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Impacto do Microambiente Hipóxico e/ou Privado de Soro sobre o Glicofenótipo de Modelos *in vitro* do Câncer de Mama

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AMANDA PINHEIRO DE BARROS ALBUQUERQUE

Impacto do Microambiente Hipóxico e/ou Privado de Soro sobre o Glicofenótipo de Modelos *in vitro* do Câncer de Mama

Tese de Doutorado apresentada ao Programa de Pós-Graduação em Inovação Terapêutica da Universidade Federal de Pernambuco, como pré-requisito para a obtenção do Título de Doutora em Inovação Terapêutica

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Aprovada em 15/03/2018

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RESUMO

O câncer de mama é responsável pelo maior número de mortes relacionadas ao câncer na população feminina mundial. Neste cenário, a elevada heterogeneidade molecular associada à aquisição de fenótipos resistentes torna a caracterização dos mecanismos envolvidos na progressão tumoral essencial para avanços no diagnóstico, prognóstico e terapêutica deste câncer. Presente em aproximadamente 40% dos tumores mamários, o microambiente hipóxico privado de nutrientes, é relacionado ao prognóstico reservado e regulação de genes associados a progressão tumoral, metástase e resistência a apoptose. Neste microambiente a regulação da glicosilação foi proposta como fator associado a aquisição de fenótipo resistente no câncer de mama. Por outro lado, a aquisição do glicofenótipo aberrante e a progressão do câncer de mama no microambiente hipóxico, quanto a regulação de fucosiltransferases e sialiltransferases, permanecem pouco exploradas. Este estudo objetivou avaliar o impacto do microambiente hipóxico e/ou privado de soro sobre a regulação de glicosiltransferases e do glicofenótipo em modelos *in vitro* do câncer de mama, bem como identificar *'housekeeping genes'* adequados para normalização de análises de expressão sobre condições de hipóxia e privação de soro. Células tumorais mamárias T47D, MCF7 e MDA-MB-231 foram submetidas as condições de hipóxia e privação de soro em diferentes períodos de exposição ao longo de seis dias. Sob condições de normóxia (controle) as células foram mantidas em estufa de CO_2. Os resultados indicaram os genes ACTB/PPIA como a mais estável combinação dos *'housekeeping genes'*. A regulação da FUT3, sobre condições de hipóxia e privação de soro, está envolvida com a aquisição da resistência a apoptose e promoção do fenótipo metastático. A hipóxia e a privação de soro induziram alterações na morfologia e na expressão de marcadores de transição epitélio-mesenquimal. Alterações significativas na expressão de fucosiltransferases e sialiltransferases, potencialmente envolvidas na síntese de antígenos carboidratos associados ao tumor, foram induzidas pela hipóxia e privação de soro. A análise bioinformática revelou que a expressão de glicosiltransferases induzidas pela hipóxia e privação de soro nas células MDA-MB-231 foi semelhante àquela observada em tumores mamários do subtipo *basal-like*. O aumento da α2,6-sialilação e da α1,3/-4/-6-fucosilação revelou os efeitos da hipóxia e da privação de soro na promoção do glicofenótipo aberrante nas células do câncer

de mama. Os resultados evidenciam a importância do microambiente hipóxico na regulação do glicofenótipo das células da câncer de mama, indicam seu envolvimento na promoção do fenótipo metastático e seu potencial na pesquisa por moléculas com aplicação diagnóstica, prognóstica e terapêutica.

Palavras-chave: Câncer de mama. Fucosiltransferases. Glicofenótipo aberrante. Hipóxia. Privação de soro. Sialiltransferases.

ABSTRACT

Breast cancer is responsible for most deaths directly related to cancer among the world female population. In this scenario, the high molecular heterogeneity associated with the acquisition of resistant phenotypes makes the characterization of the processes on a tumor progression essential to the diagnosis approaches, prognosis and cancer therapy. Found in almost 40% of breast tumors, the hypoxic microenvironment and deprived of nutrients is related to the poor prognosis and regulation of genes associated with tumor progression, metastasis and resistance to apoptosis. In this microenviroment, the hypoxic glycosylation regulation was associated with the acquisition of breast cancer resistant phenotypes. On the other hand, the acquisition of the aberrant glycophenotype in the hypoxic microenvironment during breast cancer progression, especially the fucosyltransferases and sialyltransferases regulation, remain unexplored. This study aimed to evaluate the impact of the hypoxic environment and/or serum deprivation under the glycosyltransferases and glycophenotype regulation in breast cancer models *in vitro* as well also identify appropriated housekeeping genes to normalize the analysis of expression under hypoxic and serum deprivation conditions. Breast tumor cells T47D, MCF7 and MDA-MB-231 were exposed to hypoxia and serum deprivation conditions in distinct periods for six days. Under normoxia (control) the cells were kept in CO_2 incubator. The results indicate ACTB/PPIA genes as the most stable combination of 'housekeeping genes'. The regulation of FUT3, under hypoxia and serum deprivation, is associated to the apoptosis resistance capability and the metastatic phenotype. The hypoxia and serum deprivation had induced morphological modifications and the epithelial mesenchymal transition markers expression. Fucosyltransferases and sialyltransferases alterations, potentially involved in the carbohydrate antigen associated to tumor synthesis, were induced by hypoxia and serum deprivation. The bioinformatics revealed that glycosyltransferases expression induced by hypoxia and serum deprivation in MDA-MB-231cells were like the one observed in basal-like subtype breast tumors. The increase of α2,6-sialylation e da α1,3/-4/-6-fucosylation revealed the effects of hypoxia and serum deprivation on aberrant glycophenotype promotion in breast cancer cells. The results showed the importance of hypoxic microenvironment in the glycophenotype regulation on breast cancer cells, indicate its participation on metastatic phenotype,

and its potential in researches on diagnostic, prognostic and therapeutically molecules.

