



UNIVERSIDADE FEDERAL DE UBERLÂNDIA
INSTITUTO DE GENÉTICA E BIOQUÍMICA
PÓS-GRADUAÇÃO EM GENÉTICA E BIOQUÍMICA

**IMPORTÂNCIA DA SINALIZAÇÃO DE ANEXINA A1 NO
ESTABELECIMENTO DA AGRESSIVIDADE DO CÂNCER DE MAMA**

Aluna: Mariana Alves Pereira Zóia

Orientador: Luiz Ricardo Goulart

Co-orientadora: Lara Vecchi

UBERLÂNDIA - MG

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Aluna: Mariana Alves Pereira Zóia

COMISSÃO EXAMINADORA

Presidente: Luiz Ricardo Goulart

Examinadores: Victor Piana de Andrade (A.C.Camargo Cancer Center)

Nadia Pereira de Castro (National Institute of Health USA)

Yara Cristina de Paiva Maia (Universidade Federal de Uberlândia)

Thaise Gonçalves de Araújo (Universidade Federal de Uberlândia)

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Luiz Ricardo Goulart

Dedico essa pesquisa aos pacientes acometidos por câncer, para que a ciência possa aumentar expectativa e qualidade de vida, melhorando o diagnóstico, prognóstico e tratamento dessa doença.

Também à integridade, moral e ética da comunidade científica para que não seja corrompida ou abalada e se mantenha indubitavelmente favorável aos interesses populacionais caso se contraponham a ambições econômicas.

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LISTA DE ABREVIATURAS (*List of abbreviations*)

Ac2-26	Peptídeo acetilado amino-terminal da ANXA1 (aa 2 ao 26)
AKT	Proteína quinase B (<i>Protein kinase B</i>)
ANXA1	Anexina A1 (<i>Annexin A1</i>)
ANXAV	Anexina V (<i>Annexin V</i>)
ATP	Adenosina Tri-fosfato (<i>Adenosine triphosphate</i>)
BC	Câncer de mama (<i>Breast cancer</i>)
BLBC	Câncer de mama basal-like (<i>Basal-like breast cancer</i>)
BSA	Soro fetal bovino (Bovin serum albumin)
CFSE	Carboxifluoresceína succinimidyl éster (<i>Carboxyfluorescein succinimidyl Ester</i>)
CI	Intervalo de confiança (<i>Confidence intervals</i>)
COX-2	Cicloxygenase 2 (<i>Cyclooxygenase-2</i>)
EGF	Fator de crescimento epidermal (<i>Epidermal growth factor</i>)
EGFR	Receptor do fator de crescimento epidermal (<i>Epidermal growth factor receptor</i>)
EMT	Transição epitélio-mesenquimal (<i>Epithelial-mesenchymal transition</i>)
ER	Receptor de estrógenos (<i>Estrogen receptor</i>)
ERK	Quinase regulada por sinal extracelular (<i>Extracellular-signal-regulated kinases</i>)
fMLF	N-Formilmetionina Leucil-Fenilalanina (<i>N-Formylmethionyl-leucyl-phenylalanine</i>)

FPR	Receptores para peptídeos formilados (<i>Formyl peptide receptors</i>)
HER2	Receptor tipo 2 do factor de crescimento epidérmico humano (<i>Human epidermal growth factor receptor 2</i>)
HPR	Enzima peroxidase (<i>Horseradish peroxidase</i>)
IDC NOS	Carcinoma ductal invasivo sem outra especificação (<i>Invasive ductal carcinoma not otherwise specified</i>)
IFN-γ	Interferon gama (<i>Interferon gamma</i>)
ILC	Carcinoma lobular invasivo (<i>Invasive lobular carcinoma</i>)
iNOS	Óxido nítrico sintetase induzível (<i>Inducible nitric oxide synthase</i>)
MAPK	Proteínas quinases ativadas por mitógenos (<i>Mitogen-activated protein kinases</i>)
MC	Carcinoma medular (<i>Medullary carcinoma</i>)
MSC	Carcinoma mucinoso (<i>Mucinous carcinoma</i>)
MTC	Carcinoma metaplásico (<i>Metaplastic carcinoma</i>)
NFAT	Fator nuclear de células T ativadas (<i>Nuclear factor of activated T-cells</i>)
NLS	Sinal de localização (<i>Nuclear localization signal</i>)
ON	<i>Overnight</i>
OR	<i>Odds Ratio</i>
PI3K	Fosfatidilinositol-3-quinase (<i>Phosphoinositide 3-kinase</i>)
PLA2	Fosfolipase A2 (<i>Phospholipase A2</i>)
PR	Receptor de progesterona (<i>Progesterone receptor</i>)
RT	Temperatura ambiente (<i>Room temperature</i>)
SD	Desvio-padrão (<i>Standart deviation</i>)

TC	Carcinoma tubular (<i>Tubular carcinoma</i>)
TMA	Microarranjos teciduais em matriz (<i>Tissue microarrays</i>)
TNM	Tumor/Metástase/Nódulos linfáticos (<i>Tumor/Metastasis/Lymph nodes</i>)
TNBC	Câncer de mama triplo-negativo (<i>Triple-negative breast cancer</i>)

APRESENTAÇÃO (*Geral introduction*)

Século XXI. Século da genética e do DNA. Nunca a biologia molecular esteve tão elucidada. Ao mesmo tempo, paradoxalmente, o câncer, uma doença investigada pelos mais modernos centros de tecnologia, é o segundo maior responsável pela morte da população mundial.

Para leigos, "a cura do câncer" deveria ser alcançada a partir de um *insight*, mas talvez essa seja uma das expressões menos utilizadas na comunidade científica. O motivo é que a carcinogênese, dotada de extrema heterogeneidade, não requer uma única solução, mas sim diversas "curas" personalizadas e direcionadas a específicos tipos tumorais. Assim, a missão dos pesquisadores é investigar melhores diagnósticos e tratamentos para tipos característicos de câncer, esclarecendo mecanismos pelos quais são capazes de romper a homeostasia do organismo e, a partir disso, traçar estratégias que reduzam a angiogênese e o potencial de crescimento, migração e invasão dessas células.

Diante dessa perspectiva, trabalhamos aqui a favor da ampliação do conhecimento do câncer de mama: a neoplasia mais comum entre as mulheres em todo o mundo. Atualmente, vivemos em cenários contraditórios: de um lado, a prática clínica é considerada evoluída no que diz respeito à cirurgias menos invasivas e predição da chance de surgimento do câncer de mama através de exames genéticos; porém, por outro lado, a situação é arcaica quando deparamo-nos com o fato de que os fatores prognósticos atuais não são capazes de prever a evolução da maioria das pacientes e marcadores preditivos tumorais não evitam que muitas sejam submetidas a tratamentos desnecessários. Assim, o objetivo desse trabalho foi investigar uma proteína que carrega em si o potencial de agir como fator prognóstico e/ou alvo terapêutico para o estabelecimento do fenótipo agressivo do câncer de mama: a Anexina A1.

Para melhor elucidação do nosso estudo, essa dissertação está dividida em dois capítulos: a fundamentação teórica, ou capítulo I, que objetiva expor ao leitor a base conceitual para o entendimento dessa pesquisa

com posterior esclarecimento da mesma, apresentada detalhadamente no capítulo II.

Por fim, é importante saber que esse trabalho se finalizou com a principal descoberta de que as células triplo-negativas do câncer de mama possuem vias de sinalização autócrinas constitutivas responsáveis por criar em si uma maior independência frente a estímulos corporais. Portanto, a maior contribuição dessa pesquisa para a comunidade científica é a proposta de inibição de Anexina A1 como alvo terapêutico para o câncer de mama triplo-negativo, que até hoje carece de terapia específica.

Mariana Alves Pereira Zóia

CAPÍTULO I

Fundamentação teórica

(Fundamental theories)

1. Câncer: conceito e epidemiologia (*Cancer: concepts and epidemiology*)

O câncer é definido como um grande grupo de doenças caracterizado por crescimento rápido não habitual de células anormais que carregam em si o potencial de gerarem metástases e invadirem outros órgãos corporais, descontrolando o funcionamento normal do organismo (World Health Organization, 2015). Nas últimas décadas o câncer se tornou um problema de saúde pública mundial, sendo que no atual cenário brasileiro há a transição epidemiológica de doenças populacionais mais incidentes, onde doenças parasitárias e infecciosas dão lugar a doenças crônico-degenerativas, tais como cardiológicas e neoplásicas (Schramm *et al.*, 2004). Portanto, melhorar a eficácia do diagnóstico e tratamento de câncer tem se tornado tanto prioridade econômica mundial (Rodolfo Saracci, 2015) quanto nacional, haja vista que o Ministério da Saúde incluiu, entre os 16 objetivos estratégicos no período 2011-2015, ações de controle do câncer enfatizando diagnóstico e tratamento dos cânceres de mama e do colo do útero (Ministério Da Saúde, 2011).

Estimativas nacionais do ano de 2014, válidas também para 2015, apontam a ocorrência em uma população de 206 milhões de habitantes (Countrymeters Brazil, 2015) de cerca de 580 mil casos novos de câncer, sendo majoritariamente do tipo pele não melanoma (182 mil casos), próstata (69 mil), mama feminina (57 mil), cólon e reto (33 mil), pulmão (27 mil), estômago (20 mil) e colo do útero (15 mil) (Instituto Nacional Do Câncer, 2014). Além de ser no Brasil o terceiro tipo mais incidente, acometendo 20,8% da população feminina (Figura 1), o câncer de mama é, mundialmente, o câncer mais comum entre as mulheres (Instituto Nacional Do Câncer, 2015). Assim, torna-se claro a importância do aprofundamento no estudo sobre essa doença para que a comunidade científica possa estabelecer pesquisas direcionadas à modificação positiva desse cenário.



Localização primária	casos	%	Homens	Mulheres	Localização primária	casos	%
Próstata	68.800	22,8%			Mama Feminina	57.120	20,8%
Traqueia, Brônquio e Pulmão	16.400	5,4%			Cólon e Reto	17.530	6,4%
Cólon e Reto	15.070	5,0%			Colo do Útero	15.590	5,7%
Estômago	12.870	4,3%			Traqueia, Brônquio e Pulmão	10.930	4,0%
Cavidade Oral	11.280	3,7%			Glândula Tireoide	8.050	2,9%
Esôfago	8.010	2,6%			Estômago	7.520	2,7%
Laringe	6.870	2,3%			Corpo do Útero	5.900	2,2%
Bexiga	6.750	2,2%			Ovário	5.680	2,1%
Leucemias	5.050	1,7%			Linfoma não Hodgkin	4.850	1,8%
Sistema Nervoso Central	4.960	1,6%			Leucemias	4.320	1,6%

Figura 1. Distribuição proporcional dos dez tipos de câncer mais incidentes em 2014 por sexo no Brasil, exceto pele não melanoma (Instituto Nacional Do Câncer, 2014)

2. Glândula mamária: anatomia e histologia (*Mammary gland: anatomy and histology*)

A base do estudo do câncer de mama compreende o conhecimento das estruturas anatômicas e histológicas da glândula mamária (Figuras 2 e 3). Essa glândula, considerada parte do sistema reprodutor humano, possui função básica de produzir e secretar leite para amamentação da progênie durante o início da vida. É composta em número par e se localiza simetricamente de cada lado do tórax, estendendo-se na vertical da 2^a a 6^a costela e na transversal da margem do externo à linha médio-axilar.

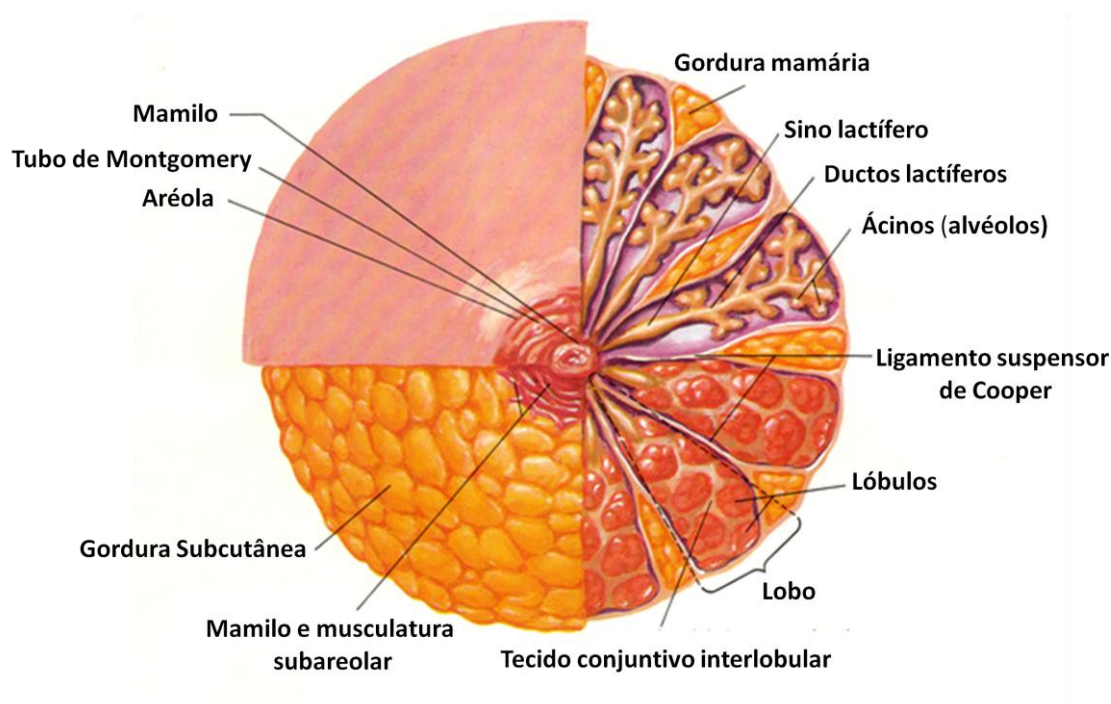


Figura 2. Cortes axiais da mama. Adaptado. (Human Body Anatomy, 2015)

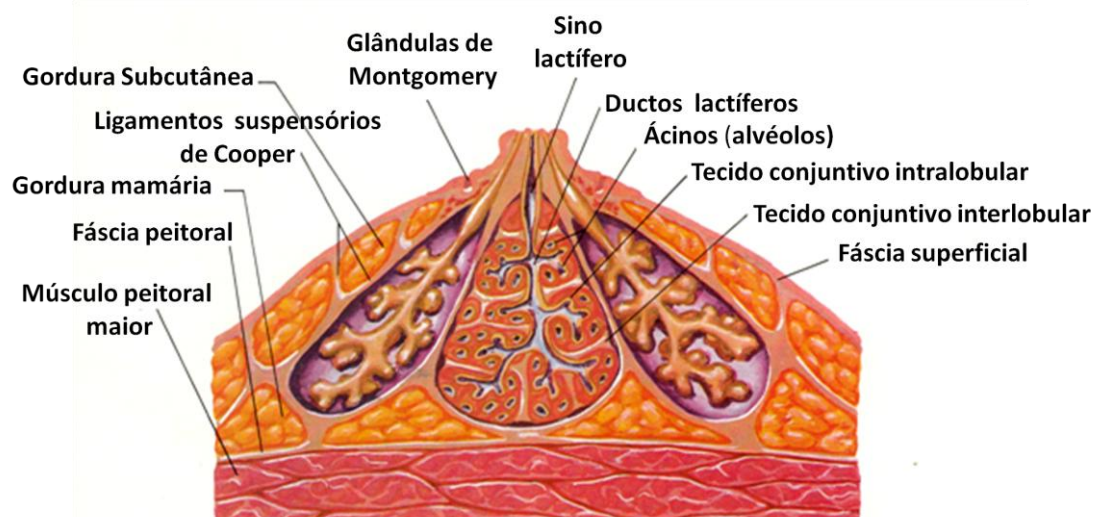


Figura 3. Corte sagital da mama. Adaptado. (Human Body Anatomy, 2015)

Partindo de uma visão geral, pode-se afirmar que a mama é formada basicamente por três tipos de tecidos: o tecido glandular (parênquima), tecido adiposo e tecido fibroso (estroma).

