was seen with GRP stimulation (Fig.2). Cell cycle and BrdU incorporation are similar assays and even though the last test is more sensitive than the first they showed similar results. Cells treated with 0.5% FBS had lower accumulation on S phase and higher on G0/G1 phase compared to cells treated

with 10% FBS. Rozengurt [47] showed that when Swiss 3T3 cells were stimulated with bombesin in serum free medium they had similar cell cycle results than cells treated with serum. But in our study we have not observed this effect. These results are consistent with other studies, which have seen that even without the presence of serum in the medium, GRPR activation has inconsistent, and only a modest effect on the growth of tumor cells, leading to the hypothesis that the GRP acts as a weak mitogen on some tumors [44]. Also, a previous study performed a MTS assay with A549, which showed no significant difference in proliferation compared to control when cells were stimulated with 100 nM for 24 hours. However, the authors do not discuss this result on the article [39]. Moreover, most studies evaluating the proliferative effect of GRP had their analysis done by not specific assays like Trypan Blue exclusion and MTT [48–51].

Previous studies investigating the effect of GRP on NSCLC cell lines have shown that GRP stimulates Akt activation primarily via c-Src activation, followed by extracellular release of the EGFR ligand amphiregulin, leading to the activation of EGFR and PI3K. Also, GRP induces rapid activation of p44/42 MAPK in lung cancer cells through EGFR [40]. But no study has ever seen ERK 1/2 activation on A549 cell line (Fig.1 C). In our study, we found that both AKT and ERK1/2 were activated after stimulation with 50 nM of GRP. These proteins are known to be involved not only in cell proliferation and migration but also in survival. Likewise, Liu *et al* found that GRP rescues A549 exposed to gefitinib, an EGFR inhibitor. This effect was mediated through release of amphiregulin and activation of the AKT pathway [39]. Therefore, we wondered if pre-treatment with GRP could rescue cells from cytotoxic effects of cisplatin, which is still one of the main treatments in NSCLC cases [43]. However, no difference was seen either in 24 or 48 hours. These might be due to the mechanism involved in the cisplatin effect, which induces cell apoptosis differently from EGFR inhibitors that are involved in the same pathways of GRPR signaling. And even thought GRP activates survival signals it is not enough to protect cells from CDDP.

Combined blockade of GRPR and EGFR pathways significantly inhibited head and neck squamous cell carcinoma (HNSCC) proliferation, invasion, and colony formation [42]. Recently, a xenograft mice model of pancreatic cancer showed significantly reduction of tumor volume and weight when treated with the combination of RC-3095 and gemcitabine [52]. Thus, we first questioned if RC-3095 alone could have cytotoxic effects since it does not interfere in the cell cycle phases (Fig.2 D). We noticed that when cells were treated with 10% FBS, 10 μM of RC-3095 had a slight but no significant cytotoxicity. However, when treated with 0.5% FBS we found a 22% reduction in cell viability in 24 hours showing that starved

cells are more sensitive to its effect. Hence, we tested whether CDDP in combination with RC-3095 could have its cytotoxic effects enhanced. We found that 500 nM with 10% FBS and 1-10 μM of RC-3095 significantly potentiated the effects of 20 μg/mL of CDDP in 24 hours. No difference was seen in 48 hours even with RC-3095 treatment alone possibly indicating that fresh medium containing RC-3095 must be added everyday to continuously exert its effects.

The regulatory development process called epithelial-mesenchymal transition (EMT) is used by tumors to acquire the ability to invade, resist apoptosis and diffuse epithelial transformed cells. This process, which can be activated transiently or stably recapitulates various steps involved in embryonic morphogenesis, wound healing and thus the neoplastic cells acquire multiple attributes that allow invasion and metastasis [53]. Therefore, the morphogenetic capacity or morphogenesis is a critical step for cell mobility during the development of invasive nature of many tumors [22]. Initial studies have suggested that GRP could play a role on NSCLC migration [54]. More recent data indicate that the GRP serves primarily as a morphogen and not as a mitogen as usually viewed [44]. In fact, in the same study they found that colon cancer cell lines had increased cell motility when treated with bombesin for 20 hours by wound healing assay but had no effect on cell number [22]. This process would be mediated by GRP upregulation of ICAM-1 via FAK [24]. Others have observed a morphological change in neuroblastomas with high expression of GRPR. In this same study, cells BE(2)-C knockdown for GRPR had inhibited the metastatic potential both *in vitro* and *in vivo* [25].

In summary, in the present study we established a relationship between GRP/GRPR and cell migration instead of proliferation and survival. Therefore, more studies are needed to address the importance of this model *in vivo* and in the promotion of NSCLC metastasis.

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Footnotes

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Figure Legends

Fig.1. *GRP-induced AKT/MAPKs phosphorylation does not promote proliferation in A549.*

A) GRPR expression in B16 and A549 cell lines was measured by qPCR. GRPR expression relative to beta-actin production. Data (mean±SEM) are representative from 3 independent experiments performed triplicates.

B-C) A549 cells were stimulated with GRP (50nM) for 5 or 15 min (red and blue histogram line, respectively) and Akt (**B**) or Erk1/2 (**C**) activation patterns were monitored by Phosflow analysis. Number of counted cells and expression of pAkt and pErk1/2 is show in the histograms. Isotype control (tinted histogram) and medium only (black histogram line) were used as negative controls. Data are representative from two independent experiments performed triplicates.

D) A549 cells were treated with GRP 50nM or 100 nM in 0.5% or 10% of FBS medium for 24 (black bars) or 48 hours (gray bars). Cell count was performed using a hemocytometer and trypan blue exclusion. Total number (mean±SEM) of counted cells per group is shown.

E) MTT proliferation assay was done with treated-cell in different concentrations of GRP (10nM, 100nM, 1000nM) in 0.5% of FBS for 24 hours (light gray bars). Medium alone with 0.5% or 10% of FBS was used as negative (black bar) or positive (dark gray bar) controls, respectively. Data (mean±SEM) are expressed as percentage of an untreated control.

F) Clonogenic assay of A549 cells treated with GRP (50nM or 100 nM) or GRP (50nM) + RC-3095 (500nM). Number of colonies per well was counted 15 day after stimuli. Data (mean±SEM) are representative from three independent experiments performed in triplicates. Statistics: one-way ANOVA with Tukey post test or Student's t-test, * = $p<0.05$.