Keywords: Breast cancer. Fucosyltransferases. Aberrant glycophenotype. Hypoxia. Serum deprivation. Sialyltransferases

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FUT3 characterization in hypoxic microenvironment: regulation and possible impact in breast cancer biology

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Identification of appropriate housekeeping genes for quantitative RT-qPCR analysis in MDA-MB-231 cells lines under hypoxic and serum deprivation conditions

Hypoxia and serum deprivation induces glycan alterations in MDA-MB-231 cells

LISTA DE ABREVIAÇÕES E SIGLAS

AAL	*Aleuria alanturia lectin*
cDNA	DNA complementar
DAPI	Do inglês *Diamidine Phenyl Indole*
ER	Receptor de estrógeno, *do inglês estrogen receptor*
Fuc	Fucose
FUT	Fucosiltrasnferases
Gal	Galactose
GalNAc	N-acetil-galactosamina
Glc	Glicose
GlcNAc	N-acetil-glicosamina
GLUT1	Transportator de Glicose 1, do inglês *Glucose transporter 1*
HER2	Receptor de fator de crescimento epidermal humano do tipo 2, do inglês *human epidermal growth factor receptor 2*
HIF	Fator Induzido por Hipóxia, do inglês *hypoxia inducible factor*
HIF-1α	Fator Induzido por Hipóxia 1α, do inglês *hypoxia inducible factor 1α*
PCR	Reação em cadeia de polimerase
PR	Receptor de progesterona, do inglês *progesteron receptor*
RNA	Ácido ribonucleico
RT-qPCR	PCR em tempo real quantitativa
sLe	Antígeno de Lewis sialilados, do inglês *sialil Lewis antigen*
SNA	*Sambucus nigra agglutinin lectin*
ST	*Sialiltransferases*
ST3Gal	β-galactosideo α-2,3-sialiltransferase
ST6GalNAc	α-N-acetilgalactosamina α-2,6-sialiltransferase
STn	Antígeno STn
T	Antígeno T
Tn	Antígeno Tn
TNBC	Do inglês *Triple Negative Breast Cancer*
TRAIL	*Do inglês* TNF-related Apoptosis-inducing Ligand

SUMÁRIO

1 INTRODUÇÃO

Com 1,7 milhões de novos casos anuais em todo o mundo, o câncer de mama é o segundo câncer mais incidente e representa a quinta causa de morte entre todos os tipos de cânceres diagnosticados (FERLAY et al., 2015). Na população feminina mundial e brasileira é o câncer mais incidente e responde pelo maior número de mortes relacionadas ao câncer (FERLAY et al., 2015; INCA, 2016).

Apesar do relativo bom prognóstico, mediante diagnóstico e tratamento oportunos, a elevada heterogeneidade molecular e metabólica dos subtipos intrínsecos, assim como as elevadas taxas de recorrência e resistência as terapias tradicionais representam um desafio terapêutico e evidenciam a necessidade de pesquisas para melhor entendimento dos fatores associados a tamanha complexidade (BLOWS et al., 2010; ANAMPA; MAKOWER; SPARANO, 2015; DAI et al., 2015; SHAJAHAN-HAQ; CHEEMA; CLARKE, 2015).

Entre os fatores especificamente envolvidos com a complexidade do câncer de mama, a hipóxia está presente em 40% dos carcinomas mamários diagnosticados e tem sido associada com prognóstico reservado, resistência terapêutica, elevado risco de metástase e recidiva, e com a diminuição da sobrevida global (LUNDGREN; LANDBERGÉ, 2007; KAYA et al., 2012; JOHNSON, 2013; JIN et al., 2016; EL GUERRAB et al., 2017).

A ativação da sinalização HIF-1 tem grande relevância na reprogramação metabólica associada ao câncer por regular eventos centrais do efeito de Warburg, entre os quais estão a glicólise aeróbica e a indução transcricional dos genes relacionados aos transportadores de açúcar e enzimas glicolíticas (NATSUIZAKA et al., 2007; ZHAO; KEATING, 2007). Em vários tipos tumorais, estas alterações metabólicas sobre condições hipóxicas têm sido associadas a indução de antígenos carboidratos associados ao tumor (KANNAGI, 2004; KOIKE et al.,2004; NATSUIZAKA, et al., 2007; NATONI et al., 2014; DEBERARDINIS; CHANDEL, 2016), fato que evidencia a relação entre este microambiente e a regulação da aberrante glicosilação associada ao câncer.

Considerada uma das mais predominantes modificações associadas ao câncer, a glicosilação aberrante regula vários eventos da progressão tumoral e frequentemente resulta de alterações na expressão e na atividade das

glicosiltransferases (PINHO; REIS, 2015; ASHKANI; NAIDOO, 2016). Entre os tipos de glicosilação aberrante observados no câncer, as alterações nos perfis de sialilação e fucosilação são cruciais durante a progressão tumoral por regular a expressão antígenos carboidratos associados ao tumor, o fenótipo invasivo e a apoptose das células tumorais (HIGAI et al., 2006; GOMES et al., 2013; CARRASCAL et al., 2017).

No câncer de mama, estudos evidenciaram o relevante papel da glicosilação aberrante por revelar que alterações nas estruturas glicanos e nos padrões de N- e O-glicosilação estão significativamente associados com a agressividade tumoral, sobrevida dos pacientes, bem como têm utilidade como marcadores para estratificação prognóstica e tratamento (POTAPENKO et al., 2015; HAAKESEN et al., 2016); e que o perfil de expressão de glicosiltransferases, especialmente fucosiltransferases e sialiltransferases, está associado com a classificação dos subtipos moleculares e correlacionado com dados clínicos e a taxa de sobrevida dos pacientes (ASHKANI; NAIDOO, 2016).

Neste contexto, Greville e colaboradores (2016) sugeriram que a hipóxia provavelmente regula genes relacionados a glicosilação bem como afeta o perfil de glicanos das células tumorais, o que pode estar envolvido com a aquisição de fenótipo maligno e resistência as terapias convencionais no câncer de mama. Além disso, embora a aberrante sialilação e fucosilação estejam significativamente correlacionadas com aumento da proliferação, infiltração linfonodal, fenótipo invasivo e prognóstico reservado no câncer de mama (KYSELOVA et al 2008; DOS SANTOS et al., 2014; WI et al.,2016), os efeitos do microambiente tumoral, particularmente do microambiente hipóxico e privado de nutrientes, sobre a regulação da glicosilação neste câncer permanecem pouco explorados.