O tecido glandular é constituído por lobos que se subdividem em lóbulos e estes, por sua vez, são compostos pelos alvéolos. Cada lóbulo, denominado unidade funcional da glândula mamária, possui um respectivo ducto lactífero, que tem como função conduzir o leite até um ducto maior, denominado sino lactífero, por onde é armazenado e, sob sucção ou mediante estímulo mecânico, é liberado no mamilo. O mamilo e a aréola são constituídos por glândulas sebáceas hipertrofiadas, denominadas glândulas de Montgomery, que protegem a epiderme deste local por meio da formação de uma camada hidrolipídica superficial (Ramsay *et al.*, 2005).

O tecido adiposo, composto tanto pela gordura mamária quanto pela gordura subcutânea, reveste toda a glândula mamária formando um envoltório de proteção. O tecido conjuntivo fibroso ajuda na sustentação de lobos pelos ligamentos suspensórios de Cooper que estão presentes por toda a fáscia superficial do músculo peitoral maior (Ane Agur, 1999). Esse tecido está presente tanto dentro dos lóbulos (denominado tecido conjuntivo intralobular) quanto entre lóbulos e ductos lactíferos (tecido conjuntivo interlobular).

No âmbito citológico, a Figura 4 ilustra claramente a estrutura do epitélio que reveste os alvéolos mamários. Nota-se que externamente há a presença da membrana basal a qual sustenta a camada mioepitelial e, durante o período feminino de gravidez, é a responsável por sofrer contrações estimuladas por impulsos succionais por meio do hormônio ocitocina. Internamente, em contato com o ducto lactífero, há a camada epitelial responsável pela produção do leite materno (Sopel, 2010). É importante esclarecer que apesar de haver expressão diferenciada de citoqueratinas e outras moléculas entre as células mioepiteliais e epiteliais, elas são clones que surgiram a partir de uma única célula-tronco mamária (Stingl *et al.*, 2005).

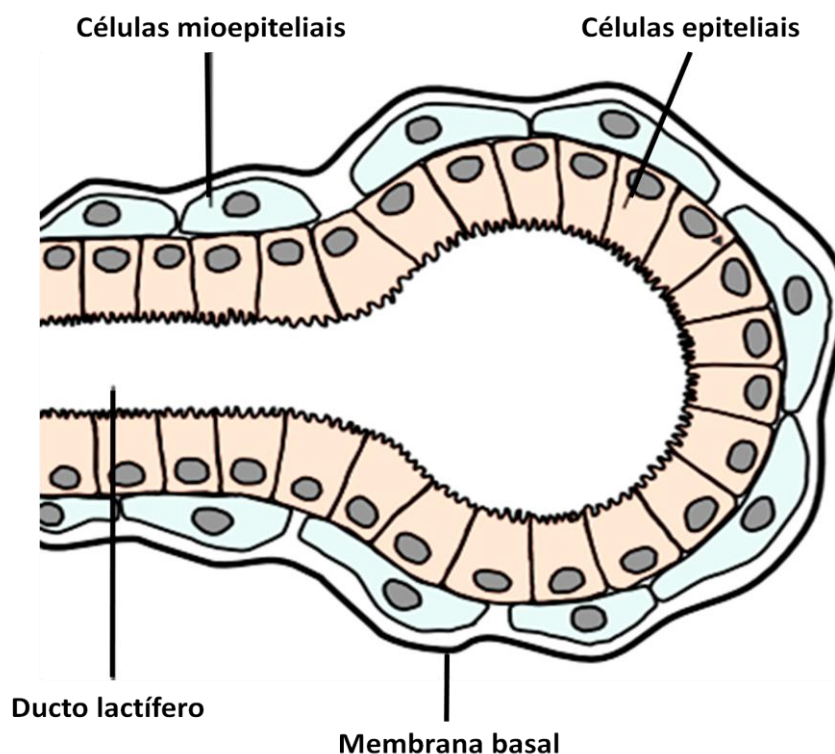


Figura 4. Visão citológica dos alvéolos da glândula mamária. Adaptado. (Radisky *et al.*, 2003)

3. Câncer de mama: classificação (*Breast cancer: classification*)

Considerando a heterogeneidade do carcinoma mamário, diagnósticos, prognósticos e definições terapêuticas confiáveis das pacientes são baseados em classificações detalhadas e rigorosas do câncer de mama. Apesar de

haver classificações já estabelecidas na prática clínica, sabe-se que os parâmetros para distinção dos diferentes tipos tumorais é alvo de inúmeras pesquisas visto a extrema importância disso em tratamentos cada vez mais específicos e personalizados.

Atualmente, as principais classificações do câncer de mama (Fattaneh Tavassoli, 2003) vão desde a classificação por tamanho, comprometimento linfático e metástases (classificação TNM); passando por classificação histológica, patológica e por fim, chegando à classificação mais atual e foco da pesquisa de nosso grupo: a classificação molecular.

3.1 Classificação TNM (*TNM classification*)

O método de estadiamento TNM é baseado em características tumorais no momento do diagnóstico que compreendem o tamanho do tumor primário (T), o comprometimento de nódulos linfáticos (N) e a presença ou ausência de metástases (M). Juntos, esses três parâmetros definem estágios tumorais que variam de I a IV (Tabelas 1 e 2) e são fortes indicadores de sobrevivência do paciente em um período de 5 anos.

Tabela 1. Critérios para classificação TNM

Tamanho do tumor	Comprometimento linfonodal	Metástase
T1 (≤ 2 cm)	N0 (sem comprometimento)	M0 (ausência)
T2 (2-5 cm)	N1 (1-3 linfonodos)	M1 (presença)
T3 (≥ 5 cm)	N2 (4 ou mais linfonodos)	

Tabela 2. Estágios tumorais a partir da classificação TNM

Estágio I (T1N0M0)	Estágio II A (T0N1M0)	Estágio II A (T1N1M0)	Estágio II A (T2N0M0)	Estágio III B (T2N1M0)
Estágio II B (T3N0M0)	Estágio III A (T1N2M0)	Estágio III A (T1N2M0)	Estágio III A (T2N2M0)	Estágio III A (T3N1M0)
Estágio III A (T3N2M0)	Estágio III B (T4N0M0)	Estágio III B (T4N1M0)	Estágio III B (T4N2M0)	Estágio III C (T2N3M0)
Estágio III C (T1N3M0)	Estágio III C (T2N3M0)	Estágio III C (T3N3M0)	Estágio III C (T4N3M0)	Estágio IV (Metastático)

Pesquisas afirmam que em torno de 90% dos pacientes pertencentes ao estágio I sobreviverão ao menos cinco anos após o diagnóstico. Para

pacientes pertencentes aos estágios II e III, a taxa de sobrevida no mesmo período de tempo é de 60 a 80% e 40 a 50%, respectivamente. Por fim, taxas inferiores a 5% de sobrevida em 10 anos acometem doentes classificados no estágio mais grave, o estágio IV (Olivotto *et al.*, 2003).

3.2 Classificação histológica (*Histologic classification*)

A graduação histológica do câncer de mama (Bloom e Richardson, 1957; Elston e Ellis, 1991) é um bom fator de prognóstico para os pacientes. De acordo esse método, instaurado em 1957 por Bloom e modificado em 1991 por Elston e Ellis, o grau de agressividade do tumor é calculado de acordo com a Tabela 3 a partir de atributos morfológicos como formação tubular, pleomorfismo nuclear e contagem mitótica das células cancerígenas. A cada parâmetro é dada uma nota que varia de um a três e, ao final da análise, a soma das notas distingue o tumor entre bem diferenciado, moderadamente diferenciado e pouco diferenciado (Tabela 4). Conceitualmente, sabe-se que tumores pouco diferenciados tendem a ser mais agressivos que tumores bem diferenciados.

Tabela 3. Parâmetros que definem a classificação histológica do câncer de mama (*10 campos ópticos de maior aumento)

Formação tubular	Escala
Maioria do tumor (>75%)	1
Grau intermediário (10 - 75%)	2
Pouca ou nenhuma (0 - 10%)	3
Pleomorfismo nuclear	
Núcleo pequeno, regular, uniforme	1
Moderado aumento no tamanho	2
Pleomorfismo acentuado	3
Contagem mitótica	
Índice baixo (0-10 figuras avaliadas em 10 CMA*)	1
Índice moderado (10-20 figuras avaliadas em 10 CMA*)	2
Índice alto (mais de 20 figuras avaliadas em 10 CMA*)	3

Tabela 4. Pontuação que define o grau tumoral de acordo com a classificação histológica do câncer de mama

Pontuação total	Grau
3 - 5 pontos	1 - Bem diferenciado
6 ou 7 pontos	2 - Moderadamente diferenciado
8 ou 9 pontos	3 - Pouco diferenciado

3.3 Classificação patológica (*Pathologic classification*)

A morfologia celular e o padrão de crescimento tumoral são os principais parâmetros que definem a classificação patológica do câncer de mama. Para o entendimento dessa classificação, deve-se ter claro que a maior parte dos tumores mamários é classificada como carcinoma pelo fato de surgir nas células epiteliais dos lóbulos que compõem a glândula mamária.

De acordo com essa classificação, o maior grupo dos carcinomas da mama corresponde a um grupo heterogêneo de tumores sem histologia específica que é diagnosticado por critério de exclusão: o carcinoma ductal invasivo (IDC) sem outra especificação (*not otherwise specified*, NOS).

Os demais subtipos patológicos incluem carcinoma lobular invasivo (ILC), carcinoma medular (MC), carcinoma mucinoso (MSC), carcinoma tubular (TC) e carcinoma metaplásico (MTC) (Fattaneh Tavassoli, 2003).

Logicamente, como o IDC NOS é diagnosticado por critério de exclusão, esse subtipo compreende uma grande variabilidade histológica e é o grupo que engloba a maior porcentagem dos casos, atingindo 75 - 80% (Fattaneh Tavassoli, 2003).

O segundo carcinoma mais incidente, com frequência que varia entre 2 e 15% (Fattaneh Tavassoli, 2003), é o ILC que se caracteriza por um padrão clássico de pequenas células redondas, com baixo grau nuclear, que crescem em "fila indiana" através de um estroma fibroso (Varga e Mallon, 2008).

Acometendo menos de 1% das pacientes com câncer de mama (Fattaneh Tavassoli, 2003), as células volumosas e com citoplasma abundante que compõem o MC possuem baixa diferenciação, núcleo com um ou mais nucléolos, padrão bem circunscrito de crescimento sincicial e um característico estroma escasso (Rakha *et al.*, 2009).

O MSC acomete cerca de 2% de todos os carcinomas da mama (Fattaneh Tavassoli, 2003) e caracteriza-se pela proliferação de células no

muco extracelular. Aparenta ser uma massa palpável com limite definido e pode mimetizar um tumor benigno (Weigelt *et al.*, 2009).

Um dos melhores prognósticos do câncer de mama é designado TC. É uma neoplasia rara que acomete 1 a 3% dos casos (Fattaneh Tavassoli, 2003). Se caracteriza por uma micro-lesão não palpável que varia entre 2mm a 1,5cm e possui ausência de necrose e/ou poucas mitoses associado a baixo índice de pleomorfismo nuclear (Volders e Van Steen, 2001).

Alterações metaplásicas variam desde focos microscópicos a substituição completa do epitélio, derivando em MTC quando acometem as células do câncer de mama. O MTC, pela própria definição de metaplasia, é um grupo heterogêneo de células tumorais que representa menos de 1% dos carcinomas mamários invasivos (Fattaneh Tavassoli, 2003) e no qual podem coexistir áreas de diferenciação escamosa, fusiforme ou mesenquimatosa (Okada *et al.*, 2010).

3.4 Classificação molecular (*Molecular classification*)

No ano 2000 novos horizontes se abriram para a classificação do câncer de mama quando Charles Perou e colaboradores publicaram um artigo na revista *Nature* contendo uma análise de milhares de genes contidos em diversos casos dessa neoplasia. Com isso, descobriram que determinados tipos de células possuíam padrões distintos e característicos de expressão gênica e concluíram que poderiam subdividir o câncer de mama de acordo com suas “assinaturas moleculares”.

Foi assim que o câncer de mama se subdividiu primeiramente em quatro tipos: luminal, basal-like, HER2 e normal-like. Segundo essa classificação, caso as células tumorais apresentassem expressão gênica semelhante às células epiteliais lumbais da glândula mamária, o câncer era tido como luminal. Seguindo o mesmo raciocínio, se a expressão gênica fosse semelhante às células basais da mama, o câncer era classificado como basal-like. Células que superexpressavam o gene do Fator de crescimento epidérmico humano tipo 2 (HER2) compunham o subtipo HER2. Por fim, caso as células apresentassem poucas alterações no perfil de expressão gênica

ocasionando em dificuldade de interpretação o câncer era designado como normal-like (Perou *et al.*, 2000).

Em 2011 ocorreu o *12th St. Gallen International Breast Cancer Conference Expert Panel* onde foi proposta uma classificação molecular de acordo com definições clínico-patológicas. Assim, a classificação simplificada do câncer de mama passou a utilizar como pilar três principais moléculas receptoras celulares: o receptor de estrógeno (ER), o receptor de progesterona (PR) e o HER-2 para identificar os subtipos tumorais (Goldhirsch *et al.*, 2011). Obviamente, é importante salientar que a perfeita sobreposição dos subtipos caracterizados em 2000 e 2011 não é perfeita uma vez que sabe-se que a expressão gênica está constantemente sujeita a regulações pós-traducionais fazendo com que perfis de expressão gênica e protéica possam ser distintos.