Fig. 2. *Lack of mitotic effect of GRP on A549 cells*

A) Gate strategy for Ki67 expression analysis. Representative FACS plots for singlets (1st and 2nd plots), viable cells, A549 cell morphology and Ki67+ cells (left to right, respectively).

B-C) Ki67 expression (MFI) of A549 treated with GRP (50nM, 100nM and 500nM) at 0.5% (black bars) or 10% FBS (gray bars) medium after 24 (**B**) or 48 hours (**C**).

D) PI-cell cycle analysis of A549 treated with GRP (50nM and 100nM) and RC-3095 (1μM). Gating strategy based on morphology (left plot) and PI staining (middle plot). Percentage of G0/G1 (white bars), S (red bars) and G2/M (blue bars) cell cycle stages are shown for groups cultured with 0.5% or 10% of FBS medium for 24 hours.

E) BrdU incorporation assay of A549 treated with GRP (50nM and 100nM). Representative FACS plots show gate strategy for singlets (top left and right plots), A549 cell morphology (bottom left plot) and BrdU/7-AAD staining (bottom right plot). Percentage of G1 (white bars), S (red bars) and G2 (blue bars) cell cycle stages are shown for groups cultured with 0.5% or 10% of FBS medium for 24 hours. Data (mean±SEM) are representative from three independent experiments performed in triplicates. Statistics: one-way ANOVA with Tukey post test or Student's t-test, * = $p<0.05$.

Fig. 3. *GRPR antagonist does not promote extensive cytotoxicity in A549*.
A) GRPR antagonist (RC-3095 10μM) cytotoxic effect on A549 was tested by MTT assay on cells cultured in 0.5% (black bars) or 10% of FBS (gray bars) medium for 24 hours. Data (mean±SEM) are representative from three independent experiments performed in triplicates c. Statistics: Student's t-test, * = $p<0.05$.

Fig. 4. *Cisplatin-mediated apoptosis is not altered by GRP treatment*
MTT survival assay of A549 cells pre-treated with GRP (50 nM) for 15 min. **A-D**) Cells cultured in 10% FBS medium were then treated with GRP (10nM, 50nM, 100nM and 500nM) for 24 (**A**) or 48 hours (**C**), or treated with RC-3095 (100nM, 500nM, 1μM and 10μM) for 24 (**B**) or 48 hours (**D**).
E-H) Alternatively, cells cultured in 0.5% FBS medium were treated with GRP (10nM, 50nM, 100nM and 500nM) for 24 (**E**) or 48 hours (**G**), or treated with RC-3095 (100nM, 500nM, 1μM and 10μM) for 24 (**F**) or 48 hours (**H**). Data (mean±SEM) are representative from three independent experiments performed in quadruplicates and are expressed as percentage of an untreated control. Statistics: one-way ANOVA with Tukey post test, *** = $p<0.001$, ** $p=<0.01$ and * = $p<0.05$, or Student's t-test # = $p<0.05$.

Fig. 5. *GRP promotes A549 cell migration*
A-B) A549 cells stimulated with GRP (50nM) in 0.5% or 10% FBS medium were subject to wound healing assay and stained with hematoxylin (blue area). **A**) Representative images at 0 (left column), 24 (middle column) or 48 hours (right column) after treatment. Microscopy images with 200x magnification.
B) Image quantification of the wounded area size. Migration is expressed as percentage of coverage of the starting wounded area (set as 0 %). Nine pictures per sample were taken and

analyzed. Data (mean+SEM) representative of three independent experiments performed in triplicates.

C-E) A549 cells were starved and placed in the upper side of transwell migration chambers and 10% FBS medium (as control) or GRP 50nM were added to the lower chamber. Cells were than allowed to migrate for 24 (**C**), 12 (**D**) or 8 hours (**E**). Transwell membranes were stained with DAPI and six pictures per sample were taken. Representative microscopy images (left side, at 200x of magnification) and quantification of cell number per field (right side) of each group are shown. Data (mean+SEM) representative of two independent experiments performed in triplicates. Statistics: Student's t-test, *** = $p<0.001$.