Em alguns tipos tumorais, os efeitos da hipóxia sobre a regulação da glicosilação durante a aquisição de fenótipos malignos foram evidenciados através da evasão a resposta imune (NONAKA et al., 2014); regulação da adesão, migração e proliferação (SHIOZAKI et al., 2011); e promoção da invasão e metástase (KOIKE et al., 2004; PEIXOTO et al., 2016). Ademais, os efeitos da hipóxia sobre a regulação de glicosiltransferases e do glicofenótipo associados ao papel da privação de soro sobre a glicosilação e a quimiorresistência das células tumorais (BRITAIN; DORSETT; BELLIS, 2017; YAKISICH et al., 2017) tornam a junção destes dois

fatores importante ferramenta para mimetizar o microambiente tumoral hipóxico em análises *in vitro* (JUNG et al., 2015; WU et al., 2015).

Nesse sentido, embora a cultura de células não possa decifrar a heterogeneidade dos glicanos em sua total complexidade (CHRISTIANSEN et al., 2014), a ampla similaridade do perfil glicoproteico e da glicosilação de superfície entre os modelos *in vitro* e os diversos subtipos moleculares do câncer de mama (TÃO et al., 2008; YEN et al., 2011) torna o uso destes modelos importante ferramenta para melhor compreensão da regulação da expressão de glicosiltransferases, glicanos de superfície celular bem como permite avaliar o impacto de fatores do microambiente tumoral sobre a aberrante glicosilação, o desenvolvimento e progressão deste tipo câncer.

Diante do exposto, a presente tese teve como objetivo avaliar o impacto do microambiente hipóxico e/ou privado de soro sobre a regulação de glicosiltransferases e do glicofenótipo em modelos *in vitro* do câncer de mama, bem como identificar *'housekeeping genes'* apropriados para normalização de análises de expressão sobre condições de hipóxia e privação de soro.

1.1 OBJETIVOS

1.1.1 Objetivo geral

Avaliar o impacto do microambiente hipóxico e/ou privado de soro sobre a regulação de glicosiltransferases e do glicofenótipo em modelos *in vitro* do câncer de mama.

1.1.2 Objetivos específicos

- Avaliar a expressão de FUT3 em células tumorais mamárias submetidas as condições de hipóxia e/ou privação de soro;
- Identificar apropriados *'housekeeping genes'* para análises de expressão gênica em células tumorais mamárias submetidas as condições de hipóxia e/ou privação de soro;

- Avaliar a expressão de marcadores da transição epitélio-mesenquimal em células tumorais mamárias submetidas as condições de hipóxia e/ou privação de soro;
- Avaliar a expressão de glicosiltransferases (10 fucosiltransferases e 6 sialiltransferases) potencialmente envolvidas na síntese de antígenos carboidratos associados ao tumor em células tumorais mamárias submetidas as condições de hipóxia e/ou privação de soro;
- Avaliar o glicofenótipo de células tumorais mamárias submetidas as condições de hipóxia e/ou privação de soro.

2 REVISÃO DA LITERATURA

2.1 CÂNCER DE MAMA: HETEROGENEIDADE, METABOLISMO E FENÓTIPOS RESISTENTES

O câncer de mama é o segundo câncer mais incidente, com 1,7 milhões de novos casos em todo o mundo, representando a quinta causa de morte entre todos os tipos de cânceres diagnosticados (FERLAY et al., 2015). Na população feminina mundial e brasileira, é o câncer mais incidente e responde pelo maior número de mortes relacionadas ao câncer (FERLAY et al., 2015; INCA, 2016). Além da elevada incidência e mortalidade, o câncer de mama é uma doença altamente complexa com características patológicas específicas e heterogêneo comportamento biológico (TANG; WANG; BOURNE, 2008; SPITALE et al., 2009).

A heterogeneidade dos fatores de risco, apresentação clínica, características histopatológicas e respostas as terapias sistêmicas observadas entre os diferentes subtipos moleculares tem ampla utilidade prognóstica, preditiva e terapêutica, assim como resultou no desenvolvimento de vários sistemas de classificação (DESMEDT; SOTIRIOU; PICCART-GEBHART, 2009; BLOWS et al., 2010; IWAMOTO; PUSZTAI, 2010; REIS-FILHO et al., 2010; VALLEJOS et al., 2010; WEIGELT et al., 2010).

O câncer de mama é comumente classificado de acordo com critérios morfológicos (ductal, lobular, invasivo ou in situ); o perfil de expressão de receptores (receptor de estrógeno (ER), receptor de progesterona (PR), receptor do fator de crescimento epidermal 2 (HER2) ou triplo negativo (TNBC)); e os subtipos intrínsecos (superexpressão de HER2, luminal A, luminal B e triplo negativo) (PEROU et al., 2000; SORLEI et al., 2001; DAI et al., 2015). Além disso, as alterações metabólicas observadas entre os subtipos moleculares podem ser úteis para o desenvolvimento de novas abordagens terapêuticas, melhorar a compreensão a respeito dos mecanismos resistência e fornecer novos métodos para o diagnóstico precoce e monitoramento deste câncer (SHAJAHAN-HAQ; CHEEMA; CLARKE, 2015).

Presente em aproximadamente 15% dos cânceres de mama diagnosticados, a amplificação e/ou superexpressão de HER2 está associada a resultados clínicos inferiores àqueles observados nos subtipos luminal e semelhantes àqueles observados no subtipo TNBC (CAREY et al., 2006; WIRAPATI et al., 2008; HOWLADER et al., 2014). Em geral, pacientes com superexpressão de HER2 são

tratados com terapia anti-HER2 combinada com quimioterapia (SERKUS et al., 2013). Recentemente foi evidenciado que o tratamento com terapia endócrina pode aumentar a expressão de HER2 (ELIA et al., 2016) e que o tratamento com trastuzumab só é eficaz em aproximadamente 30% dos pacientes, fato que evidencia a necessidade de terapias mais eficazes dado a elevada complexidade deste subtipo e a aquisição de fenótipo resistente (ROMOND et al., 2005; VU; CLARET, 2012).

Metabolicamente, modelos tumorais *in vitro* com superexpressão de HER2 exibem elevado metabolismo glicolítico (WALSH et al., 2013; O'NEAL et al., 2016). Em amostras tumorais deste subtipo molecular a expressão do transportador de glicose 1 (GLUT1) foi reduzida quando comparada aquela observada no subtipo TNBC e maior do que aquela observada no subtipo luminal (DOYEN et al., 2014).