Apesar de tudo, mesmo com todas as limitações, chegou-se a uma classificação molecular padrão que é muito utilizada na prática clínica uma vez que os subtipos moleculares podem ser associados a tratamentos específicos (Tabela 5). Assim, a pesquisa científica caminha no rumo de refinar cada vez mais essa classificação buscando moléculas que possam ser marcadores moleculares do câncer de mama, como por exemplo as citoqueratinas, a proteína p63, Receptor do fator de crescimento epidérmico humano (EGFR) (Badve *et al.*, 2011) e ki67 (Cheang *et al.*, 2009).

Tabela 5. Classificação molecular do câncer de mama com perfis de expressão gênica e tratamentos indicados.

Subtipo	Expressão gênica	Tratamento
Luminal A	ER +	Hormonioterapia
	PR +/-	+ Cirurgia
	HER2 -	+/- Radioterapia +/- Quimioterapia
Luminal B		Hormonioterapia
	ER +	+ Cirurgia
	PR +/-	+/- Radioterapia
HER 2	HER2 +	+/- Quimioterapia
	ER -	Trastuzumab + Cirurgia
	PR -	+/- Radioterapia
Basal-like	HER2 +	+/- Quimioterapia
		Carece de tratamento específico
	ER -	+ Cirurgia
	PR -	+/- Radioterapia
	HER2 -	+/- Quimioterapia

A Tabela 5 mostra uma complexa questão: o câncer de mama triplo negativo não possui terapia direcionada disponível e, conseqüentemente, como já abordado, possui menor taxa de sobrevivência. Nota-se, portanto, a necessidade de pesquisas na identificação de marcadores biológicos prognósticos dessa doença que possam ser alvos para possível terapia. Com base nisso, esse trabalho tem como abordagem principal explorar ao máximo as diversas abordagens de uma proteína que carrega em si o potencial de agir como fator prognóstico e/ou alvo terapêutico para o câncer de mama triplo negativo: a Anexina A1 (ANXA1).

4. Anexina A1 (*Annexin A1*)

O entendimento e elucidação da estrutura e função da ANXA1 passa pela caracterização da superfamília das Anexinas. Por definição, uma proteína denominada Anexina deve cumprir dois critérios: primeiramente, deve ser capaz de se ligar a fosfolipídios carregados negativamente de maneira dependente de Ca^{2+} e depois deve conter um elemento estrutural evolutivamente conservado composto por 70 a 80 aminoácidos.

Estruturalmente, como representado na Figura 5, as Anexinas são compostas por dois domínios: a extremidade C-terminal que contém quatro repetições de uma seqüência conservada de 70 a 80 aminoácidos e constitui a estrutura de ligação a Ca^{2+} , fosfolipídios e adenosina tri-fosfato (ATP) (Raynal e Pollard, 1994) e o domínio N-terminal, que varia em tamanho e composição de aminoácidos pois é distinto e responsável pelas propriedades específicas de cada membro da família. Em razão disso, o N-terminal se caracteriza pelas modificações pós-traducionais das Anexinas englobando fosforilação, glicosilação, ação de peptidases e clivagem proteolítica seletiva (Kim *et al.*, 2001; Rosengarth *et al.*, 2001).



Figura 5. Representação esquemática das anexinas. Adaptado. (Rescher e Gerke, 2004)

Tendo em vista a afinidade dessas proteínas a fosfolipídios, as Anexinas podem atuar como pontes de ligação entre membranas e membrana e citoesqueleto. Algumas outras atividades já descritas para as Anexinas incluem participação em mecanismos de anticoagulação, endocitose e exocitose, formação de citoesqueleto e participação em transdução de sinais (Pepinsky *et al.*, 1988; Rescher e Gerke, 2004).

A ANXA1 foi o primeiro membro caracterizado da superfamília das Anexinas sendo descrita em 1979 pelos pesquisadores Flower e Blackwell. Originalmente foi conhecida como macrocortina (Blackwell *et al.*, 1980), renocortina (Rothhut *et al.*, 1983), lipomodulina (Hirata *et al.*, 1981) e lipocortina antes de ser nomeada como ANXA1, uma proteína de 37KDa codificada pelo gene localizado no cromossomo 9 (posição 9q-12-21.2). Primordialmente, teve como função o envolvimento ativo na inibição da síntese de eicosanóides e fosfolipase A2 (PLA2) induzida por glicocorticóides e hoje sabe-se que está relacionada a uma série de processos que incluem transporte de membrana, proliferação, diferenciação e apoptose (Scannell e

Maderna, 2006) e, além disso, sugere-se que possa contribuir para mitogênese e metástase do câncer mamário (Parente e Solito, 2004).

A ANXA1 sinaliza na célula por meio de receptores da família dos peptídeos formilados (FPRs). FPRs são um grupo de proteínas de sete domínios transmembrânicos acoplados a proteína G, que são expressos por células fagocitárias e epiteliais/endoteliais de mamíferos exercendo função importante na defesa do hospedeiro e inflamação. Em humanos, foram descritos três FPRs que se ligam a um grupo numeroso composto por diferentes agonistas (De Graauw *et al.*, 2010). Sabe-se que após interação com FPRs, a ANXA1 é capaz de induzir ativação da via de sinalização de proteínas quinases ativadas por mitógenos (MAPK), resultando em mudanças no cálcio intracelular e no “shedding” de L-selectina dos neutrófilos, promovendo alteração do potencial de migração celular e redução da resposta inflamatória (Croxtall *et al.*, 2003).

Ao longo do tempo, a pesquisa focada na elucidação das funções dessa proteína foi possível graças à biologia molecular que permitiu o estudo em camundongos nocauteados para o gene da ANXA1 e a possibilidade de induzir a expressão dessa proteína em animais a partir da administração de dexametasona ou hidrocortisona (Peers *et al.*, 1993). Atualmente, a ANXA1 possui diversas atribuições e esse trabalho dá enfoque em sua atividade antiinflamatória e sua atuação nos diversos tipos de câncer, principalmente no câncer de mama.

4.1 Atividade antiinflamatória (*Anti-inflammatory activity*)

A ANXA1 é descrita como uma proteína essencialmente antiinflamatória e, assim, a elucidação dessa função tem como base o entendimento da expressão da ANXA1 em componentes da resposta imunológica. Estudos indicam que a ANXA1 está expressa, entre os leucócitos, discretamente nos linfócitos e predominantemente nos neutrófilos, eosinófilos e monócitos (Goulding *et al.*, 1995; Morand *et al.*, 1995).

Os neutrófilos humanos são ricos em ANXA1, chegando a 1 a 2% do total de proteínas citosólicas, e são capazes de externalizar grande quantia dessa proteína quando aderidos na monocamada de células endoteliais

(Perretti e Flower, 2004). Sabe-se que, quando os neutrófilos intravasculares estão aderidos ao endotélio, a ANXA1 intacta, 37 KDa, é predominantemente encontrada em suas membranas plasmáticas enquanto que quando estão no espaço extravascular a maior parte de ANXA1 se encontra clivada na região N-terminal, caracterizada por ter propriedade antiinflamatória (Perretti e Gavins, 2003).

Nos mastócitos, tanto de mucosa como de tecido conjuntivo, a ANXA1 apresenta um distinto grau de expressão e susceptibilidade na modulação após diferentes estímulos. É importante destacar que uma das principais funções de ANXA1 exógena nessas células corresponde à liberação histamínica (Tasaka *et al.*, 1994).

Em geral, nos leucócitos, os níveis de ANXA1 aumentam em resposta a injeção de glicocorticóide e de algumas citocinas, tais como fator de necrose tumoral, IL-1 e IL-6 (Perretti e Gavins, 2003). Há uma constatação de que essa proteína pode retardar o processo de extravasamento dos leucócitos (Allcock *et al.*, 2001) para o local de inflamação (Yang *et al.*, 2004; Damazo *et al.*, 2005; Souza *et al.*, 2007). Tal função representa um dos principais papéis antiinflamatórios exercidos pela ANXA1.

O provável mecanismo pelo qual a ANXA1 atua é inibindo a atividade das moléculas de adesão das interações leucócito-endotélio, principalmente integrinas e selectinas (Solito *et al.*, 2000). Além disso, outro papel antiinflamatório relacionado a essa proteína engloba a inibição de ciclooxigenase 2 (COX-2) e de óxido nítrico sintetase induzível (iNOS) e se relaciona com a liberação de IL-10 em fagócitos ocasionando a indução de apoptose das células inflamatórias (Parente e Solito, 2004).

4.2 ANXA1 e câncer (*ANXA1 and cancer*)

Além de possuir papel antiinflamatório, a ANXA1 tem funções importantes e distintas no desenvolvimento e progressão de diversos tumores visto que a sua expressão é diferenciada em cada tipo de tumor.

Em alguns tipos de câncer a ANXA1 possui expressão reduzida como é o caso do carcinoma oral (Koike *et al.*, 2005), de esôfago (Huang, Z. Y. *et al.*, 2007), da nasofaringe (Cheng *et al.*, 2008), de colo uterino (Liu *et al.*, 2011),

mieloma múltiplo (Xiao *et al.*, 2009), em melanoma (Caputo *et al.*, 2011), de tireóide (Yamazaki *et al.*, 2010) e intestino (Souza *et al.*, 2007). Em câncer de próstata, a perda da expressão de ANXA1 contribui para o aumento da expressão de PLA2 e IL-6 acarretando em progressão tumoral enquanto que o aumento de expressão de ANXA1 favorece a apoptose das células tumorais (Patton *et al.*, 2005).

Em câncer de pulmão (Brower, 2009), pâncreas (Bai *et al.*, 2004), fígado (Seth *et al.*, 2003), rins e bexiga (Facio *et al.*, 2011), astrocitomas e glioblastomas (Schittenhelm *et al.*, 2009) a ANXA1 está correlacionada positivamente com efeito pró-tumoral e contribui para o potencial agressivo do tumor.

Em câncer de mama, a literatura é muito controversa, configurando possibilidades de que a ANXA1 poderia estar agindo de maneira pró e anti-tumoral. Por um lado, algumas pesquisas afirmam que há a perda de ANXA1 na fase precoce da tumorigênese contribuindo para a transformação maligna e que a transfecção de ANXA1 inibe a metástase em células de tumores invasivos (Maschler *et al.*, 2010). Em contrapartida, há estudos que demonstram que o aumento da expressão de ANXA1 está correlacionado positivamente com a progressão do câncer, colaborando para metástase pulmonar e resistência a terapias (Wang *et al.*, 2004; De Graauw *et al.*, 2010). Nosso grupo de pesquisa, baseado em inúmeras evidências elucidadas no Capítulo II, posiciona esse trabalho favorável à teoria de que a ANXA1 tem papel essencialmente pró-tumoral no câncer de mama

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CAPÍTULO II

Sinalização autócrina de Anexina A1 e seu crosstalk com o Receptor do fator de crescimento epidermal são essenciais para o fenótipo agressivo do câncer de mama*

(Annexin A1 autocrine signaling and its crosstalk with epidermal growth factor receptor are essential for breast cancer aggressive phenotype)

*Este capítulo está formatado de acordo com as normas da revista *Cancer Research* com algumas alterações para a Dissertação.

Annexin A1 autocrine signaling and its crosstalk with epidermal growth factor receptor are essential for breast cancer aggressive phenotype

Running title

Crosstalk between Annexin A1 and EGFR pathways in breast cancer

Authorship

Mariana Alves Pereira Zóia^{1,#}, Lara Vecchi^{1,*,#}, Cristiano Colaço Ramos², Bruna França Matias Colombo¹, Yara Cristina Paiva Maia¹, Victor Piana de Andrade³, Fernando Soares³, Sonia Maria Oliani⁴ and Luiz Ricardo Goulart^{1,*}.

Affiliation

¹ Laboratory of Nanobiotechnology, Institute of Genetics and Biochemistry, Federal University of Uberlandia, Uberlandia, MG, Brazil.

² Department of Life Sciences, University of Coimbra, Portugal

³ Department of Surgical and Investigative Pathology, AC Camargo Cancer Center, São Paulo, Brazil

⁴ Immunopathology Laboratory, Department of Biology, Instituto de Biociências, Letras e Ciências Exatas (IBILCE), UNESP, São Jose do Rio Preto, São Paulo, Brazil

Both authors contributed equally to this work.

*** Corresponding authors**

Luiz R. Goulart and Lara Vecchi, Laboratory of Nanobiotechnology, Institute of Genetics and Biochemistry, Federal University of Uberlandia, Campus Umuarama, Bloco 2E, Sala 248, 38400-902, Uberlandia, MG, Brazil. Phone: +55 34 3225 8440. lrgoulart@ufu.br; lrgoulart@ucdavis.edu; laravecchi7@yahoo.it.

Palavras-chave: Câncer de mama, anexina A1, EGFR, FPR1, sinalização autócrina

Key words: Breast cancer, annexin A1, EGFR, FPR1, autocrine signaling.

Resumo

O papel da Anexina A1 (ANXA1) no câncer de mama ainda não está elucidado completamente, visto que há indícios contraditórios demonstrando que essa proteína pode exercer funções tanto pró-tumorais quanto antitumorais. Nesse trabalho, nós analisamos a importância da via da sinalização de ANXA1 e o que ela acarreta nas células de câncer de mama por meio de quantificação da sua expressão, localização celular e sinalização através dos Receptores Peptido-Formilados (FPRs). O trabalho foi conduzido com duas linhagens de câncer de mama (MCF-7 e MDA-MB-231) e uma linhagem de células normais de mama (MCF-10A). Primeiramente, tivemos que ANXA1 demonstrou-se muito expressa, altamente secretada e majoritariamente localizada no compartimento nuclear de células MDA-MB-231 (oriundas de câncer de mama do tipo triplo-negativo). Utilizando amostras de tecidos de câncer de mama de humanos, nós confirmamos os nossos resultados *in vitro* de que ANXA1 estaria predominantemente expressa em tecidos de câncer de mama do tipo triplo negativo, quando comparados com outros. Além disso, verificamos que tal expressão está associada com a positividade de metástases linfonodais. Vimos também *in vitro* que a secreção de ANXA1 em MDA-MB-231 estava correlacionada com o aumento de expressão de FPR1 e, subsequentemente, nós demonstramos que a sinalização de ANXA1 por meio do FPR1, promovia a ativação da enzima fosfatidilinositol-3-cinase (PI3K) e tal fato se correlacionava com um aumento de agressividade das células MDA-MB-231 (aumento do potencial de invasão e migração). Outro resultado importante que tivemos foi quando demonstramos que a sinalização de ANXA1 acarretava em aumento de secreção de interleucina 2 (IL-2) e esta sinalizava de maneira autócrina em MDA-MB-231, contribuindo adicionalmente para a ativação de PI3K. Sabendo que a atividade de PI3K é essencial para a migração de MDA-MB-231, concluímos que IL-2 aparentemente seria uma das moléculas principais envolvidas na migração de células triplo-negativas. Por fim, nós demonstramos que o FPR-1 realizava um cross-talk com o receptor do fator de crescimento epidérmico (EGFR) e isso ativava outra cascata de sinalização (a sinalização das proteíno-quinases ativadas por mitógenos ou MAPK) e colaborava para o aumento do potencial de sobrevivência celular de MDA-MB-231. Adicionalmente, uma elevada correlação entre a expressão de ANXA1 e EGFR foi demonstrada em amostras de tecidos de câncer de mama. Como conclusão, temos que nossos resultados mostram um papel crucial da sinalização de ANXA1 em células de câncer de mama do tipo triplo negativas enfatizando a possibilidade da inibição dessa via para o controle do câncer de mama triplo negativo.