Paper Figures

Figure 1

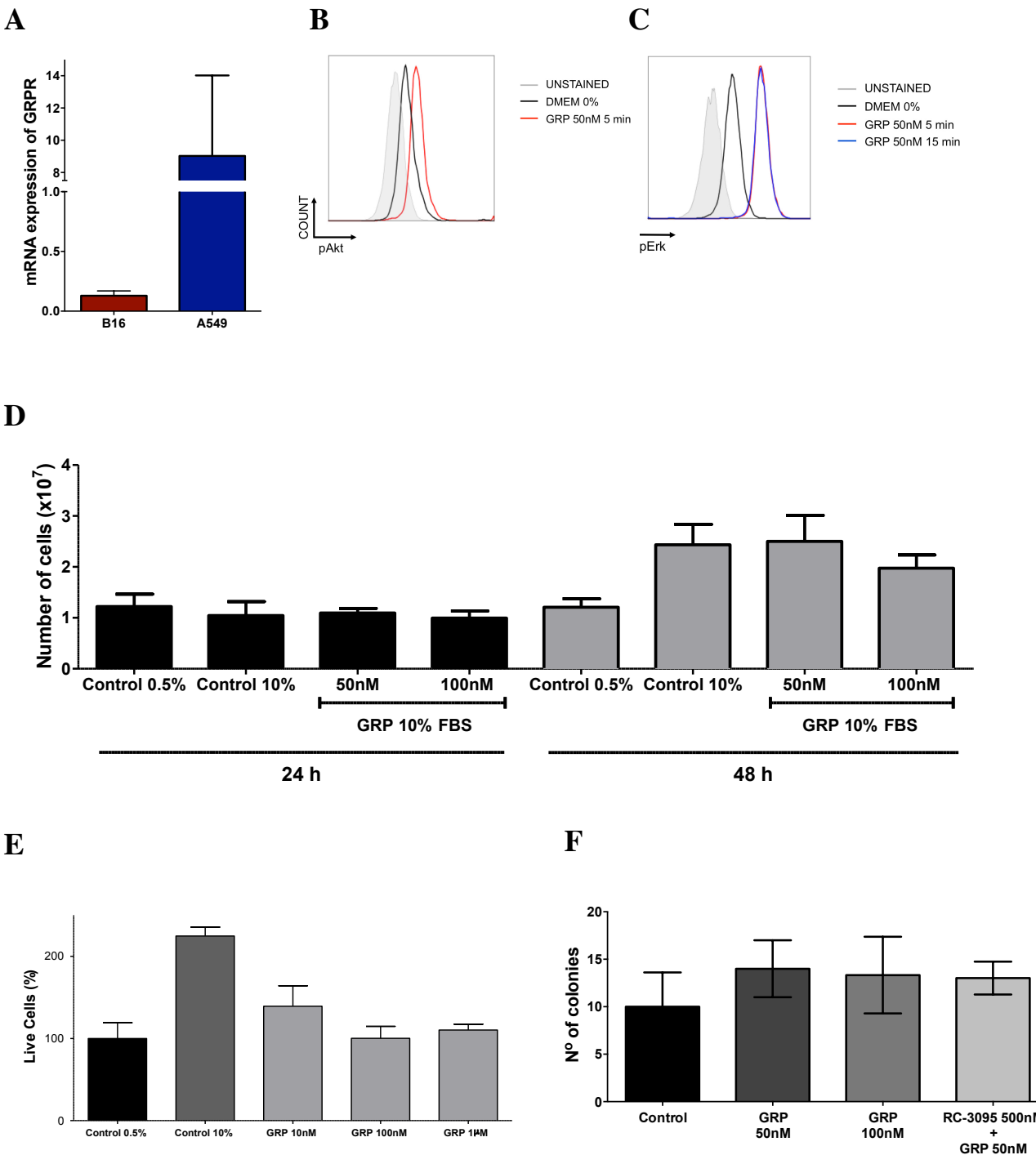

A
mRNA expression of GRPR
14
12
10
8
1.0
0.5
0.0
B16
A549
B
COUNT
pAkt
UNSTAINED
DMEM 0%
GRP 50nM 5 min
C
pErk
UNSTAINED
DMEM 0%
GRP 50nM 5 min
GRP 50nM 15 min
D
Number of cells (x10^7)
4
3
2
1
0
Control 0.5%
Control 10%
50nM
100nM
GRP 10% FBS
24 h
Control 0.5%
Control 10%
50nM
100nM
GRP 10% FBS
48 h
E
Live Cells (%)
200
100
0
Control 0.5%
Control 10%
GRP 10nM
GRP 100nM
F
Nº of colonies
20
15
10
5
0
Control
GRP 50nM
GRP 100nM
RC-3095 500nM + GRP 50nM