O subtipo luminal representa 70% dos cânceres de mama diagnosticados (HOWLADER et al, 2014) e pode ser dividido nos subgrupos intrínsecos luminal A e luminal B (SORLIE et al., 2001). Os tumores Luminal A são geralmente ER, PR e HER2 positivos, enquanto os tumores luminal B são comumente ER e/ou PR positivos e HER2 negativos (DAI et al., 2015). Os tumores do subtipo luminal são em sua maioria bem diferenciados, menos agressivos e associados a um prognóstico mais favorável e melhor resultado pós cirúrgico (PUTTI et al., 2005; DUNNWALD; ROSSING; Li, 2007) quando comparado aos subtipos HER2 e TNBC (ONITILO et al., 2009).

Comumente, pacientes diagnosticados com o subtipo luminal são tratados com terapia endócrina associada ou não a quimioterapia (SERKUS et al., 2013). No entanto, embora a terapia endócrina seja eficaz em bloquear a proliferação tumoral através de alterações na sinalização ou biossíntese do estrógeno (DUFFY, 2006; RUSSO; RUSSO, 2006), a ineficácia do tratamento com tamoxifeno é observada em 66% dos tumores ER+/PR-, 55% dos tumores ER-/PR+ e 25% dos tumores ER+/ PR+ (CLARKE te al., 2003).

Além do seu envolvimento na proliferação tumoral, o estrógeno também regula o metabolismo das células do subtipo luminal de modos que sob elevada concentração de glicose ele estimula a glicólise e suprime o ciclo do ácido tri-carboxílico; sob concentrações fisiológicas de glicose ele suprime a glicólise e estimula o ciclo do ácido tri-carboxílico (O'MAHONY et al., 2012); e sob reduzida

concentração de glicose há indução da resistência à apoptose através da regulação da autofagia (KRETOWSKI et al., 2016).

Em comparação com o TNBC, o subtipo luminal exibe distintas características metabólicas, especialmente relacionada a indução do efeito reverso de Warburg, onde as células tumorais induzem a produção de lactato e piruvato em fibroblastos associados ao câncer para manter o metabolismo glicolítico (PAVLIDES et al., 2009). Esta reprogramação metabólica promove a liberação de peróxido de hidrogênio pelos fibroblastos, o qual por sua vez, regula a glicólise aeróbica e a produção de lactato através da ativação do fator de transcrição induzido por hipóxia 1 α (HIF-1α) (MARTINEZ-OUTSCHOORN et al., 2010; MARTINEZ-OUTSCHOORN et al., 2011).

Com elevada frequência em pacientes mais jovens e resultados clínicos reservados (FOULKES; SMITH; REIS-FILHO, 2010), os tumores triplo negativos representam aproximadamente 10 a 25% dos cânceres de mama diagnosticados (PEROU, 2011; HO-YEN; BOWEN; JONES, 2012; HOWLADER et al., 2014), são ER, PR e HER2 negativos e, em geral são subclassificados como *basal-like* e *normal-like* ou *claudin-low* (RAKHA et al., 2009; HO-YEN; BOWEN; JONES, 2012).

O subtipo *basal-like* representa cerca de 70% dos tumores TNBC (RAKHA et al., 2009), caracteriza-se pela elevada expressão de genes associados as citoqueratinas, integrinas e a transição epitélio-mesenquimal, bem como apresentam elevada expressão de fatores associados à hipóxia (RAKHA et al., 2009; HO-YEN; BOWEN; JONES, 2012). O subtipo *claudin-low* representa cerca de 7% a 14% dos tumores TNBC e caracteriza-se pela reduzida expressão de claudinas e elevada expressão de genes associados a remodelação da matriz extracelular, a transição epitélio-mesenquimal, a população de células tronco tumorais e ao fenótipo invasivo (PRAT et al., 2010). Ao comparar estes subtipos intrínsecos, o subtipo *basal-like* foi positivamente associado ao *status* de mutação do BRCA1, apresentou melhor resposta à quimioterapia e menor taxa de sobrevida (RAKHA et al., 2009; PRAT et al., 2010).

Até então, devido à ausência da expressão dos receptores hormonais, o subtipo TNBC é clinicamente tratado com quimioterapia e radioterapia (SERKUS et al., 2013). Em geral, vários regimes quimioterápicos são utilizados, os quais geralmente incluem combinações de ciclofosfamida, metotrexato, 5-fluorouracil, taxanos e/ou antraciclinas, e embora a eficácia destes compostos seja observada

em muitos pacientes, elevadas taxas de recorrência e resistência terapêutica ainda são observadas (LANCET, 2005; ANAMPA; MAKOWER; SPARANO, 2015; LEE; DJAMGOZ, 2018).

Metabolicamente, o subtipo TNBC geralmente exibe o efeito de Warburg (KIM et al., 2013), aumento dos transportadores e da captação de glicose (GROHEUX et al., 2011; CHOI et al., 2013; KOO et al., 2014), aumento dos transportadores e da secreção de lactato (DOYEN et al., 2014) e elevada expressão de genes glicolíticos quando comparado aos outros subtipos de câncer de mama (PALASKAS et al., 2011). Neste subtipo, o elevado metabolismo glicolítico está correlacionado com a proliferação e fenótipo agressivo (ELIA et al., 2016) e deve-se, em parte, à amplificação e/ou à superexpressão do fator de transcrição c-Myc (Myc), o qual é superexpresso quando comparado com os subtipos luminal e HER2 e ativa diretamente o GLUT1 e os genes envolvidos na glicólise (OSTHUS et al., 2000; VAN-DAN et al., 2010).

A heterogeneidade metabólica associada aos subtipos moleculares do câncer de mama podem explicar mecanismos de sensibilidade ou resistência terapêutica bem como servir como novos alvos terapêuticos (SHAJAHAN-HAQ; CHEEMA; CLARKE, 2015). Entre os fatores do microambiente tumoral com grande relevância na progressão do câncer de mama e elevado impacto durante a reprogramação metabólica associada ao câncer, a hipóxia intratumoral está diretamente envolvida em diversos eventos que serão descritos nos tópicos subsequentes.