Abstract

Annexin A1 (ANXA1) protein role in breast cancer (BC) has not been well elucidated since contradictory reports suggest both tumor-promoting and tumor-suppression activities. We analyzed ANXA1 expression, localization and signaling through FPR receptors in BC cell lines and in normal breast cell line (MCF-10A). ANXA1 is highly expressed, massively secreted and intensively localized in the nuclear compartment in MDA-MB-231 triple negative BC cell line (TNBC). ANXA1 secretion in MDA-MB-231 cells is accompanied by FPR1 expression, through which, ANXA1 activates PI3K/Akt and MAP kinases (MAPK) signaling pathways. By using human BC tissue samples we also confirmed that ANXA1 is predominantly expressed in TNBCs and its expression associates with lymph node metastasis. Subsequently, we demonstrated that ANXA1/FPR1 signaling induced an increase in endogenous ANXA1 translocation to the nuclear compartment, which was dependent on PI3K activation and correlated with increased MDA-MB-231 aggressiveness. Furthermore, we demonstrated that ANXA1 signaling leads to IL-2 secretion which, in turns, through its autocrine signaling in MDA-MB-231, contributes to PI3K/Akt axis activation. Since PI3K activity is essential for MDA-MB231 migration, IL-2 is apparently one of the pivotal molecules involved in TNBC cells migration. Finally, we demonstrated that FPR1 signaling trans-activates the epidermal growth factor receptor (EGFR) leading to MAPK cascade activation and MDA-MB-231 cells survival. The association between ANXA1 and EGFR expression was also demonstrated in human BC tissue samples. Altogether, our results shed light on the crucial role of ANXA1/FPR1 signaling in TNBCs aggressiveness emphasizing the possibility of inhibiting ANXA1 signaling for amelioration of breast cancer management.

Introduction

Breast cancer (BC) is the most common type of cancer worldwide, followed by prostate and lung cancer (Society, 2013). The molecular classification, based on gene expression profile, divides BCs into luminal A, luminal B, normal breast-like, HER2 positive and basal-like (BLBC) groups (Sorlie *et al.*, 2001; Sorlie *et al.*, 2003). Among them, the BLBC group, mostly represented by triple negative breast cancer (TNBC), (Rakha *et al.*, 2008), displays the highest invasive potential and correlates with a poor clinical outcome (Rouzier *et al.*, 2005).

The association between inflammation and cancer is unquestionable (Hussain e Harris, 2007). Although chronic inflammation may be detrimental and frequently predispose cells to oncogenic transformation (Schetter *et al.*, 2010), an acute inflammatory response is usually beneficial and may present a tumor suppression role (Mantovani *et al.*, 2008). A key mediator of anti-inflammatory response, the 37KDa protein Annexin A1 (ANXA1), has been linked to BC development and progression with contradictory roles. On one side, ANXA1 is described for displaying a protective role against epithelial-mesenchymal transition (EMT) (De Graauw *et al.*, 2010), a process exploited by tumor cells to exacerbate their invasive potential (Sarrio *et al.*, 2008). On the other side, ANXA1 has been described to promote TGF- β /Smad signaling, thus being responsible for EMT (De Graauw *et al.*, 2010).

The controversial role of ANXA1 led us to focus our attention on the ANXA1/FPRs (formyl peptide receptors) signaling, in an attempt of providing a better understanding of its role in BC. ANXA1 exerts an anti-inflammatory activity by signaling, upon its secretion, through FPR1 or ALXR/FPR2 (Gavins *et al.*, 2003), inhibiting PLA2 (Parente e Solito, 2004; Sakaguchi *et al.*, 2007) and leukocyte transmigration (Perretti *et al.*, 1996; Perretti e Flower, 2004; Perretti e D'acquisto, 2009). FPRs are Gi protein-coupled receptors involved in chemotaxis of leukocytes towards the bacterial chemotactic peptide N-formyl-methionyl-leucyl-phenylalanine (fMLF). By binding to FPRs, N-formylated peptides elicit a signal cascade involving PI3K and MAPK (Le *et al.*, 2002; Paruch *et al.*, 2006; Babbin *et al.*, 2007). ANXA1 also regulates different

processes including membrane trafficking, proliferation, differentiation and apoptosis (Gerke e Moss, 2002; Rescher e Gerke, 2004; Petrella *et al.*, 2005; Scannell e Maderna, 2006). ANXA1 can be found in its 37KDa intact form that displays an anti-inflammatory activity and in two cleaved form of 33 and 36KDa. The 33KDa cleaved form was described to be pro-inflammatory (Perretti e Flower, 2004; Blume *et al.*, 2012) whereas the 36KDa cleaved form was associated with monocyte recruitment and prevention of inflammation (Blume *et al.*, 2012).

ANXA1 expression positively correlates with progression of astrocytomas, lung, melanoma and pancreatic cancers (Bai *et al.*, 2004; Biao xue *et al.*, 2014; Boudhraa *et al.*, 2014) whereas, ANXA1 expression negatively correlates with thyroid cancer progression (Petrella *et al.*, 2005). Moreover, an association between ANXA1 nuclear localization and worst prognosis in gastric cancer and esophageal squamous cell carcinoma was described (Zhu *et al.*, 2010; Han *et al.*, 2014). By using ANXA1 null mice some of the tumor promoting features of ANXA1, such as metastasis, angiogenesis and wound healing, were discovered (Yi e Schnitzer, 2009). Reports demonstrated that in glioblastoma, apoptotic cells-derived ANXA1 activates FPR1 on surrounding glioblastoma cells. The FPR1 activates a non-receptor tyrosine kinase, named Src (Aligayer *et al.*, 2002; Dehm e Bonham, 2004; Wheeler *et al.*, 2009) which, in turns, activates EGFR by phosphorylating its tyrosine residues 845, 992 and 1086. Once activated, EGFR leads to ERK activation thus contributing to cell survival (Huang, J. *et al.*, 2007). EGFR is expressed in approximately 18% of BC cases and is a marker of poor clinical outcome (Bhargava *et al.*, 2005; Rimawi *et al.*, 2010).

Here we show that ANXA1 signals through FPR1 leading to increased nuclear localization of endogenous ANXA1 in the TNBC cell line, MDA-MB-231. Such increase is due to PI3K/Akt activation followed by NFAT activation. The NFAT active form in complex with ANXA1 migrates to the nucleus. In the nucleus, the NFAT/ANXA1 complex induces IL-2 expression, with a subsequent increase of IL-2 autocrine signaling. IL-2 signaling increases the PI3K/Akt axis activation contributing to an additional increase of ANXA1 nuclear localization in MDA-MB-231. Nuclear ANXA1 associates with

increased migration. Since EGFR seems to display an important role in BC aggressiveness, we subsequently showed that FPR1 activation by ANXA1, in an autocrine fashion, is responsible for EGFR activation. EGFR activation contributes to cell survival by activating the MAPK pathway. Our cell line results were further confirmed by analyzing ANXA1 and EGFR expression in tissue microarrays (TMAs) containing BC patient samples.

Materials and methods

Cell Culture and treatments.

Human breast cell lines MCF-10A and human BC cell lines MCF-7 (ER+, PR+ cell line) and MDA-MB-231 (triple negative cell line) were obtained from the American Type Culture Collection. MCF-10A were maintained in DMEM F12 (Invitrogen) supplemented with EGF 20ng/mL, hydrocortisone 0.5µg/mL and insulin 10µg/mL (Invitrogen) while MCF-7 and MDA-MB-231 were maintained in IMDM (Invitrogen) with 5% FBS.

Cell were treated with the following chemicals and antibodies: Boc-MLF (FPR1 inhibitor, used at 10µM), FR180204 (ERK inhibitor, used at 10µM) and Ac2-26 (ANXA1 mimetic peptide, used at 1µM) were purchased from Tocris; LY294002 and Triciribine Hydrate, respectively PI3K and Akt inhibitors, (both used at 25µM) and Cyclosporin A (used at 10µM) were purchased from Sigma. Neutralizing anti IL-2 (Invitrogen) was used at 2µg/mL.

Cell lysis, Immunoblotting and immunoprecipitation.

By using NE-PER Nuclear and Cytoplasmic Extraction Reagent kit (Thermoscientific) nuclear and cytoplasmic extracts of 24h-treated cells were prepared. Identical amounts of protein extracts and supernatants were subjected to Western blotting analysis. For details see Supplementary Material.

Cytoplasmic and nuclear protein extracts (50µg) were immunoprecipitated for 2h-treated at RT using 50 µL of 50% protein A-Sepharose CL-4B beads (Amersham Biosciences) in TNN buffer, and 2µL of anti NFAT antibody (Abcam). Protein-A Sepharose beads were washed three times with TNN buffer, twice with PBS and finally resuspended in 20 µL of loading buffer to elute NFAT-interacting proteins. Immunoprecipitated samples were subjected to SDS-PAGE and immunoblotting using anti-ANXA1 antibody.

Wound healing assay.

Cell migration was measured in a classical wound healing assay (Liang *et al.*, 2007). Pictures of the wounds (10X) were taken at time 0 and 24h (EVOS ®, Advanced Microscopy Group). Closure of the wound was measured by using Image J software. For details on the formula used see Supplementary Material.

Matrigel invasion assay.

For invasion assay BD BioCoat Matrigel 24-well Invasion Chambers, with 8.0 µm pore PET membrane (BD Pharmingen™) were used. Accordingly to manufacturer's instructions, 5% FBS medium was used as chemo attractant and added to wells. Cells (2.5×10^5) were seeded in the upper chamber in serum free medium containing treatment or vehicle only. Upon 24-h incubation, invasive cells were fixed, stained and counted manually (EVOS ®, AMG). For details on the formula used see Supplementary Material.

Immunofluorescence.

MCF-10A, MCF-7 and MDA-MB-231 cells were fixed with 3.7% para-formaldehyde, permeabilized with 0.1% Triton X-100 and blocked with 1% BSA. Cells were subsequently stained with anti ANXA1 antibody (1:200, Invitrogen) and anti IgG-FITC secondary antibody (1:200, Invitrogen). Nuclei were counterstained with TO-PRO®3 Iodide (1:1000, Invitrogen). Cell staining was analyzed by confocal microscopy (Zeiss LSM510).

Flow cytometry.

Cells were stained with anti-FPR1 and anti-FPR2 (Sigma) for membrane surface expression evaluation. 24-h treated cells were stained with anti-EGFR phospho Y⁹⁹², anti-ERK1 (phospho T²⁰²/Y²⁰⁴) + ERK2 (phospho T¹⁸⁵/Y¹⁸⁷) and anti-Akt1 phospho S⁴⁷³ (all from Abcam) upon fixation and permeabilization using Cyto Fix/Cyto Perm kit (BD Pharmingen™). The following secondary antibodies were used: anti-rabbit IgG-FITC (Sigma) and anti-mouse IgG-PE (Invitrogen). Secreted IL-2 and IL-6 were measured by using Th1-Th2-Th17 CBA kit (BD Pharmingen™). Cell proliferation was

measured by staining cells with 0,1 μ M Carboxyfluorescein succinimidyl ester (CFSE). Treatments were applied to cells every other day for a total of five days. Apoptosis was measured upon 24 hours of treatment by using Annexin V (ANXAV) PE/7-AAD apoptosis kit (BD PharmingenTM). Cell staining, cell proliferation and apoptosis were analyzed by flow cytometry (Accuri C6, BD PharmingenTM).

TMA.

Tissue microarray (TMA) of human breast cancer tissues (Biomax and AC Camargo Cancer Center) containing 144 cases, 49 of which, with matched lymph node metastasis, were used. All cases included clinical-pathological features description (Scarff Bloom-Richardson grading, TNM system staging and ER, PR and HER2 status). The Ethics Committee of the Institutional Research Board of the Federal University of Uberlandia approved all procedures, under the number 176/2008.

Immunohistochemistry.

TMA slides containing 1mm diameter and 4mm thickness tissue samples were incubated o.n. with anti-EGFR (Sigma) or anti-ANXA1 (Invitrogen), respectively at 1:20 and 1:500 dilutions, subsequently incubated with HRP polymer (HRP Multimer) and the reaction was developed with DAB (Sigma). Slides were counterstained with Hematoxylin (Ventana) and mounted on Tissue-Tek film (Sakura). Staining was quantified using the Aperio ScanScope XT digital scanner (Leyca Biosystems) using the PixelCount V9.0 and IHC nuclear algorithms. For details on quantifications see Supplementary Material and Supplementary Table

Statistical analysis.

Significant differences between means were determined by the Student t test, while correlations were analyzed by Pearson's coefficient, McNemar test and Odds ratios (OR). OR with 95% confidence intervals (CI) were estimated using unconditional logistic regression. Statistical significance was considered when $P < 0.05$. The statistical analyses were performed by using GraphPad

Prism 5 (GraphPad Software Inc., La Jolla, CA) and SPSS version 17.0 (SPSS; Chicago, IL).

Results

MDA-MB-231 express high levels of ANXA1 and FPR1.