Figure 2

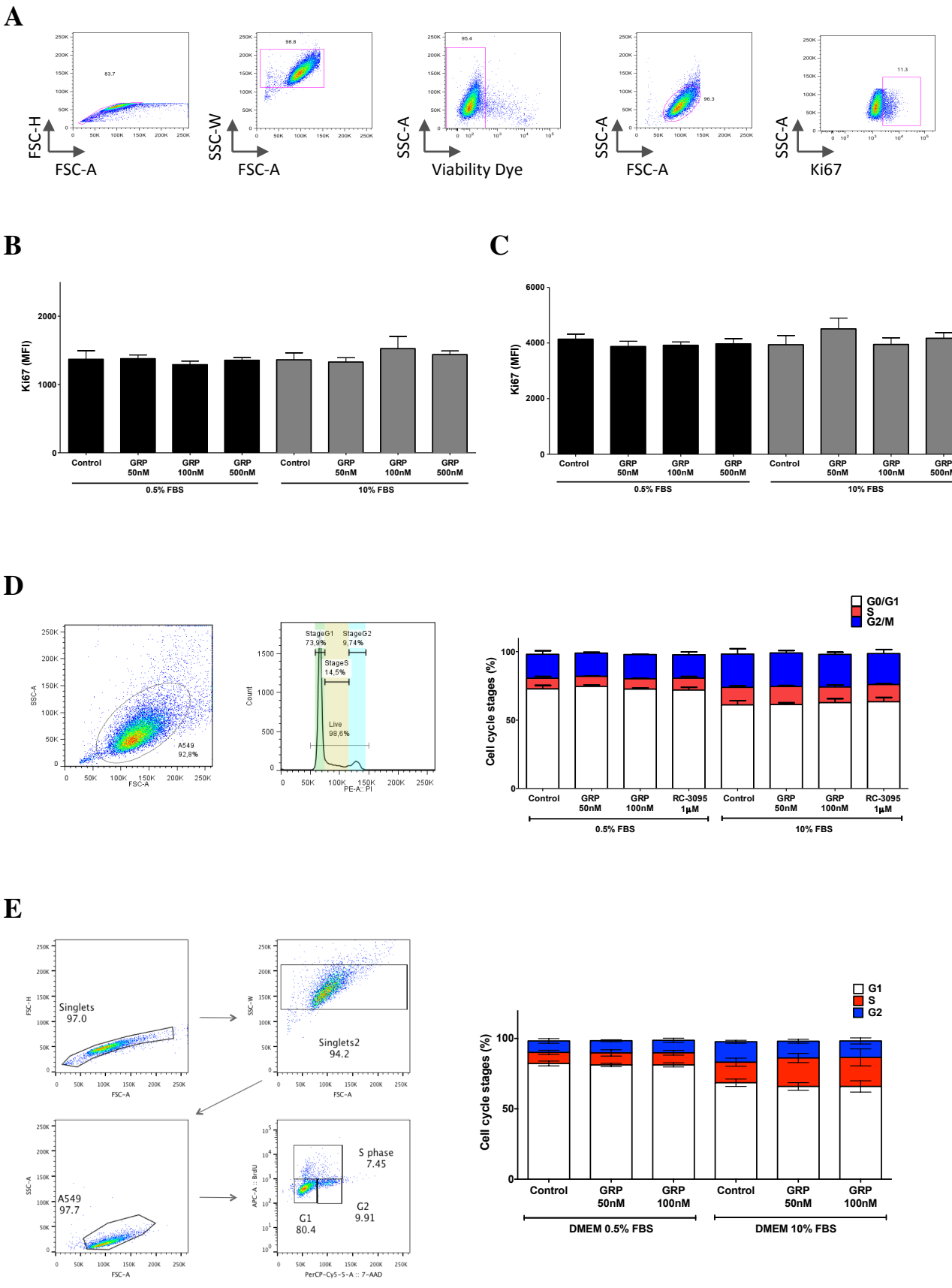

A
FSC-H
FSC-A
SSC-W
FSC-A
SSC-A
Viability Dye
SSC-A
FSC-A
SSC-A
Ki67
B
Ki67 (MFI)
Control
GRP 50nM
GRP 100nM
GRP 500nM
0.5% FBS
10% FBS
C
Ki67 (MFI)
Control
GRP 50nM
GRP 100nM
GRP 500nM
0.5% FBS
10% FBS
D
SSC-A
FSC-A
A549 92,8%
Count
PE-A :: PI
StageG1 73,9%
StageG2 9,74%
StageS 14,5%
Live 98,6%
G0/G1
S
G2/M
Cell cycle stages (%)
Control
GRP 50nM
GRP 100nM
RC-3095 1µM
0.5% FBS
10% FBS
E
FSC-H
FSC-A
Singlets 97.0
SSC-W
Singlets2 94.2
SSC-A
A549 97.7
APC-A :: BrdU
PerCP-Cy5-5-A :: 7-AAD
S phase 7.45
G2 9.91
G1 80.4
G1
S
G2
Cell cycle stages (%)
Control
GRP 50nM
GRP 100nM
DMEM 0.5% FBS
DMEM 10% FBS

Figure 3

A

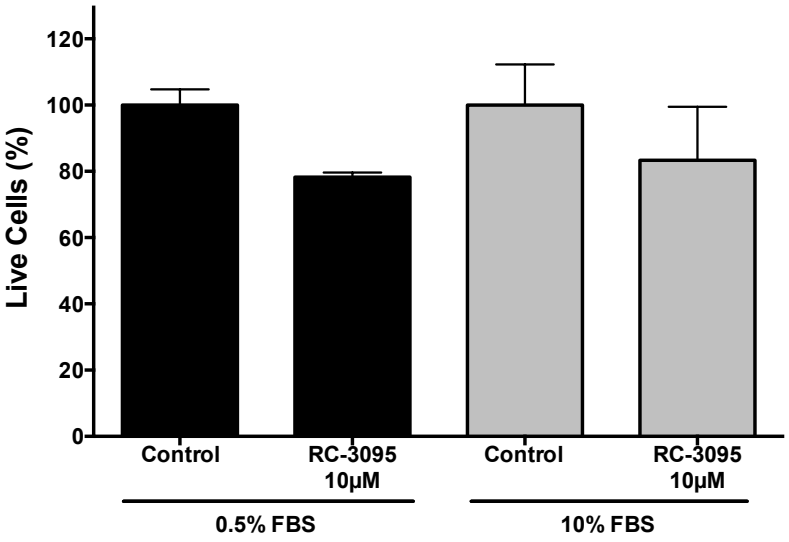

Live Cells (%)
120
100
80
60
40
20
0
Control
RC-3095
10μM
Control
RC-3095
10μM
0.5% FBS
10% FBS