2.2 HIPÓXIA INTRATUMORAL: REPROGRAMAÇÃO METABÓLICA E ABERRANTE GLICOSILAÇÃO

O microambiente tumoral é um complexo sistema composto por um microambiente químico, constituído por pH, PO_2, pequenas moléculas e metabólitos; e um microambiente celular, formado por células tumorais, células estromais e componentes da matriz extracelular (SEMENZA, 2016). Neste sistema, anormalidades estruturais na vasculatura e o rápido crescimento da massa tumoral resultam no estabelecimento de um microambiente hipóxico onde as células tumorais são submetidas a baixa disponibilidade de oxigênio, nutrientes e pH ácido (SEMENZA, 2012).

As estratégias de sobrevivência ao estresse hipóxico são reguladas pela família de fatores de transcrição induzidos por hipóxia (*do inglês, hypoxia inducible factor* (HIF)). Os HIFs são proteínas diméricas compostas por uma subunidade α cuja estabilização é dependente da concentração de O_2 (HIF-1α, HIF-2α, or HIF-3α) e uma subunidade β constitutivamente expressa (HIF-1β). Em condições de normóxia, a subunidade α é degradada via ubiquitina- proteossomo ao passo que sobre condições de hipóxia a estabilização desta subunidade permite sua translocação para o núcleo e subsequente dimerização com a subunidade β para formar o HIF-1 ativo, o qual por sua vez induz a expressão de numerosos genes envolvidos na regulação da angiogênese, do metabolismo e diversos eventos da progressão tumoral (SEMENZA, 2002; SEMENZA, 2012).

Entre os eventos regulados pela sinalização HIF-1 no microambiente tumoral, destacam-se: a renovação das células tronco tumorais (ZHANG et al., 2016); a indução da resistência a quimioterapia (EL GUERRAB et al., 2011); a resistência a apoptose via regulação da autofagia e da sinalização TRAIL (BAGINSKA et al., 2013; KNOLL et al., 2016); a indução da transição epitélio mesenquimal, invasão e metástase (TERRY et al., 2016; YE et al., 2016); e a reprogramação metabólica (LUO et al., 2012; SEMENZA, 2017).

Considerada como novo *hallmark* do câncer, a reprogramação metabólica promove a sobrevivência, proliferação e invasão das células tumorais durante a transformação maligna (DEBERARDINIS; CHANDEL, 2016; PAVLOVA; THOMPSON, 2016; VANDER HEIDEN; DEBERARDINIS, 2017). Esta reprogramação pode manifestar-se através da regulação de vias metabólicas ou oncogenes (HU et al., 2013; SON et al., 2013) e comumente é representada pelo efeito Warburg, no qual muitas células tumorais induzem a glicólise, independente da disponibilidade de oxigênio, resultando no aumento da expressão de transportadores de glicose, enzimas glicolíticas e na elevada produção de lactato (KANNAGI, 2004; LU; TAN; CAI, 2015).

Entre as vias de sinalização envolvidas no efeito de Warburg, a ativação da sinalização HIF-1, seja pelo estabelecimento da glicólise aeróbica ou pela reduzida disponibilidade de O_2 no microambiente tumoral, é conhecida por regular a glicólise aeróbica e alterar o perfil de expressão de várias moléculas glicolíticas específicas (LU; FORBES; VERMA, 2002; MARIN-HERNANDEZ et al., 2009), entre as quais

está a indução transcricional dos genes relacionados aos transportadores de açúcar e enzimas glicolíticas (NATSUIZAKA et al., 2007; ZHAO; KEATING, 2007).

As alterações metabólicas associadas ao efeito de Warburg sobre regulação da hipóxia têm sido recentemente associadas com a indução da expressão de fucosiltransferases e sialiltransferases envolvidas na síntese dos antígenos sialilados de Lewis (sLe) em vários tipos tumorais (KANNAGI, 2004; KOIKE et al.,2004; NATSUIZAKA, et al., 2007; NATONI et al., 2014; DEBERARDINIS; CHANDEL, 2016), fato que evidencia a relação entre este microambiente e a regulação da aberrante glicosilação associada ao câncer.

Aos poucos, os efeitos da hipóxia sobre a regulação da glicosilação durante a aquisição de fenótipos malignos foram evidenciados através da evasão a resposta imune (NONAKA et al., 2014); regulação da adesão, migração e proliferação (SHIOZAKI et al., 2011); e promoção da invasão e metástase (KOIKE et al., 2004; PEIXOTO et al., 2016) em alguns tipos tumorais. Ademais, os efeitos da hipóxia sobre a regulação de glicosiltransferases e do glicofenótipo associados ao papel da privação de soro sobre a glicosilação e a quimiorresistência das células tumorais (BRITAIN; DORSETT; BELLIS, 2017; YAKISICH et al., 2017) tornam a junção destes dois fatores importante ferramenta para mimetizar o microambiente tumoral hipóxico em análises *in vitro* (JUNG et al., 2015; WU et al., 2015).

No entanto, apesar da identificação das alterações na glicosilação induzidas por hipóxia ser considerada uma promissora via para descoberta de novos biomarcadores e intervenções terapêuticas mais eficazes (SILVA-FILHO et al., 2017), ainda é escassa a análise dos efeitos do microambiente hipóxico sobre o glicofenótipo de diversos tipos tumorais, incluindo o câncer de mama.

2.3 HIPÓXIA E SUAS IMPLICAÇÕES NO CÂNCER DE MAMA

As adaptações celulares ao estresse hipóxico envolvem modificações transcricionais associadas a promoção de fenótipo maligno e da resistência a quimioterapia e radioterapia (VAUPEL; MAYER, 2007; EL GUERRAB et al., 2017). No câncer de mama, a ativação dos genes induzidos por hipóxia desempenha importante papel na progressão tumoral por regular a proliferação celular, diferenciação, sobrevivência, angiogênese, metabolismo, migração e metástase

(FAVARO et al., 2011; CHU et al., 2013; GILKES; SEMENZA, 2013; SEMENZA, 2017).