We analyzed the expression of ANXA1 and its receptors, FPR1, FPR2 and FPR3, in normal breast cell line (MCF-10A) and BC cell lines (MCF-7 and MDA-MB-231). In this experiment we demonstrated that ANXA1 protein is expressed in both cytoplasm and nuclei of MCF-10A and MDA-MB-231, whereas, almost no expression of ANXA1 was seen in MCF-7 cells. MDA-MB-231 cells display a more intensive expression of ANXA1 in either cytoplasm or nuclei when compared to MCF-10A cells (Fig 1A and B). A great amount of ANXA1 was detected in supernatants of MDA-MB-231 cells when compared with MCF-10A cells, which presented only a slight ANXA1 secretion. MCF-7 cells did not secrete ANXA1 at all. In both MCF-10A and MDA-MB-231 cells it was possible to visualize ANXA1 in its intact (37KDa) and cleaved form (33KDa) with a predominance of the 37KDa form in cytoplasmic and nuclear extracts, and a predominance of the 33KDa cleaved form in supernatants. We also tested EGFR expression in these cell lines demonstrating that MDA-MB-231 cells express high levels of EGFR, while MCF-10A expresses low levels of EGFR and MCF-7 cells expresses only tiny amounts of this receptor (Figure 1A).

With regard to FPRs expression, MCF-7 cells do not express either FPR1 or FPR2, MCF-10A cells express both FPR1 and FPR2 whereas MDA-MB-231 cells express FPR1 only (Figure 1C). None of these cell lines express FPR3.

Therefore, high expression of EGFR, accompanied by high expression and secretion of ANXA1, and expression of FPR1, but not FPR2, is a characteristic of the TNBC cell line MDA-MB-231.

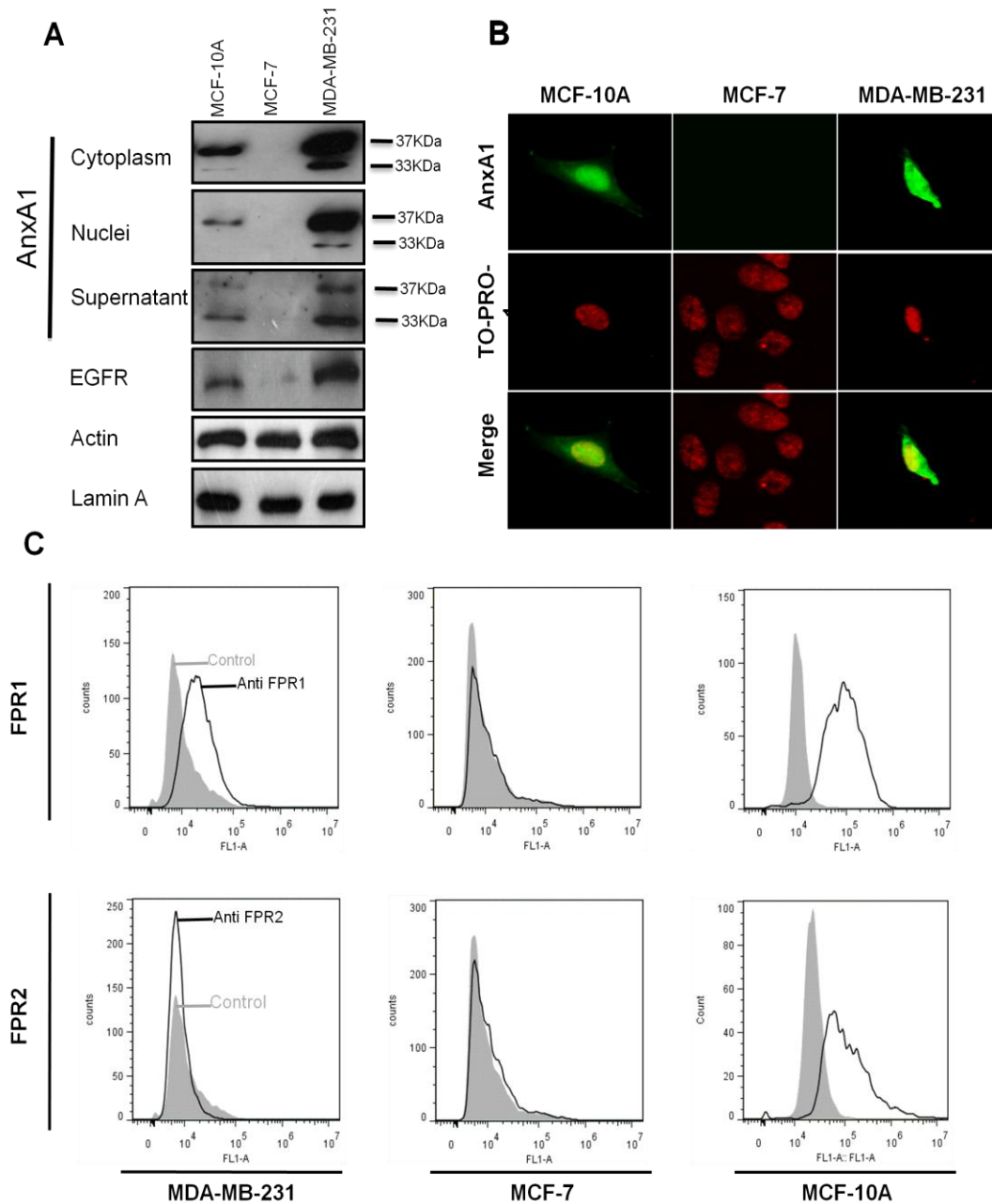


Figure 1. ANXA1 and FPRs expression in normal (MCF-10A) and BC cell lines (MCF-7 and MDA-MB-231). A. Western blotting analysis of ANXA1 expression in cytoplasmic, nuclei and supernatant fractions. EGFR expression was tested in total protein extract. Actin and Lamin A were used as loading controls of cytoplasmic and nuclear protein extracts respectively. B. ANXA1 localization was analyzed by staining cells with anti ANXA1 (green) whereas nuclei were counter-stained with YO-PRO-1 (red). Merged images (yellow) confirmed nuclear localization of ANXA1 in MCF-10A and MDA-MB-231 cells C. Cell surface expression of FPR1 and FPR2 was analyzed by flow cytometry (black peak). As negative control, secondary antibody alone was used (solid grey peak).

FPR1 inhibition is essential for low nuclear ANXA1 expression in MDA-MB-231 cells.

Since nuclear localization of ANXA1 was associated with the aggressiveness of several types of cancer (Zhu *et al.*, 2010; Han *et al.*, 2014), we wanted to verify whether FPR1 autocrine activation by secreted ANXA1 correlates with nuclear localization of ANXA1 in MDA-MB-231 cells. Thus, we treated MDA-MB-231 cells with N-terminal ANXA1 peptide, Ac2-26, and with FPR1 inhibitor, Boc-MLF. We found that Ac2-26 stimulation increases ANXA1 nuclear expression and, consistently, Boc-MLF leads to a decrease of ANXA1 nuclear expression (Fig. 2A). We next investigated the effect of ANXA1 activation of FPR1 on the migration and invasion capacities of MDA-MB-231 cells by classical wound healing assay and invasion assays. We found that Ac2-26 significantly ($p < 0.05$) increases the invasion and migration potential of TNBC cells. Although not significantly, such properties were reduced by Boc-MLF (Fig. 2B and C). Our data suggest that FPR1 autocrine activation in MDA-MB-231 cells correlates with increased nuclear expression of ANXA1 and with invasion and migration properties of MDA-MB-231 cells.

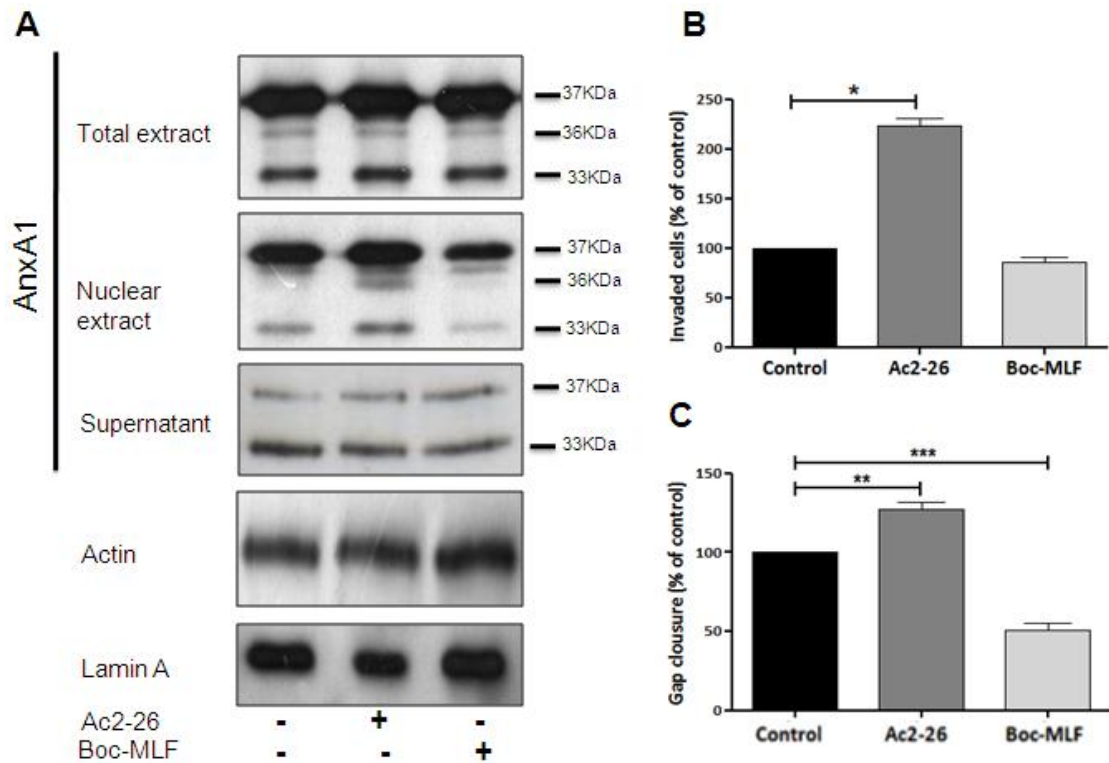


Figure 2. Autocrine ANXA1 signaling through FPR1 is essential for increased nuclear expression of intracellular ANXA1. A. MDA-MB-231 cells were pre-incubated with ANXA1 mimetic peptide (Ac2-26 1 μ M) or with FPR1 inhibitor (Boc-MLF 10 μ M). The cell lysates were measured for ANXA1. Immunoblotting for Actin and Lamin A was used to verify equivalent protein loading. B. Invasion assay of MDA-MB-231 stimulated with Ac2-26 or Boc-MLF measured using BD BioCoat Matrigel 24-well Invasion Chambers. C. Wound healing assay of MDA-MB-231 treated with Ac2-26, Boc-MLF or vehicle only. Data are representative of experiments run in triplicate. Bars represent SD*, p<0.05; **, p < 0.01; ***, p<0.001.

ANXA1 autocrine signaling induces Akt activation leading to increased ANXA1 nuclear localization and cell migration.

We further investigated the ANXA1 signaling pathway downstream FPR1 activation is involved in aggressive potential of MDA-MB-231. Since FPR1 activation commonly leads to PI3K/Akt pathway activation (Le *et al.*, 2002), we treated MDA-MB-231 cells with Boc-MLF to inhibit ANXA1 autocrine signaling through FPR1. Such treatment determined a drop in Akt phosphorylation levels (Fig 3A). Moreover we wanted to reveal whether PI3K/Akt pathway could be responsible for nuclear localization of ANXA1. As shown in figure 3B, inhibition of FPR1 downstream signaling molecules,

through PI3K inhibitor, LY294002, (LY, 25 μ M) and Akt inhibitor, Triciribine Hydrate, (TCN, 25 μ M), strongly decreased ANXA1 nuclear localization. By classical wound healing, invasion and proliferation assays, we demonstrated that inhibition of PI3K and Akt also significantly affected the proliferation, migration and invasive potential of cells when compared to non-treated cells (Fig. 3C-E). These results were consistent with our previous results illustrated in Figure 2, and support the influence of FPR1 signaling pathway on nuclear localization of ANXA1, migration and invasion potential of MDA-MB-231 cells. Thus, autocrine stimulation of FPR1 by secreted ANXA1 seems to exacerbate aggressiveness of MDA-MB-231 cells probably by increasing nuclear ANXA1.

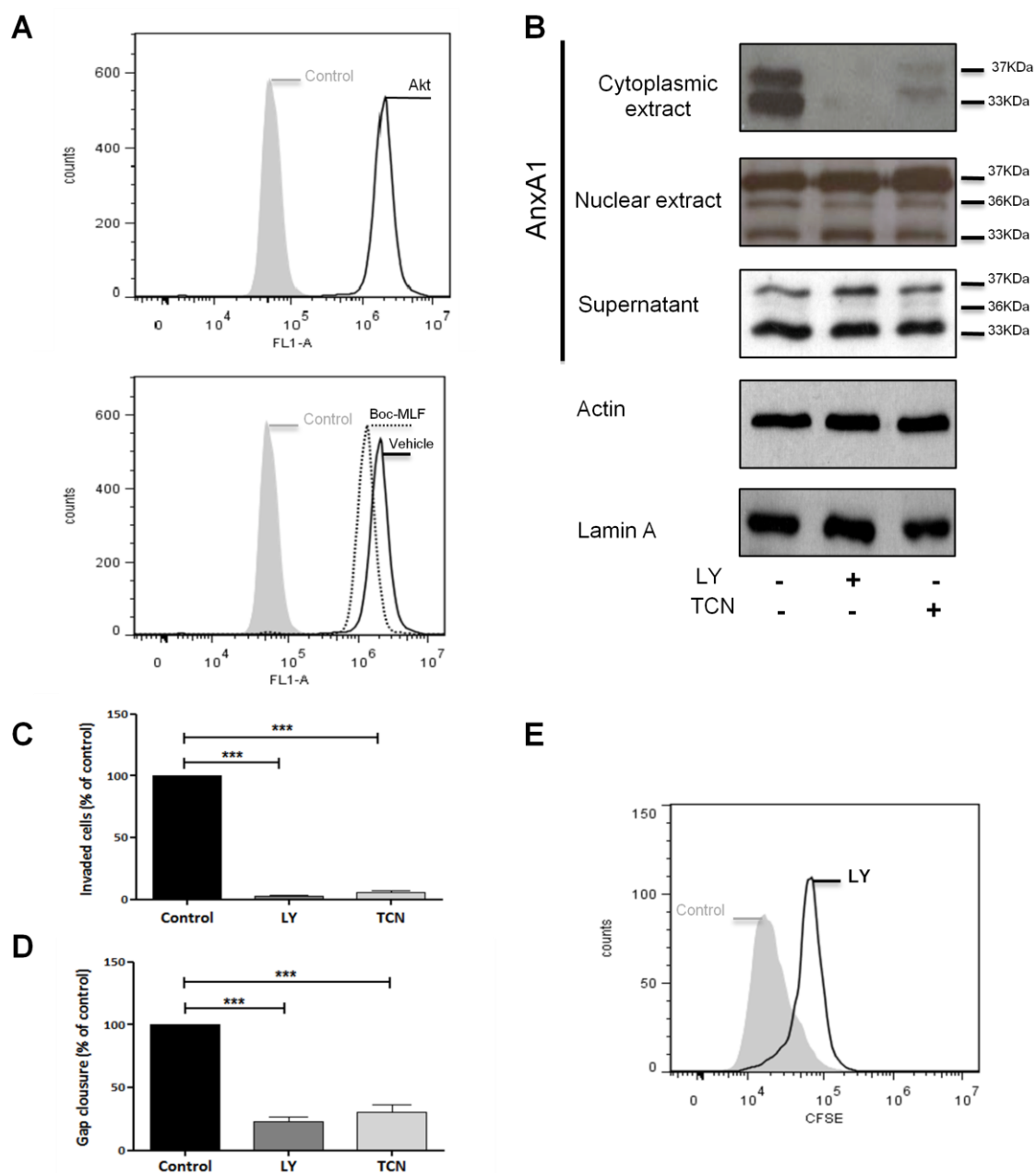


Figure 3. ANXA1 autocrine signaling through FPR1 induces activation of PI3K/Akt pathway, increased ANXA1 nuclear localization and cell migration. A. Flow cytometry assay for detection of AKT1 phospho S⁴⁷³ levels in MDA-MB-231 treated with Boc-MLF (dotted black peak) or cells treated with vehicle only (black peak). Staining with secondary antibody alone was used as control (solid grey peak). B. ANXA1 expression analysis by western blotting of supernatants, cytoplasmic and nuclear protein extracts of MDA-MB-231 treated with LY or TCN (PI3K and AKT inhibitors respectively, 25 μ M). Actin and Lamin A were used as loading controls of cytoplasmic and nuclear protein extracts respectively. C. Invasion assay of MDA-MB-231 treated with LY or TCN measured using BD BioCoat Matrigel 24-well Invasion Chambers. D. Wound healing assay of MDA-MB-231 treated with LY, TCN or vehicle only. E. Proliferation analysis of MDA-MB-231 cells treated with

FR180204 (black peak) or vehicle only (grey solid peak). Data are representative of experiments run in triplicate. Bars represent SD***, $p < 0.001$.