Figure 4

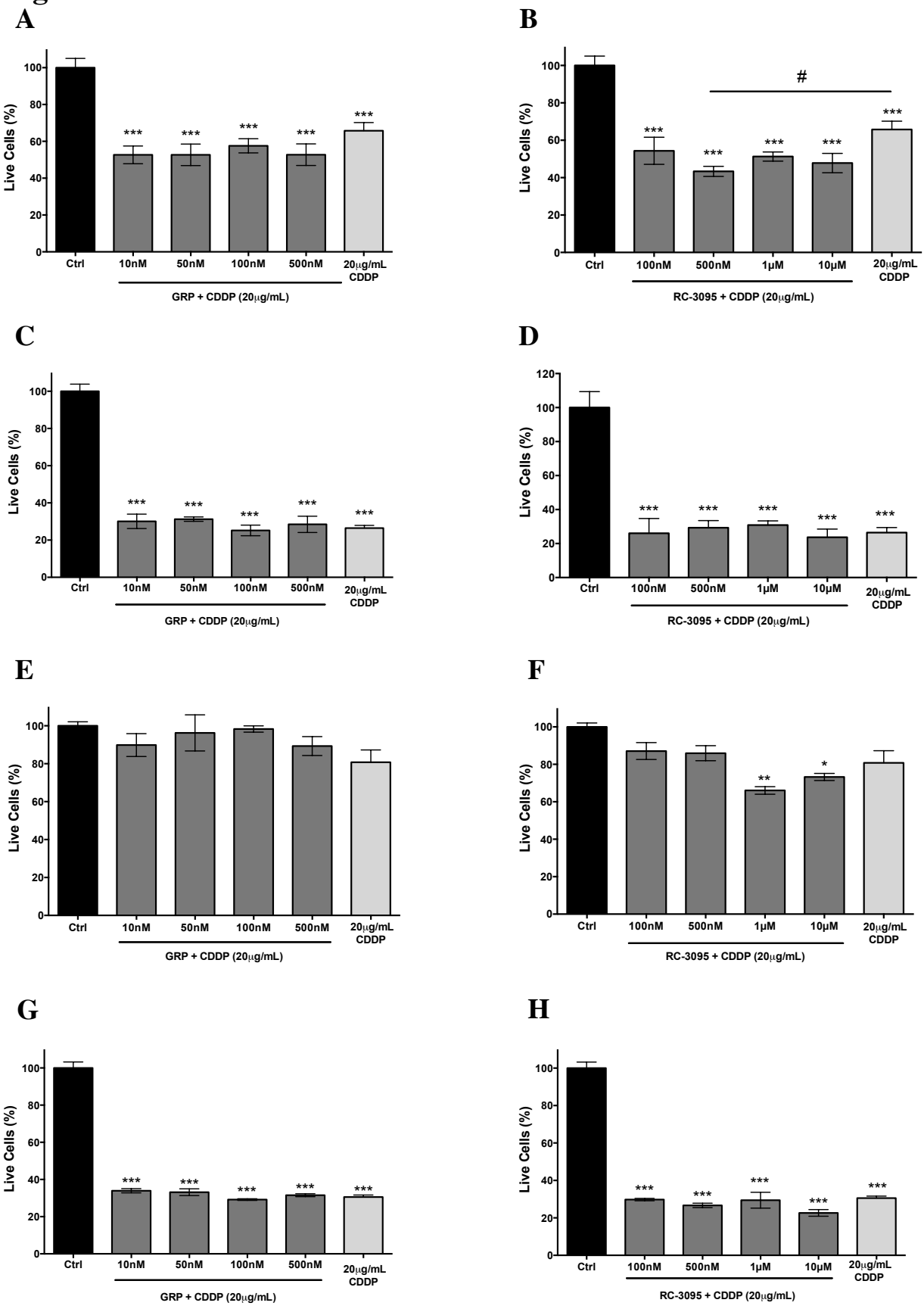
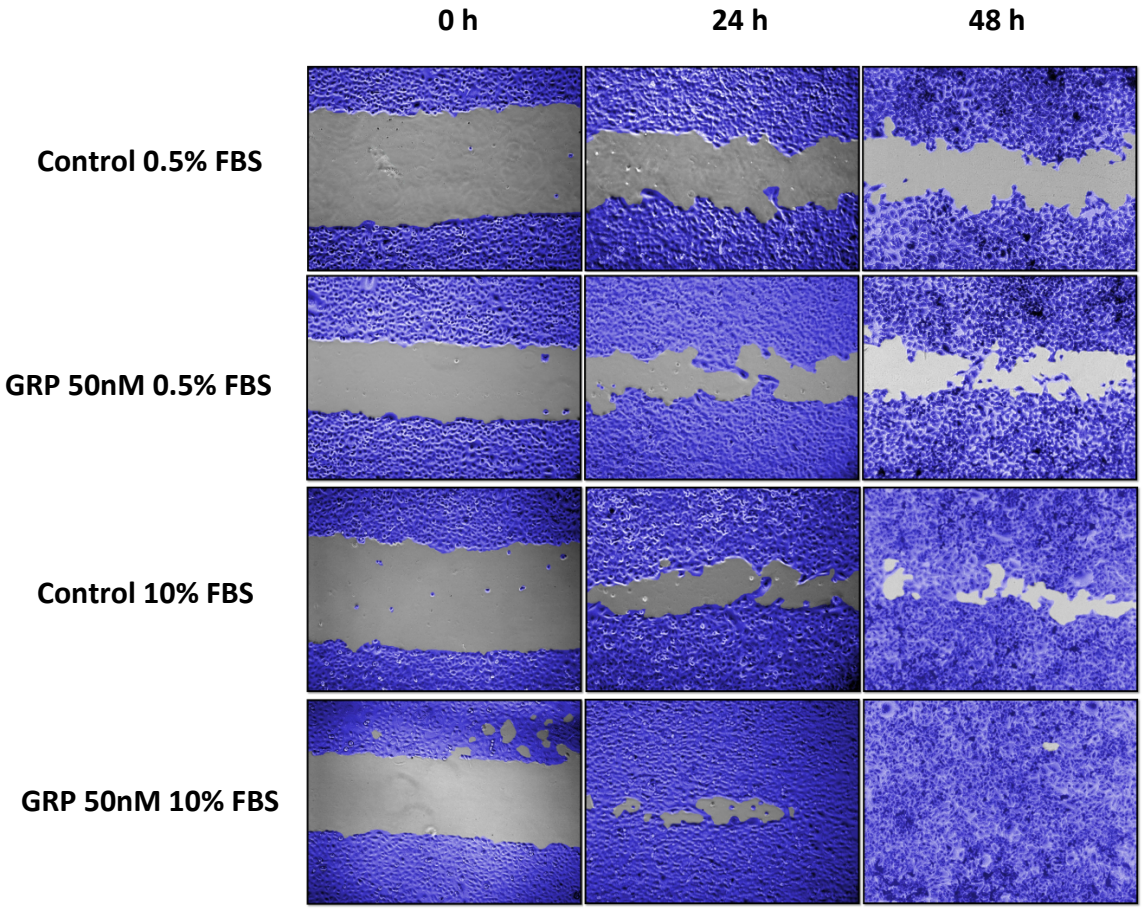
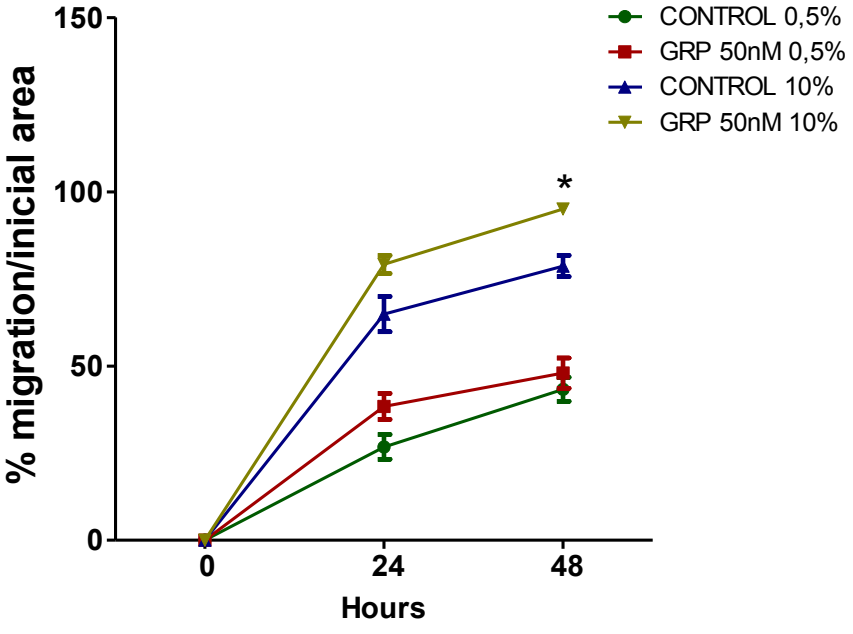

A
B
C
D
E
F
G
H
Live Cells (%)
Ctrl
10nM
50nM
100nM
500nM
100nM
500nM
1μM
10μM
20μg/mL CDDP
GRP + CDDP (20μg/mL)
RC-3095 + CDDP (20μg/mL)
***
**
*
#