Além disso, análises *in vitro* utilizando linhas tumorais mamárias e modelos animais demonstraram que o aumento da expressão do HIF-1α e a ativação da sinalização HIF-1 induzem a resistência a terapias convencionais por promover a manutenção das células tronco tumorais (AMERI et al., 2010; ZHANG et al., 2015; SAMANTA et al., 2016; WIGERUP, PAHLMAN, BEXELL, 2016; ZHANG et al., 2016; RANKIN, GIACCIA, 2016).

Outros estudos revelaram que o aumento na porcentagem das células tronco tumorais foi dependente da ativação HIF-1 induzida após tratamento com quimioterapia citotóxica (CAO et al, 2013; SAMANTA et al, 2014; LU et al, 2015) e de forma geral, quando os diferentes subtipos moleculares do câncer de mama são tratados com quimioterápicos observa-se um aumento da expressão do HIF-1α (SAMANTA et al., 2014).

Presente em cerca de 40% dos tumores mamários invasivos (LUNDGREN; HOLM; LANDBERG, 2007), o elevado perfil de expressão dos HIFs é similar entre subtipos moleculares de câncer de mama (YEHIA et al., 2015). No entanto, apesar desta similaridade, vários estudos descrevem interações específicas entre hipóxia e a resposta as terapia convencionais observadas entre os subtipos moleculares do câncer de mama (SAMANTA et al., 2014; YANG et al., 2015; WOLFF et al., 2017).

Em condições de hipóxia, as linhas tumorais mamárias MCF7 e T47D (ER+) apresentam reduzida expressão dos receptores de estrógeno (WOLFF et al., 2017), enquanto que a suplementação com estrógeno sobre hipóxia induz a elevada expressão do HIF-1α neste subtipos moleculares (YANG et al., 2015). Uma vez que as vias sinalização HIF-1 e ER co-regulam a expressão de diversos genes envolvidos na glicólise, o tratamento com o antagonista do ER interfere com a sinalização HIF-1 e metabolismo glicolítico por reduzir a expressão do HIF-1α e do GLUT1, os quais normalmente são induzidos sobre condições de hipóxia (YANG et al., 2015). Além disso, a elevada expressão do HIF-1α em células ER positivas induz a resistência ao tratamento com tamoxifeno (YANG et al., 2015).

Em células MDA-MB-231 (subtipo TNBC) o tratamento com fulvestranto não teve efeito sobre a expressão do HIF-1α (YANG et al., 2015), ao passo que o tratamento de várias células do subtipo TNBC com paclitaxel induziu a expressão e

atividade transcricional do HIF-1α bem como o aumento da população de células tronco tumorais de maneira dependente do HIF-1α (SAMANTA et al., 2014).

Análises *in vivo* revelaram que o bloqueio da sinalização HIF-1 resultou na redução da população de células tronco tumorais, do crescimento do tumor primário, da angiogênese, da formação do nincho pré-metastático, da invasão local, da metástase linfonodal e pulmonar, bem como na redução da recidiva (SCHITO et al., 2012; WONG et al., 2012; ZHANG et al., 2012; SAMANTA et al., 2014; XIANG et al., 2014).

Em acordo com estes modelos, o aumento da expressão do HIF-1α e a ativação da sinalização HIF-1 em pacientes diagnosticados com câncer de mama têm sido associados com resistência à terapia endócrina combinada com quimioterapia (GENERALI et al., 2006); prognóstico reservado (KAYA et al., 2012; JIN et al., 2016); elevado risco de metástase, recidiva e diminuição da sobrevida global (RUNDQVIST, JOHNSON, 2013; EL GUERRAB et al., 2017). Juntos, os resultados das análises *in vitro*, *in vivo* e em amostras tumorais evidenciam que a análise da expressão gênica relacionada a hipóxia pode ter utilidade diagnóstica, prognóstica e terapêutica para o câncer de mama.

Neste contexto, Greville e colaboradores (2016) sugeriram que a hipóxia provavelmente regula genes relacionados a glicosilação bem como afeta o perfil de glicanos das células tumorais, o que pode estar envolvido com a aquisição de fenótipo maligno e resistência as terapias convencionais no câncer de mama.

Além disso, o relevante papel das alterações na glicosilação no câncer de mama foi evidenciado por estudos onde: várias estruturas glicanos foram significativamente associadas com a agressividade tumoral e a sobrevida dos pacientes (HAAKESEN et al., 2016); as diferenças nos padrões de N- e O-glicosilação observadas entre subtipos luminal e basal foram sugeridas como marcadores para estratificação prognóstica e tratamento dos pacientes (POTAPENKO et al., 2015); e o perfil de expressão de glicosiltransferases, especialmente fucosiltransferases e sialiltransferases, foi associado com a classificação dos subtipos luminal e basal, correlacionado com dados clínicos e a taxa de sobrevida dos pacientes (ASHKANI e NAIDOO, 2016).

Todavia, embora as alterações na glicosilação e a hipóxia tenham relevância prognóstica e terapêutica para o câncer de mama, os efeitos do microambiente

hipóxico sobre a glicosilação deste tipo de câncer ainda permanecem pouco explorados.

2.4 GLICOSILAÇÃO ABERRANTE: A DOCE VIA DA PROGRESSÃO TUMORAL

Alterações no perfil de glicosilação das células tumorais estão intimamente envolvidas em todos os eventos considerados '*hallmarkrs*' do câncer (HANAHAN; WEINBERG, 2011). Vários estudos revelam a associação entre as alterações no perfil de glicosilação com a insensibilidade a sinais anti-proliferativos, autossuficiência de sinais de crescimento, evasão da morte celular, imunorresistência, ilimitado potencial replicativo, vias inflamatórias pró-tumorais, ativação da invasão e metástase, angiogênese, instabilidade genômica e mutação e, desregulação do metabolismo energético (revisado em MUNKLEY; ELLIOTT 2016). Além disso, o envolvimento das alterações na glicosilação durante a iniciação, progressão e metástase tumorais sugerem o seu grande potencial diagnóstico e terapêutico (REIS et al., 2010; VAJARIA; PATEL, 2017).