ANXA1 signaling through FPR1 induces Akt, EGFR and ERK1/2 activation in MDA-MB-231.

Due to the relevant communication between FPR1 and EGFR signaling pathways described for glioblastoma (Huang, J. *et al.*, 2007), we next examined whether this finding was applicable for EGFR-expressing TNBC cells. Thus, we investigated the activation of EGFR and its downstream signaling molecule ERK1 and ERK2 upon FPR1 inhibition in MDA-MB-231 cells. By analyzing cells treated or not treated with Boc-MLF (10 μ M) by flow cytometry, we found that inhibition of FPR1 resulted in decreased EGFR and ERK1/2 activation by means of EGFR Tyr⁹⁹² and ERK1 Tpr²⁰² Tyr²⁰⁴ and ERK2 Trp¹⁸⁵ and Tyr¹⁸⁷ phosphorylation levels decay (Fig 4A and B). These results indicate that activation of FPR1 by ANXA1 autocrine signaling leads to trans-activation of EGFR signaling pathway. In order to test the importance of ERK1/2 activation in cells survival, we performed an apoptosis assay. The use of the ERK inhibitor (FR180204) induces apoptosis cell death in 20% of cells (Fig 4C and D).

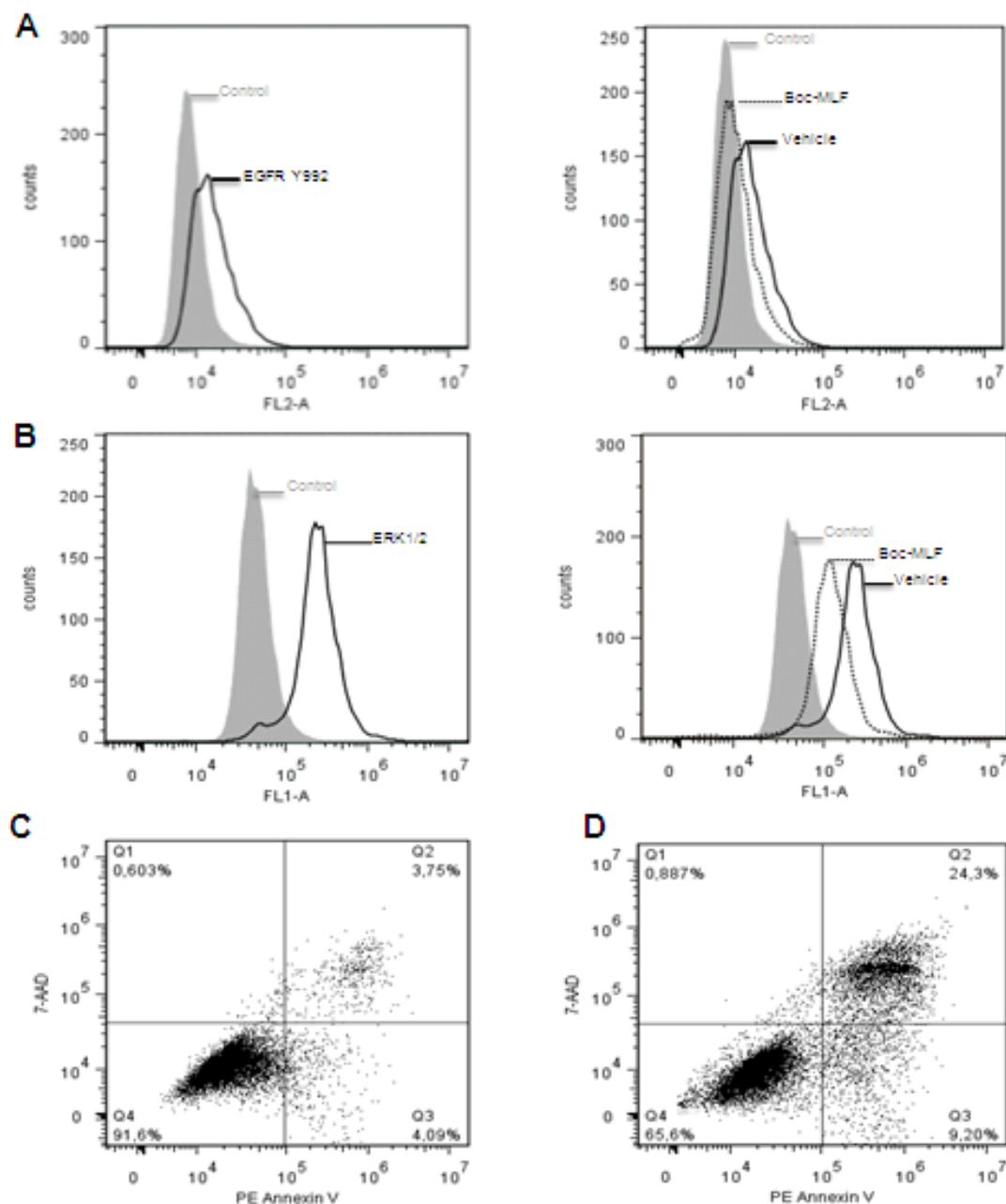


Figure 4. Autocrine ANXA1 signaling through FPR1 induces EGFR and ERK1/2 phosphorylation in MDA-MB-231. A. Flow cytometry assay for detection of EGFR-phospho Tyr⁹⁹² levels in MDA-MB-231 treated with Boc-MLF (dotted black peak) or cells treated with vehicle only (black peak). Staining with secondary antibody alone was used as control (solid grey peak). B. Flow cytometry assay for detection of ERK1 phospho Tpr²⁰² Tyr²⁰⁴ and ERK2 phospho Tpr¹⁸⁵ and Tyr¹⁸⁷ in MDA-MB-231 untreated cells (black peak) and in Boc-MLF treated cells (dotted black peak). Secondary antibody alone was used as control (solid grey peak). Apoptosis assay of vehicle control (C) and FR180204 treated cells (D).

Nuclear ANXA1 is a co-activator of NFAT-dependent IL-2 expression.

To investigate the effect of ANXA1 signaling on cytokines production by MDA-MB-231 cells, we analyzed supernatants of cells treated with Ac2-26 (1 μ M), Boc-MLF (10 μ M) or vehicle only. FPR1 activation by Ac2-26 resulted in an increase of IL-2 and a decrease of IL-6; the opposite effect was visualized when cells were treated with Boc-MLF (Figure 5A). These results demonstrated that ANXA1 signaling results in an anti-inflammatory activity, by virtue of IL-6 decay. Due to the effect of IL-2 increase, we investigated the role of ANXA1 in activating IL-2 expression. In an attempt to correlate IL-2 expression with nuclear localization of ANXA1, we inhibited the IL-2 transcription factor, NFAT, by treating cells with Cyclosporin A (10 μ M). In particular, we wanted to verify whether ANXA1 could be transported to nuclei by activated NFAT. As shown in figure 5B, the amount of nuclear ANXA1 in Cyclosporin A treated cells were much lower when compared to control cells. This finding probably indicates that ANXA1 could migrate to nuclear compartment once bound to the activated NFAT. Cyclosporin A also induces an increased secretion of ANXA1 probably as a compensatory event.

In order to verify whether ANXA1 could help the NFAT transcription activity, we performed an immunoprecipitation analysis using anti-NFAT antibody of both cytoplasmic and nuclear extracts. By staining immunoprecipitated samples with anti-ANXA1, we confirmed the formation of ANXA1-NFAT complexes both in cytoplasm and nuclei, and we confirmed that the ANXA1 form that interact with NFAT is mostly the 37KDa form (Figure 5C).

These findings probably indicate that ANXA1 could migrate to nuclear compartment once bound to the activated NFAT, aiding the transcription of IL-2.

In order to verify whether ANXA1 could activate an IL-2-dependent autocrine loop, we inhibited secreted IL-2 by using a neutralizing monoclonal antibody against IL-2. As shown by flow cytometry (figure 5D), IL-2 neutralization led to a decrease, although not complete, of Akt activation confirming the existence of an autocrine signaling of IL-2 in TNBC cells. Since

PI3K/Akt pathway displays an essential role in nuclear localization of ANXA1 and for TNBC cells migration, we tested whether IL-2 neutralization could lead to a decrease of nuclear ANXA1. Although with a lower efficiency compared to PI3K and Akt inhibitors, IL-2 neutralization caused a slight decrease of nuclear ANXA1 and a decay of migration (Figure 5E and 5F, respectively).

Altogether these experiments indicate that both ANXA1 and IL-2 autocrine signaling acts on the amplification of PI3K/Akt pathway in MDA-MB-231 cell line.

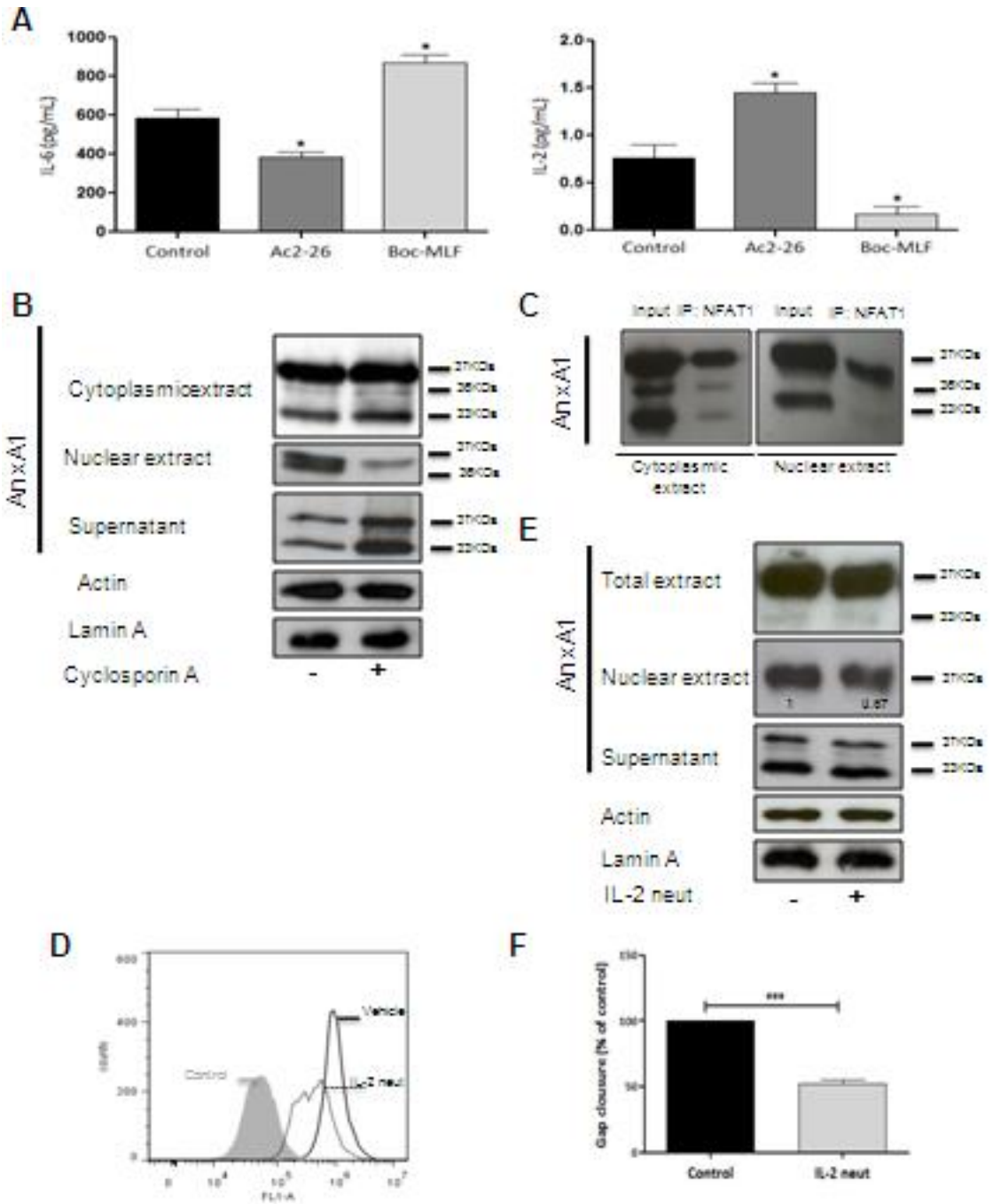


Figure 5. Autocrine ANXA1 signaling through FPR1 induces an IL-2 /PI3K/Akt autocrine loop in MDA-MB-231 cells. A. IL-2 and IL-6 expression by MDA-MB-231 cells treated with Ac2-26, Boc-MLF or by untreated cells was analyzed by flow cytometry by using Th1-Th2-Th17 CBA kit (BD Pharmingen TM). B. Western blotting analysis of ANXA1 expression in supernatants, cytoplasmic and nuclear protein extracts in MDA-MB-231 treated with Cyclosporin A (10 μ M) or with vehicle only. C. Immunoprecipitation analysis of interaction of ANXA1 with NFAT. Immunoprecipitation was performed using anti NFAT antibody. NFAT/ANXA1 interaction was revealed by Western blotting analysis using anti ANXA1 antibody. D. Levels AKT1 phospho S⁴⁷³, in cells treated with neutralizing anti IL-2 antibody or with vehicle only, were measured by flow cytometry. E. Western blotting ANXA1 expression analysis in cytoplasm, nuclei and supernatants of cells treated with neutralizing anti IL-2 antibody or with vehicle only. Numbers represent nuclear ANXA1 quantification by using Image J software. F. Wound healing assay of MDA-MB-231 treated with neutralizing anti IL-2 antibody or vehicle only. Data are representative of experiments run in triplicate. Bars represent SD*, p<0.05.