Figure 5

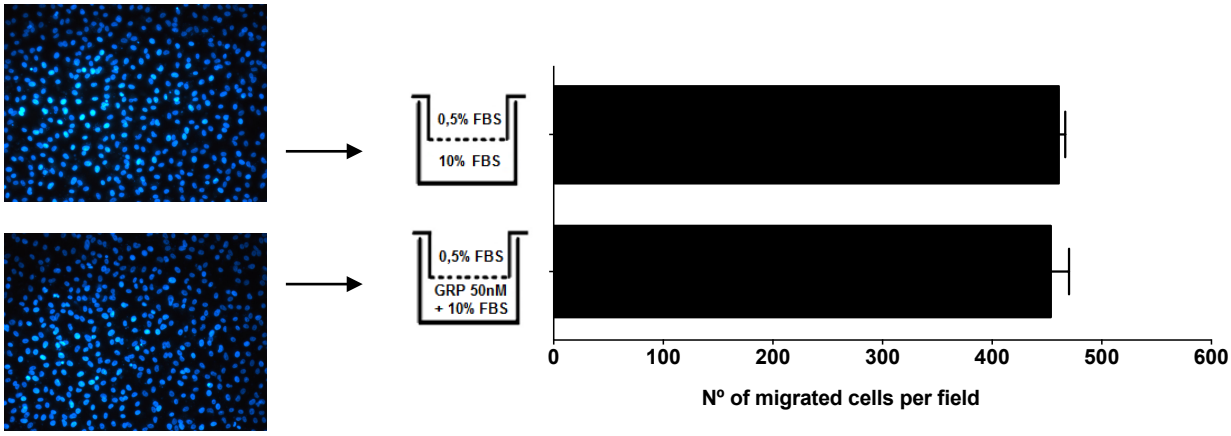
A


0 h
24 h
48 h
Control 0.5% FBS
GRP 50nM 0.5% FBS
Control 10% FBS
GRP 50nM 10% FBS

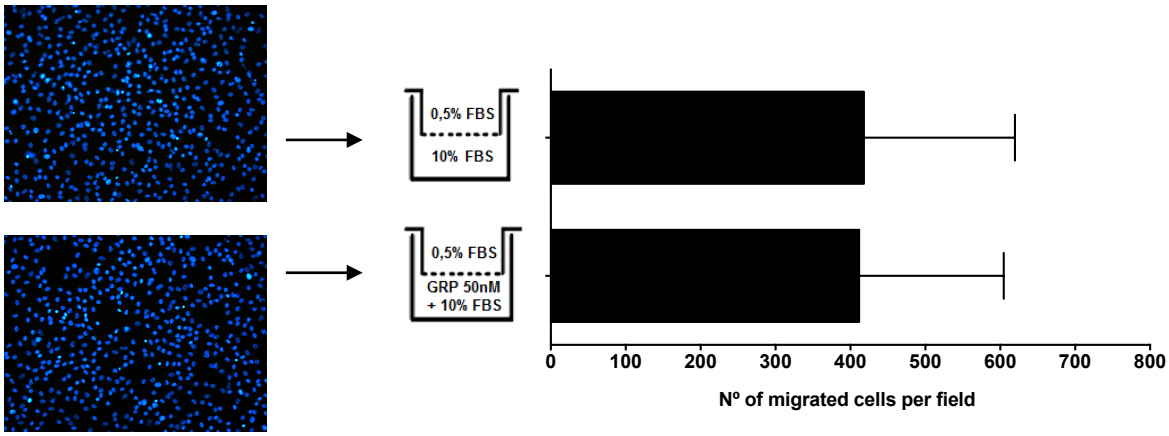
B


150
100
50
0
% migration/inicial area
0
24
48
Hours
*
CONTROL 0,5%
GRP 50nM 0,5%
CONTROL 10%
GRP 50nM 10%

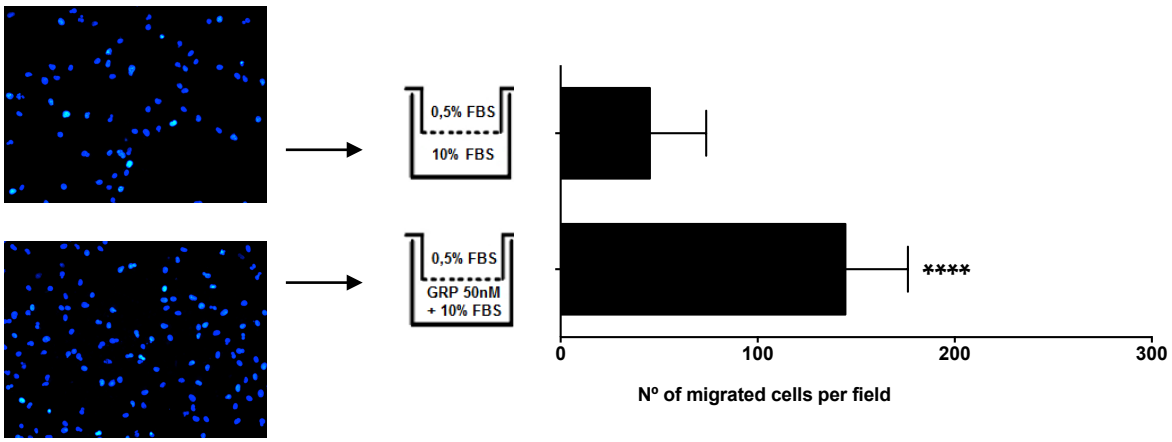
C


0,5% FBS
10% FBS
0,5% FBS
GRP 50nM
+ 10% FBS
0
100
200
300
400
500
600
Nº of migrated cells per field

D


0,5% FBS
10% FBS
0,5% FBS
GRP 50nM
+ 10% FBS
0
100
200
300
400
500
600
700
800
Nº of migrated cells per field

E


0,5% FBS
10% FBS
0,5% FBS
GRP 50nM
+ 10% FBS
****
0
100
200
300
Nº of migrated cells per field

3.1 Considerações Finais

Neste trabalho caracterizamos um novo papel da interação do GRP ao seu receptor específico, GRPR, em uma linhagem de adenocarcinoma pulmonar. Observamos que o neuropeptídeo GRP pode induzir a migração destas células em dois métodos diferentes de avaliação de migração celular (Fig.5). Vimos que o tratamento com o GRP ativa as quinases AKT e ERK1/2 (Fig.1) as quais estão envolvidas nas vias de proliferação, sobrevivência e migração celular.

Toi-Scott e colaboradores já haviam descrito que o GRPR é o receptor para o GRP mais comumente expresso em pacientes com câncer de pulmão, sendo detectado em 85% dos cânceres de pequenas e não pequenas células (TOI-SCOTT; JONES; KANE, 1996). Recentemente, a expressão de GRPR foi avaliada em uma grande população de pacientes com câncer de pulmão e os resultados demonstraram uma maior expressão nos casos de adenocarcinoma (MATTEI et al., 2014). Assim como Liu *et al* (LIU et al., 2007), nosso estudo verificou que a linhagem A549, representativa de um tumor de não pequenas células de pulmão, possui uma alta expressão de GRPR, porém utilizamos PCR de tempo real ao invés de RT-PCR (Fig.1 A).