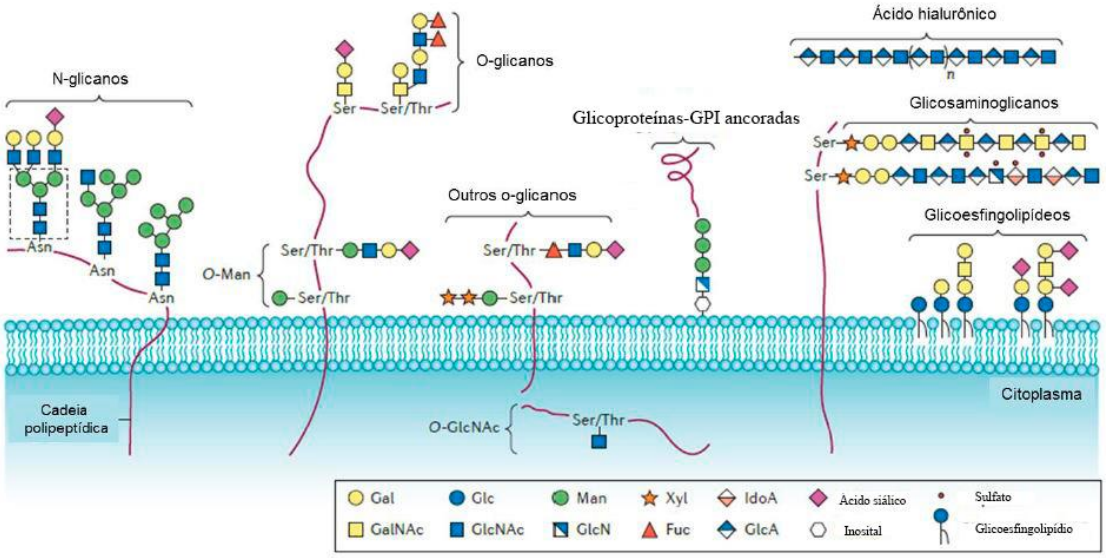
Em células não transformadas, a complexa diversidade dos glicanos resulta de diferenças na composição dos monossacarídeos, na ligação entre os monossacarídeos, no estado anomérico, nas estruturas de ramificação e na ligação com o componente não glicano (Figura 1) (VARKI *et al.,* 2009). Em células transformadas, a síntese incompleta e a síntese *de novo* dos glicanos são os principais mecanismos envolvidos com a aberrante glicosilação associada ao câncer (HAKAMORI; KANNAGI, 1983).

A síntese incompleta resulta na biossíntese de O-glicanos truncados e ocorre mais frequentemente em estágios iniciais do câncer (JULIEN et al., 2006; Marcos et al., 2011); enquanto que a síntese *de novo* resulta da indução de genes envolvidos na expressão de determinantes carboidratos e comumente é observada em estágios avançados do câncer (KANNAGI et al., 2008).

Entre os principais fatores responsáveis pela aberrante glicosilação associada ao câncer, destacam-se: (1) alterações na conformação terciária do esqueleto peptídico e na cadeia do glicano nascente; (2) alterações na expressão dos glicoconjugados receptores juntamente com a disponibilidade e abundância dos

açúcares nucleotídeos e seus transportadores; (3) alterações no aparelho de Golgi onde muitas glicosiltransferases estão localizadas; e (4) alterações na expressão de glicosiltransferases devido a desregulações a nível epigenético, transcricional, pós-transcricional, bem como alterações na função das chaperonas e na atividade das glicosidases (VAJARIA; PATEL, 2017).

Figura 1 - Classes comuns de glicoconjugados em células de mamíferos. Gal: galactose, GalNAc: N-acetilgalactosamina, Glc: glicose, GlcNAc: N-acetilglicosamina, Man: manose, GlcN: N-glicosamina, Xyl: xilose, Fuc: fucose, GlcA: ácido glicurônico, Sialic acid: ácido siálico, GPI: Glicosilfosfatidilinositol.



Fonte: PINHO; REIS, 2015.

Durante a transformação maligna, o aumento na sialilação, na fucosilação e na síntese de O-glicanos truncados são as mais frequentes alterações na glicosilação associadas ao câncer (PINHO; REIS, 2015; NATONI; MACAULEY; DWYER, 2016). No câncer de mama, estas alterações são atribuídas a alterada atividade de glicosiltransferases, resultam na elevada expressão dos antígenos T, STn e de Lewis, particularmente Le^{y} e $sLe^{a/x}$, e estão associadas a progressão tumoral (CHRISTIANSEN et *al.,* 2014).

Dada a relevância da sialilação, fucosilação e dos O-glicanos truncados durante a progressão do câncer de mama estas alterações serão analisadas em detalhes nos tópicos subsequentes.

2.5 O-GLICANOS TRUNCADOS: O ‘STOP’ PARA A TRANSFORMAÇÃO MALIGNA

A O-glicosilação, também conhecida como glicosilação do tipo mucina, ocorre no aparelho de Golgi e resulta na transferência de uma N-acetilgalactosamina (GalNAc) a hidroxila (-OH) de um resíduo de serina ou treonina (Ser/Thr) através da atividade de N-acetilgalactosaminiltransferase (ppGalNAcT) (Bennett et al., 2012). A adição de GalNAc a Ser/Thr forma o antígeno Tn (GalNAcα1-O-Ser/Thr), o qual pode ser estendido pela adição de galactose (Gal), N-acetilglicosamina (GlcNAc) ou GalNAc aos grupos 3-OH e/ou 6-OH resultando na formação do antígeno T (Galβ1-3GalNAcα1-O-Ser/Thr, núcleo 1) e múltiplas estruturas estendidas. Estes antígenos geralmente são modificados por adição de um grupo sulfato, GalNAc, ácido siálico e fucose, o que resulta na formação dos antígenos sialil-Tn (STn) sialil-T (ST), sialil Lewis x (SLe^x) e sulfo-SLe^x (Figura 2) (JU et al., 2013; CORFIELD; BERRY, 2015; CORFIELD, 2015).