Association between ANXA1 and EGFR pathways in BC patient samples.

By using human TMAs containing 144 BC tissue samples with 49 cases of primary tumors and matched lymph node metastasis, we found that in normal breast tissue both ANXA1 and EGFR are mainly expressed in the myoepithelial cell layer (Figure 6A). With the aid of pathologist's visual score we choose a lavg cut-off of 185 to determine positive and negative staining. The ANXA1 expression is significantly higher in TNBC when compared to ER+ BCs (p= 0.0014) with a percentage of ANXA1 positive cases of 83.33% in TNBCs, 68.42% in HER2+ BCs and 53.19% in ER+ cases. (Figure 6B-G; Figure S1A; Table 1). On the contrary, EGFR is expressed at similar levels among all three BC subtypes with 70% positive cases in TNBCs, 54% in HER2+ and 85.1% in ER+ (Figure 6B-G; Figure S1B; Table 2). Among all BC subtypes, we found both double positive (ANXA1+/EGFR+) and double negative cases (ANXA1-/EGFR-). Interestingly, we found that there is a positive correlation between expression of ANXA1 and EGFR in TNBC and HER2+ cases (r=0.60; p<0.0001, McNemar χ^2 p=0.001) with a 10-fold chance of having double positive cases (OR=10.53; p<0.0001; Figure S1C; Table 3).

This result suggests that a cross-talk between ANXA1 and EGFR signaling pathways is indeed very important for BC.

Table 1. ANXA1 expression in breast cancer subtypes. ANXA1 expression was analyzed in human breast cancer tissues by using Iavg system. Percentage of positive cases was determined by using a Iavg cut-off of 185.

Molecular subtypes	Cases	Iavg	Positive cases (Iavg<185)	p value
TN	60 (41.67%)	168.5 ± 2.233	83.33% (50/60)	
HER2	37 (25.70%)	174.5 ± 2.718	68.42% (26/38)	0.0952 (compared to TN) 0.0353 (compared to ER+)
ER +	47 (32.64%)	181 ± 1.667	53.19% (25/47)	<0.0001 (compared to TN)
Total	144			

Table 2. EGFR expression in breast cancer subtypes. EGFR expression was analyzed in human breast cancer tissues by using Iavg system. Percentage of positive cases was determined by using a Iavg cut-off of 185.

Molecular subtypes	Cases	Iavg	Positive cases (Iavg<185)	p value
TN	60 (41.67%)	174.1 ± 1.948	70% (42/60)	
HER2	37 (25.70%)	175.4 ± 3.193	54.05% (20/37)	0.7302 (compared to TN) 0.1561 (compared to ER+)
ER +	47 (32.64%)	170 ± 2.2	85.10% (40/47)	0.1591 (compared to TN)
Total	144			

Table 3. ANXA1 vs EGFR expression in TNBC and HER2+ primary tumors. Human primary breast cancer tissues were analyzed for correlation between ANXA1 and EGFR by using Iavg system. Percentage of single positive, double positive and double negative cases was determined by using a Iavg cut-off of 185.

Status	EGFR Positive	EGFR Negative	Total	OR	p value (OR)	CI95%	Pearson coefficient	Mc Nemar χ^2
AnxA1 Positive	79	21	100					
AnxA1 Negative	5	14	19	10.53	p < 0.0001	3.4 to 32.6	R = 0.6	p = 0.001
Total	84	35	119					

Moreover, by using TMAs of BC primary tumors and matched lymph node metastasis, we found that ANXA1 is significantly higher in metastasis ($p=0.001$; OR=6.53; McNemar χ^2 $p=0.0001$, Figure S1D; Table 4) with 63.26% and 91.84% of ANXA1 positive cases in primary tumors and metastasis, respectively. On the contrary, EGFR expression suffers a slight decrease in metastasis comparing to primary tumor (63.26% vs 69.38%, OR= 0.6233; Table 5). Indeed, despite the majority of metastasis samples being EGFR and ANXA1 positive (OR=19.55, $p=0.05$, McNemar χ^2 $p<0.0001$, Table 5), ANXA1 expression increases in metastasis (13.3% of ANXA1+/EGFR- cases in primary tumor compared to 29.16% in metastasis), probably indicating a percentage of tumors where ANXA1 signaling become independent of EGFR signaling.

In addition, we found that nuclear localization of ANXA1 is significantly higher in metastasis than in primary tumors ($p=0.0095$, Figure S1E), showing the pivotal role of nuclear ANXA1 in metastasis and consistently with our *in vitro* experiments with the TNBC cell line derived from pleural effusion, MDA-MB-231.

By analyzing primary tumors and comparing metastatic to non metastatic samples (based on TNM staging), we found that ANXA1 can be considered a prognostic marker of metastasis development (OR= 3.25, McNemar χ^2 $p=0.0001$, Table 6).

Table 4. ANXA1 in primary tumor vs metastasis. Human primary breast cancers and matched metastatic tissues were analyzed to determine an increase in ANXA1 expression in metastasis by using lavg system. For correlation between metastasis and ANXA1 expression the number of ANXA1 positive and negative cases was determined by using a lavg cut-off of 185.

Status	Primary and matched metastatic carcinoma cases	lavg	Positive cases (lavg<185)	p value	Positive cases	Negative cases	OR	p value (OR)	CI 95%	Mc Nemar χ^2
	49									
Primary tumor		177.4 ± 2.282	63.26% (31/49)	0.001	31	18	6.53	p = 0.001	2.0 - 21.17	p = 0.0001
Metastasis		166.3 ± 2.377	91.84% (45/49)		45	4				

Table 5. ANXA1 vs EGFR expression in metastasis. Human metastatic breast cancer tissues were analyzed for correlation between ANXA1 and EGFR by using lavg system. Percentage of single positive, double positive and double negative cases was determined by using a lavg cut-off of 185.

Status	EGFR Positive	EGFR Negative	Total	OR	p value (OR)	CI 95%	Mc Nemar χ^2
AnxA1 Positive	31	14	45				
AnxA1 Negative	0	4	4	19.55	p = 0.05	0.98 - 387.74	p < 0.0001
Total	31	18	49				

Table 6. ANXA1 as prognostic marker of metastasis development. Human primary breast cancers were divided in metastasis positive and metastasis negative cases by using TNM staging. ANXA1 positive and negative cases were determined by using a lavg cut-off of 185.

Metastasis (based on TNM)	Cases	AnxA1 positive	AnxA1 negative	Positive cases (lavg<185)	OR	p value (OR)	CI 95%	Mc Nemar χ^2
Positive	55	45	10	81.82 (45/55)	3.25	0.007	1.39 - 7.61	p = 0.0001
Negative	62	36	26	58% (36/62)				

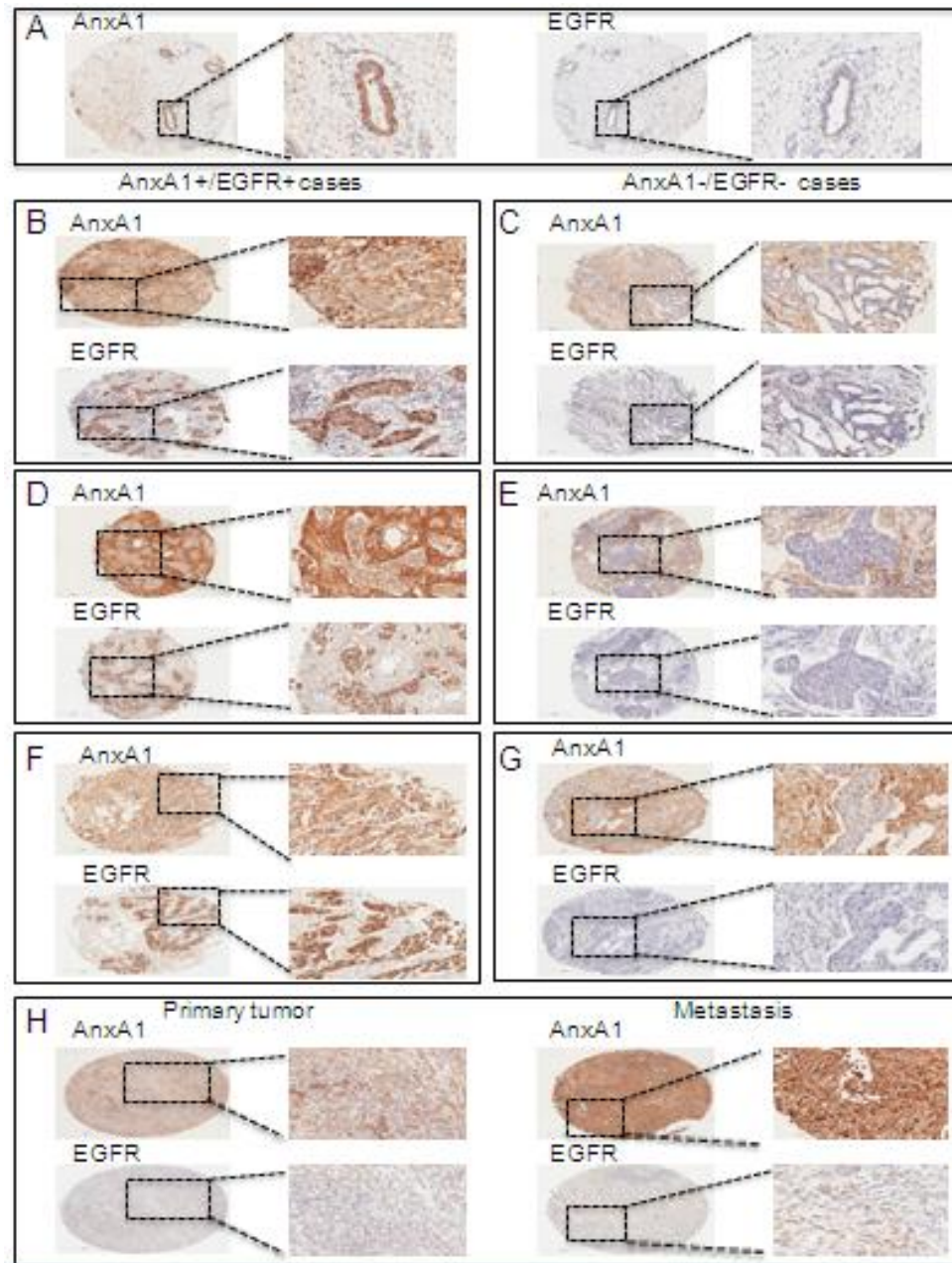


Figure 6. Association between ANXA1 and EGFR expression. Immunohistochemistry of ANXA1 and EGFR expression in normal breast tissue (A), ER+ (B,C), HER2+ (D,E), and TNBC (F, G) BCs. Immunohistochemistry analysis of ANXA1 and EGFR expression in primary tumor (H) and matched metastasis (I). Dotted squares represent areas magnified on the right.

Discussion

A high expression, secretion and nuclear localization of ANXA1 are characteristics of the TNBC cell line MDA-MB-231.

In this work we describe the importance of ANXA1 signaling in TNBC cells. TNBC is the more aggressive type among BCs displaying a high incidence of metastasis and deaths. For these reasons, the search for new drug targets with the intention of inhibiting TNBC cells dissemination is extremely important in order to change the clinical outcomes of these patients. Although existing reports indicate a role of ANXA1 in migration and invasion of BC cells, evidences about how the TNBC subtype takes advantage of ANXA1 signaling are missing. Moreover, considering the activation of pathways in BC, the need for unraveling ANXA1 signaling is still very strong.

In this work we focused our attention on the expression, localization and function of ANXA1 in BC cell lines and patients tissue samples. We found that ANXA1 is highly expressed in the TNBC cell line MDA-MB-231, is strongly localized in the nuclear compartment and massively secreted. We also found that these cells express the FPR1, through which, secreted ANXA1 elicits an autocrine signaling. ANXA1 expression in breast cell lines also correlates with EGFR expression. Up to now, our study is the solely demonstration that an active secretion of ANXA1 followed by a constant activation of FPR1 cascade, is a peculiar characteristic of TNBC cell line MDA-MB-231.

We also confirmed in *ex vivo* the predominance of ANXA1 expression in TNBCs (83.33% of cases) and the positive correlation between ANXA1 and EGFR expression in TNBC and HER2+ cases. In addition, we demonstrated that ANXA1 expression is significantly higher in metastasis compared to primary tumors and that expression of ANXA1 in primary tumors correlates with metastasis development. The 3.25 odds ratio value of ANXA1 expression between metastatic vs non-metastatic cancers, towards metastatic cases, suggests that ANXA1 represents an independent prognostic marker in BC. By using TMAs of primary tumors and matched lymph node metastasis and, accordingly to the fact that MDA-MB-231 cells are derived from pleural effusion, we confirmed that nuclear ANXA1 could exert a predominant function

in BC metastasis. Altogether, our findings about expression and localization of ANXA1 demonstrate its relevance as a marker of aggressiveness of BC. ANXA1 could be used as a prognostic marker of metastasis formation and could help pathologists to distinguish, among BC subtypes, patients with better or worse prognosis.

ANXA1 autocrine signaling is essential for the activation of a PI3K/Akt/IL-2 loop in the TNBC cell line MDA-MB-231.