GRP é usualmente descrito como um fator de crescimento de tumores (revisado em FLORES; LENZ; ROESLER, 2009; PATEL; SHULKES; BALDWIN, 2006). Para avaliar a proliferação celular utilizamos diversos métodos já descritos na literatura. Inicialmente, testamos diferentes concentração de GRP, incluindo aquelas já antes utilizadas em outros estudos como a de 100 nM. Também tratamos as células com duas concentrações diferentes de soro fetal bovino: 0.5% e 10%. O meio com baixa concentração de soro foi utilizado, pois estudos anteriores que adicionaram soro e GRP concomitantemente verificaram uma menor eficácia do GRP como mitógeno. Esta diminuição da eficácia do GRP na presença de soro foi explicada pela hipótese de que o ligante poderia estar sendo degradado por peptidases presentes no soro; por fatores interferindo na ligação ao GRPR ou até mesmo na sua função; ou pelo fato de que a produção endógena de GRP e/ou de outros fatores de crescimento estariam presentes e assim ativariam completamente o GRPR expresso (CARNEY et al., 1987b; NELSON et al., 1991; VANGSTED et al., 1991). No entanto, não verificamos diferença na proliferação celular nas diferentes concentrações de GRP, de soro e ensaios (Fig.1 e Fig.2).

Estes resultados são compatíveis a outros estudos os quais viram que mesmo sem a presença de soro no meio, a ativação do GRPR tem um efeito modesto e inconsistente sobre o crescimento de células tumorais, levando a hipótese de que o GRP atua como um mitógeno fraco sobre tumores (JENSEN; CARROLL; BENYA, 2001). Neste mesmo ensaio, verificamos se o antagonista RC-3095 seria capaz de diminuir a proliferação celular, como descrito na literatura (CHATZISTAMOU et al., 2001; KIARIS et al., 1999; KOPPAN et al., 1998; QIN et al., 1994). Verificamos que somente em doses altas (10 μM) o antagonista diminui a viabilidade celular em 20 % em relação ao controle (Fig. 3).

Estudos mostraram que o GRP atua na sobrevivência celular e o pré-tratamento com GRP aumenta o IC_{50} do gefitinib, que também é utilizado no tratamento deste câncer. No entanto, os autores não testaram a possibilidade de combinar os tratamentos (LIU et al., 2007). Em nosso estudo, vimos que a combinação de RC-3095 com Cisplatina apresentou uma maior perda da viabilidade celular quando comparada a estes tratamentos sozinhos (Fig.4). E este efeito foi mais significativo nas células tratadas com baixa concentração de soro (0.5%).

Dados recentes sugerem que o GRP atua primeiramente como um morfógeno e não como um mitógeno. Sabe-se que a morfogênese é um passo importante para a mobilidade celular durante o desenvolvimento da natureza invasiva de diversos tumores (GLOVER et al., 2005; LEE et al., 2012). A fim de verificar o potencial migratório do GRP sobre a linhagem A549 utilizamos dois ensaios de migração diferentes: o ensaio de *wound healing* e o de *transwell*. O ensaio de *transwell* avalia a migração celular tridimensional o que o torna mais perto do que realmente acontece *in vivo*. Já o *wound healing* mostra a migração bidirecional após a mimetização de uma injúria.

Além disso, estamos no momento realizando novos experimentos para melhor entender o mecanismo envolvido no efeito migratório do GRP nestas células. Resultados preliminares indicam que a migração induzida por GRP é dependente de GRPR e ERK1/2 através de pré-tratamento das células com antagonista e inibidor, respectivamente (Anexo Fig 2 A,B).

Além da linhagem A549, estamos no momento realizando experimentos com uma linhagem murina de melanoma (B16/F10) a fim de verificar se o GRP é também capaz de induzir a migração destas células, uma vez que elas são altamente agressivas e costumam formar metástases quando inoculadas *in vivo*. Após alguns experimentos realizados no laboratório, verificamos que o GRP atua como um fator de crescimento nesta linhagem, triplicando o número de células em relação ao controle em apenas 24 horas (Anexo Fig.1 B,

C) e o tratamento por 24 horas com RC-3095 tem efeito citotóxico nas doses e 500 e μM (Anexo Fig.1 D). Curiosamente, esta linhagem expressa níveis muito mais baixos de GRPR do que a linhagem A549 (Anexo Fig.1 A). No entanto, a expressão do GRPR parece ter um papel importante na biologia deste tumor e muito pode ser explorado ainda, através de novos ensaios como o BrdU, uma vez que pouco se sabe sobre o papel do GRP/GRPR em melanomas. Ainda, por ser uma linhagem murina podemos melhor entender o efeito desta via na progressão do tumor, assim como no infiltrado de células do sistema imune.

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5 Anexos

Supplemental Figures Legends

Fig. 1. *RC-3095 prevents the migratory effect of GRP in A549*

A-B) A549 cells were pre-treated with RC-3095 (500nM) for 30 min and stimulated with GRP (50nM) in 0.5% or 10% FBS medium were subject to wound healing assay and stained with hematoxylin (blue area). **A**) Representative images at 0 (left column), 24 (middle column) or 48 hours (right column) after treatment. Microscopy images with 200x magnification.

B) Image quantification of the wounded area size. Migration is expressed as percentage of coverage of the starting wounded area (set as 0 %). Nine pictures per sample were taken and analyzed. Data (mean+SEM) representative of three independent experiments performed in triplicates.

C) A549 cells were starved, pre-treated with RC-3095 (500nM) or PD-98059 ERK-inhibitor (300nM) for 30 min and placed in the upper side of transwell migration chambers. Medium with 10% of FBS (control) or GRP 50nM were added to the lower chamber. Cells were than allowed to migrate for 8 hours. Transwell membranes were stained with DAPI and six pictures per sample were taken. Representative microscopy images (left side, at 200x of magnification) and quantification of cell number per field (right side) of each group are shown. Data (mean+SEM) representative of one experiment performed in triplicates.