We found that FPR1 autocrine stimulation by ANXA1 activates PI3K/Akt pathway, which is essential for cytoplasmic ANXA1 to migrate to the nucleus. Therefore, the rate of ANXA1 secretion is directly correlated with the amount of ANXA1 localized into nuclei. As verified by bioinformatics tools (Goldberg *et al.*, 2014), ANXA1 lacks a nuclear localization signal (NLS) which is consistent with what reported for ANXA2 (Madureira *et al.*, 2012). Therefore, our hypothesis was that ANXA1 needs to form a complex with other proteins displaying an NLS to migrate to the nucleus. In this work, we discovered one of the possible ways through which ANXA1 localizes in the nuclear compartment. Indeed, it seems that ANXA1 takes advantage of the nuclear localization domain of NFAT to migrate to the nucleus, where it probably aids the NFAT transcription factor activity resulting in increased IL-2 expression. IL-2, in turns, activates even more the PI3K/Akt pathway by eliciting an additional autocrine signaling (Figure 7). The activity of ANXA1 in inducing IL-2 secretion seems to be peculiar of TNBC cells since no increase of IL-2 was observed in MCF-7 cells.

The activity of ANXA1 in increasing IL-2, as well as IFN- γ , was already been described to be important for Th0 lymphocyte to differentiate in Th1 lineage (D'acquisto *et al.*, 2007). Moreover, the existence of an autocrine signaling of IL-2 in tumor cells was already demonstrated for cervical cancer (Rangel-Corona *et al.*, 2010). Although with a poor understood scenario, indications about the participation of IL-2 in an autocrine loop in BC existed. Interestingly, it has been shown a correlation of hypoxia-dependent induction of IL-2 and TNF- α in MCF-7 cells with the activation of an autocrine signaling capable of stimulating MCF-7 cells migration (Voss *et al.*, 2011). Differently

from ER+ BC (MCF-7 cells), we demonstrated that TNBC cells probably present a constitutively activated IL-2 autocrine signaling induced by ANXA1. Although IL-2 is a cytokine used to activate the immune system against cancer cells (Payne *et al.*, 2014; Rosenberg, 2014), based on our results IL-2 therapy wouldn't be a good option for metastatic TNBC treatment. In the context of TNBCs, IL-2 could potentially induce metastasis formation due to the activation of PI3K.

Consistently with other studies indicating the essential role of PI3K activation in BC (Zhao *et al.*, 2014), we can conclude that the effect of ANXA1 signaling on the aggressiveness of these cells profoundly depends on PI3K/Akt pathway.

FPR1 activation transactivates EGFR in the TNBC cell line MDA-MB-231.

EGFR constitutive activation and Src overexpression are common features of carcinomas (Mao *et al.*, 1997). Several reports indicate the essential role of the tyrosine kinase Src in the phosphorylation and activation of EGFR in cancer (Huang, J. *et al.*, 2007; Formisano *et al.*, 2014). In particular, tyrosine residues 845, 992 and 1086 are involved in Src-dependent EGFR activation (Mueller *et al.*, 2008). As reported for glioblastoma (Huang, J. *et al.*, 2007), we discovered that in TNBC cells FPR1 activation by ANXA1 activates the EGFR by phosphorylating tyrosine 992, such phosphorylation, in turns, lead to ERK phosphorylation and increased cell survival. Based on our finding about EGFR and ANXA1 positive correlation in BC tissue samples, we suggest that the crosstalk between EGFR and ANXA1 is essential in BC aggressiveness, resembling *in vitro* conditions (Figure 7). The effect of ANXA1 signaling in inducing ERK phosphorylation in TNBC is consistent with the finding that in heat-induced growth arrest conditions, ANXA1 protects MCF-7 cells from growth arrest through ERK activation (Nair *et al.*, 2010). The difference in comparison to ER+ BCs is that in the TNBC subtype the FPR1-EGFR-ERK pathway and FPR1-PI3K-IL-2 loop are constitutively activated without the need of heat-induction.

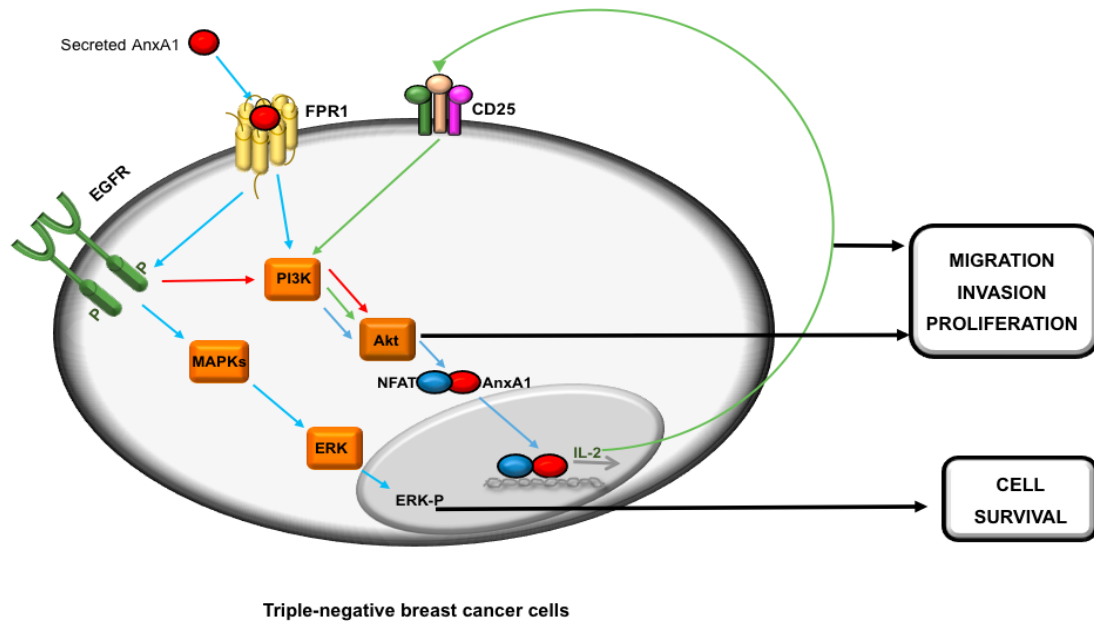


Figure 7. Schematic representation of ANXA1 signaling in TNBC cells. ANXA1 secreted by TNBC cells acts in an autocrine fashion by stimulating FPR1 and the subsequent activation of PI3K/Akt. PI3K activation results in ANXA1 nuclear localization together with NFAT transcription factor. NFAT-ANXA1 complex is able to transcribe the IL-2 gene; the following increase in IL-2 autocrine signaling leads to a further activation of PI3K/Akt pathway. On the other side, activated FPR1 activates EGFR leading to ERK activation and to an ulterior activation of PI3K/Akt pathway.

Conclusion

In this work we have discovered that TNBC cells display two constitutive autocrine signaling pathways. It is likely that ANXA1 signaling, involving FPR1, acts first by increasing the IL-2 autocrine signaling. In this way, the constitutive ANXA1 autocrine signaling acts amplifying the activation of PI3K/Akt pathway. The fact that these signaling pathways are activated in an autocrine manner possibly explains the independence of TNBCs aggressiveness from environmental conditions. In addition, the parallel and constitutive transactivation of the EGFR by FPR1 makes TNBC cell survival independent of growth factor stimulation.

Because of the essential role of PI3K activation in malignancy of BC, innovative therapies using PI3K inhibitors have started. Unfortunately, a lot of side effect frequently manifest upon PI3K inhibitors treatment (Chia *et al.*, 2015). Taking into consideration the essential role of ANXA1 autocrine signaling in activating the MAPK and PI3K/Akt pathways in TNBC cells, we speculate about the possibility of using neutralizing anti-ANXA1 antibodies or FPR1 inhibitors as a less toxic alternative to PI3K inhibitors. In conclusion, this study shed light not only about the importance of the ANXA1/FPR1 signaling pathway in advanced BC, but it also demonstrates that ANXA1 detection in BC may be considered as an independent prognostic factor. Finally, we propose the inhibition of ANXA1 secreted form as a good target to improve patients clinical outcome.

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Supplementary material

Western blotting.

To prepare nuclear and cytoplasmic extracts NE-PER Nuclear and Cytoplasmic Extraction Reagent kit (Thermoscientific) was used. Supernatants of cells treated for 24 hours were collected. Identical amounts of protein extracts and supernatants were separated eletrophoretically on 10% SDS-PAGE and transferred onto nitrocellulose membrane (Hi-Bond C; Amersham GE). Membrane were blocked in 5% milk for 1 hour and incubated with anti ANXA1 (1:1000, Invitrogen), anti EGFR (1:1000, Sigma) anti Actin (1:3000, Sigma) and anti Lamin A (1:1000, Abcam). Anti mouse and anti rabbit HRP-conjugated secondary antibodies were used (1:3000, Sigma).

Formulas used.

Closure of the wound in wound healing assay was measured by using Image J software and the formula F1 was used (G0 = wound size at time 0 h; G24= wound size at time 24 h).

$$F1: \text{Wound closure (\% of control)} = \frac{G0 - G24h \text{ (cultured cells)}}{G0 - G24h \text{ (control)}} \times 100$$

The inhibition of invasiveness potential in invasion assay was calculated using the formula F2.

$$F2: \text{Invaded cells (\% of control)} = \frac{\text{Number of treated invasive cells}}{\text{Number of invasive control cells}} \times 100$$

Quantification of Immunohistochemistry staining (Iavg) with PixelCount V9.0 algorithm.

The algorithm, PixelCount V9.0, quantifies the brown stain intensity pixel by pixel in a continuous scale from 0 to 256 that represents light absorbance. In such way it classifies stainings as negative (>220), weak (220-

175), moderate (175-100) and strong (100-0) according to default input parameter indicated by the manufacturer. Averages of positive and negative pixel intensities are used to calculate the Intensity average level of tumor selected area (F3).

$$F3: \quad I_{avg} = \frac{I_{wp} + I_p + I_{sp}}{N_{wp} + N_p + N_{sp}}$$

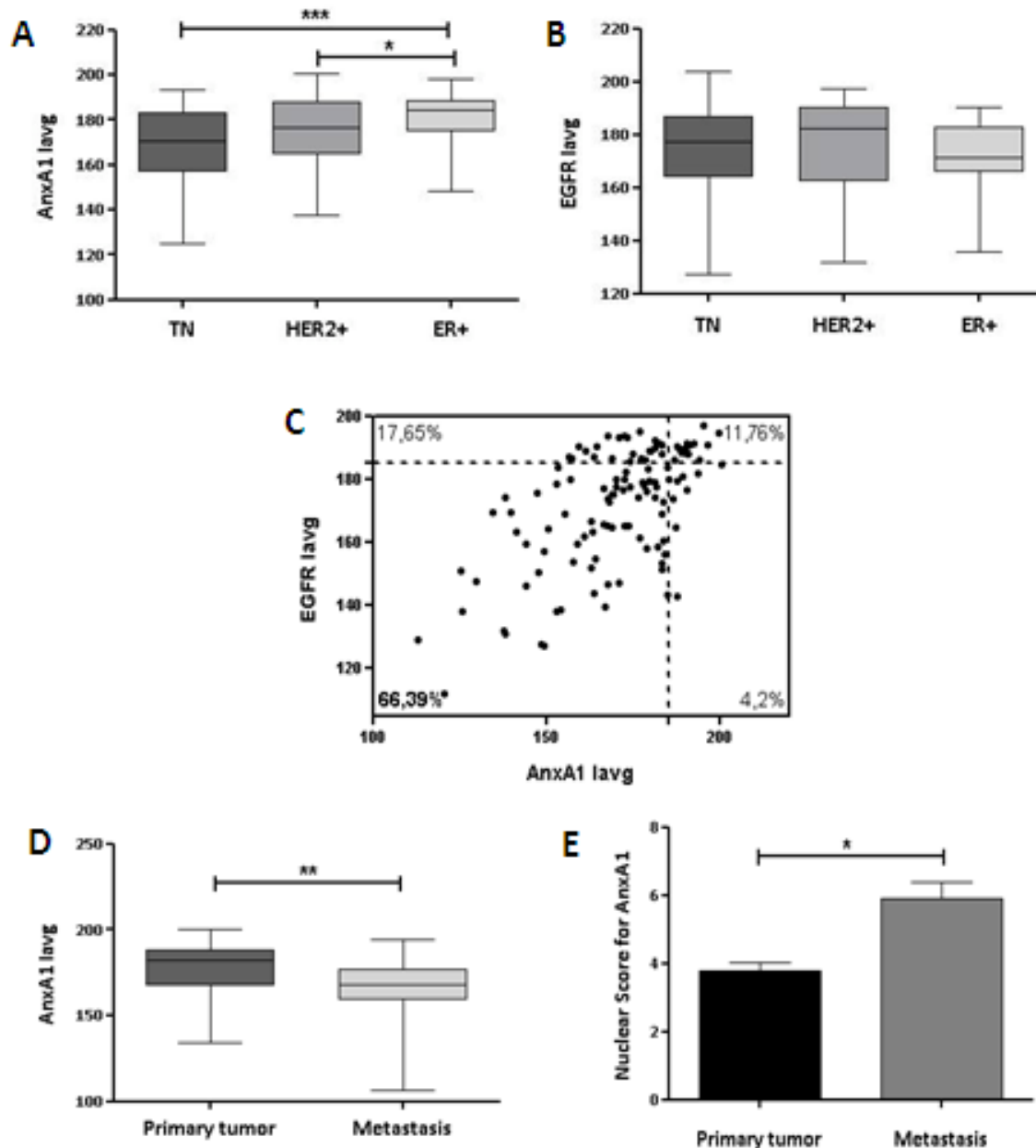
(I_{wp} = Intensity of weak positive pixels, I_p= Intensity of positive pixels, I_{sp}= Intensity of strong positive pixels, N_{wp}= Number of weak positive pixels, N_p= Number of positive pixels, N_{sp}= Number of strong positive pixels)

Quantification of Immunohistochemistry staining (Iavg) with IHC Nuclear V1 algorithm.

The intensity of nuclear expression of Annexin A1 was determined by using the algorithm IHC Nuclear V1 that quantifies pixels contained in round-oval structures recognized for being nuclei. Intensity values (1-3) and percentages of cells with positive nuclear staining were used for calculating nuclear score accordingly to matrix of Supplementary Table.

Supplementary Table. Matrix for nuclear score determination

SCORE	Percent of cells	Intensity			
			1	2	3
		0-25%	3	6	9
		25 - 100%	4	7	10



Supplementary Figure: Human TMAs staining analysis by using lavg measurement.

Analysis in breast cancer tissue samples of ANXA1 (A) and EGFR staining (B), expressed as lavg, comparing breast cancer subtypes. lavg values are indicated by Box-Whisker plot. *, $p < 0.05$, ** $p < 0.01$ compared to TNBCs C. Correlation between ANXA1 and EGFR in Her2 positive and TNBC primary tumors, $p < 0.001$ D. ANXA1 expression analysis between primary tumor and matched metastasis is indicated by Box-Whisker plot. *, $p < 0.05$. E. Nuclear ANXA1 expression analysis between primary tumor and matched metastasis. *, $p < 0.05$. Higher lavg levels corresponds to lower staining and viceversa.