Fig. 2. *B16F10 proliferate upon GRP stimuli and is affected by RC-3095*

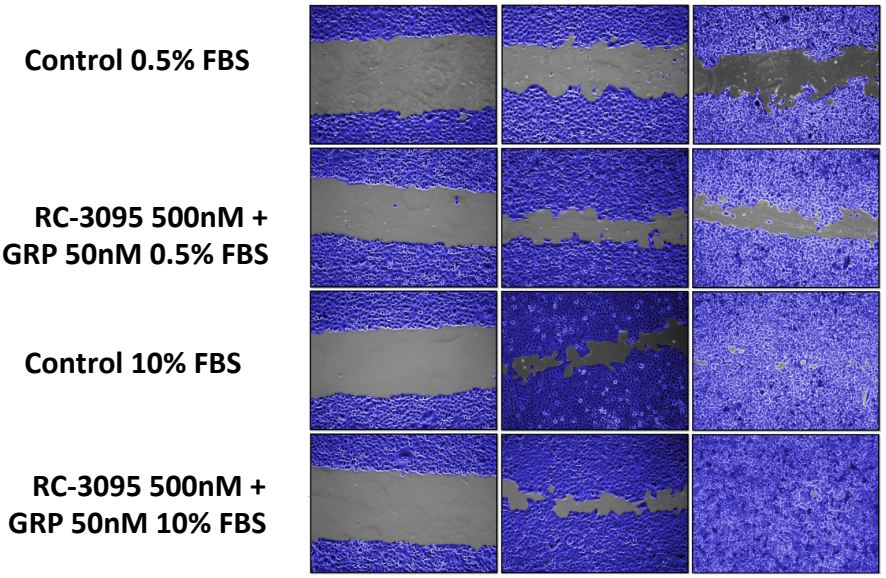
A) B16F10 cells were treated with GRP 50nM or 100 nM in 0.5% (gray bars) or 10% (black bars) of FBS medium for 24 hours. Cell count was performed using a hemocytometer and trypan blue exclusion. Total number (mean±SEM) of counted cells per group is shown. **B**) Fold over control is shown for each group of the data in **A**).

C) RC-3095 (50, 100, 250, 500nM and 1, 5 and 10μM) cytotoxic effect on B16F10 was tested by MTT assay on cells cultured in 10% of FBS medium for 24 hours. Data (mean±SEM) are representative from two independent experiments performed in quadruplicates and are expressed as percentage of an untreated control. Statistics: one-way ANOVA with Tukey post test, *** = $p<0.001$, ** $p=<0.01$ and * = $p<0.05$.

Supplemental Figures

Supplement Figure 1

A



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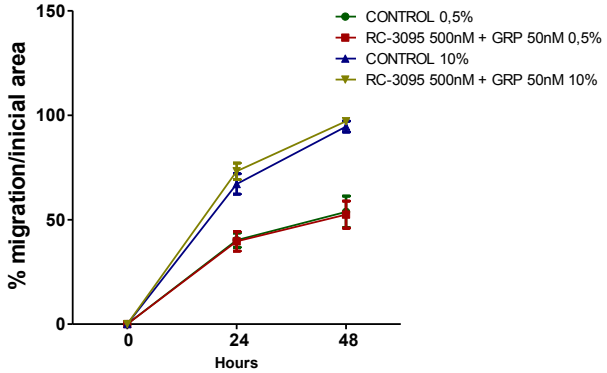

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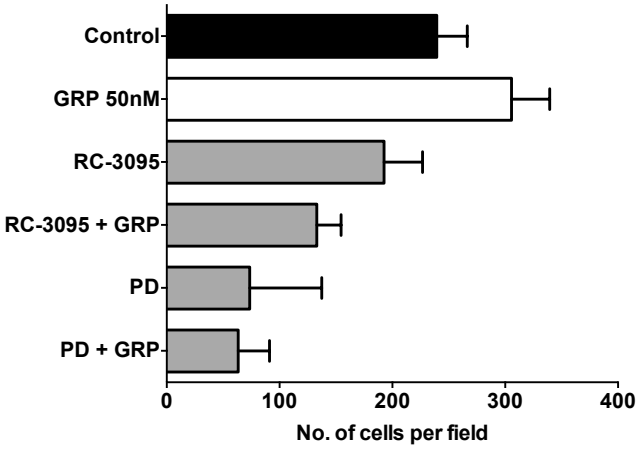
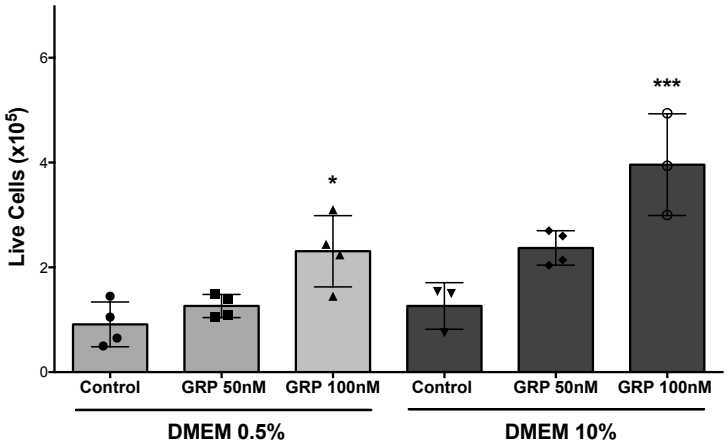


Supplement Figure 2

A



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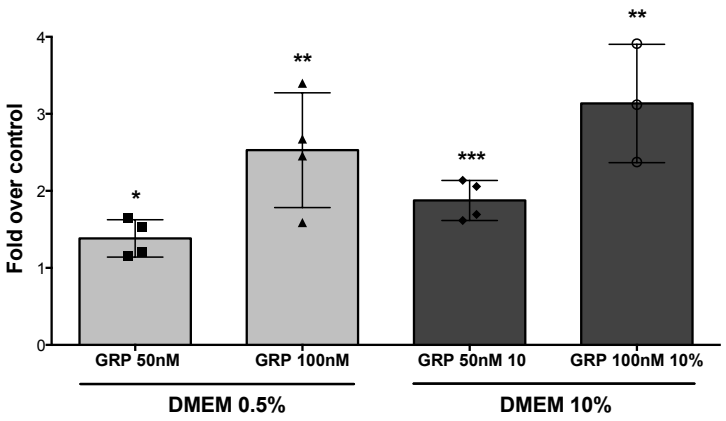

C