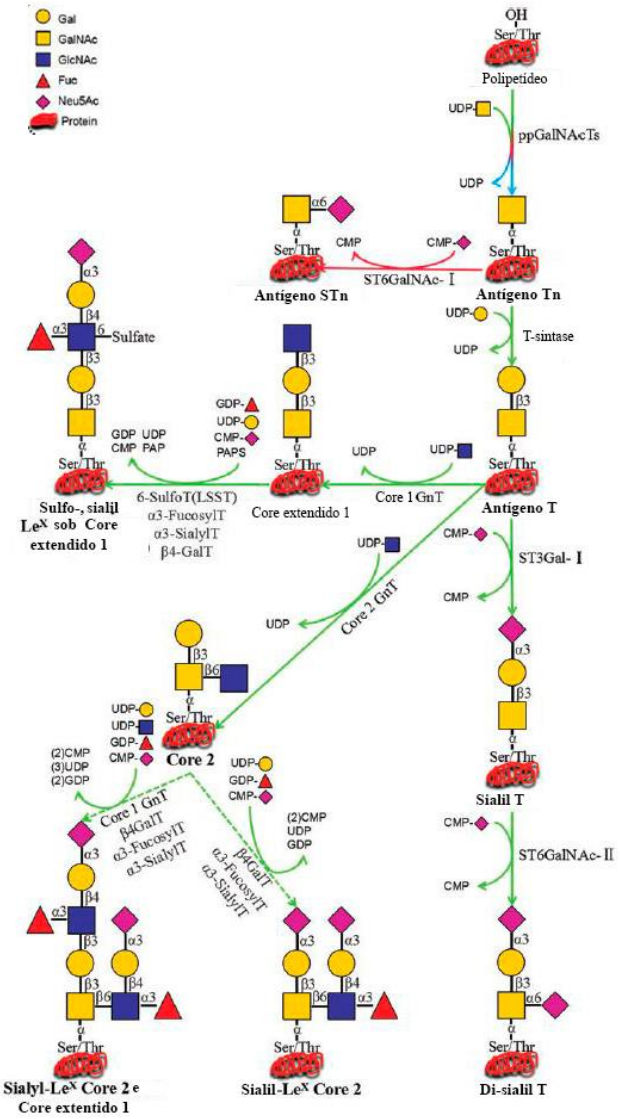
Em tecidos normais, os o-glicanos truncados são ausentes ou possuem reduzida expressão ao passo que durante a transformação maligna são comumente aumentados e envolvidos em diversas vias da progressão tumoral (CHRISTIANSEN et al., 2014; FU et al., 2016). Estudos em diversos tipos tumorais revelaram a elevada expressão dos antígenos T, Tn e STn como a mais frequente alteração associada à transformação maligna (revisado em CHRISTIANSEN et al., 2014).

Com reduzida ou ausente expressão em células não transformadas, o antígeno Tn é comumente expresso durante a transformação maligna e correlacionado com o potencial metastático, prognóstico reservado e reduzida sobrevida em vários tipos tumorais, incluindo o câncer de mama (KONNO et al., 2002; WELINDER et al., 2013; KOLBL et al., 2016). Apesar da necessidade de avanços sobre o papel deste antígeno durante a progressão tumoral, alguns estudos demonstraram o seu envolvimento em eventos imunossupressivos através da ligação com a lectina de ligação a galactose do tipo C presente em macrófagos e células dendríticas (NAPOLETANO et al., 2007; JU et al., 2013). No câncer de mama, a ligação do antígeno Tn a galectina-3 favorece a extravasão metastática ou modula a resposta imune (KOLBL et al., 2016).

Semelhante ao antígeno Tn, a expressão do antígeno STn é ausente ou reduzida em células não transformadas e associada ao prognóstico reservado,

doença metastática, recidiva e reduzida sobrevida (revisado em SCHULTZ; SWINDALL; BELLIS, 2012;). No câncer de mama, a elevada expressão deste antígeno está presente em 30% dos carcinomas, é principalmente atribuída a atividade da sialiltransferase ST6GalNAc-I (SEWELL et al., 2006; JULIEN et al., 2006) e associada a inibição da apoptose mediada por células dendríticas e *natural killer*, prognóstico reservado e resistência a quimioterapia (BEATSON et al., 2015). Testes clínicos com vacinas anti-STn têm revelado a importância da expressão deste antígeno como modulador do desenvolvimento e progressão do câncer de mama metastático (BLIXT et al., 2011; MILES et al., 2011).

Figura 2 - Principais O-glicanos sialilados.



Fonte: FU et al., 2016.

Com relevante papel durante a metástase e a modulação da resposta imune (GLINSKY et al., 2001; BIAN et al., 2011; BUTCHER et al., 2014), a expressão do antígeno T é regulada pela atividade da T-sintase (CHEN et al., 2012) e varia entre vários tipos tumorais (KARSTEN; GOLETS, 2015). No câncer de mama, a expressão deste antígeno é associada ao potencial metastático, está presente em células tumorais circulantes, modula a metástase via interação com a galectina-3 e desempenha importante papel na imunoterapia (KHALDOYANIDI et al., 2003; YU et al., 2007; ANDERGASSEN et al., 2013; FERGUSON et al., 2014).

Todavia, embora alguns efeitos regulatórios da hipóxia sobre a expressão dos O-glicanos truncados tenham sido descritos (PEIXOTO et al., 2016), a relação entre o microambiente hipóxico e a regulação da expressão e atividade destes glicanos no câncer de mama permanece desconhecida.

2.6 ABERRANTE SIALILAÇÃO E CÂNCER DE MAMA

Os ácidos siálicos são predominantemente adicionados a região terminal não redutora de N- e O-glicanos sobre proteínas ou lipídios através de uma ligação covalente a Gal, GalNAc, GlcNAc ou outro ácido siálico mediada pela atividade das sialiltransferases (ST).

Em humanos, a família de ST é composta por vinte enzimas (HARDUIN-LEPERS et al., 2001; DATTA, 2009; TAKASHIMA; TSUJI, 2011) cuja classificação é baseada no tipo de ligação do ácido siálico ao resíduo sacarídeo receptor: (1)- enzimas ST6Gal- as quais catalisam a transferência de ácido siálico ao 6'-OH de um resíduo de Gal; (2)- enzimas ST3Gal- as quais catalisam a transferência de ácido siálico ao 3'-OH de um resíduo de Gal; (3)- enzimas ST6GalNAc- as quais catalisam a transferência de ácido siálico ao 6'-OH de um resíduo de GalNAc; e (4) enzimas ST8Sia- as quais catalisam a transferência de ácido siálico ao 8'-OH de outro resíduo de ácido siálico (DATTA, 2009; TAKASHIMA; TSUJI, 2011). Estas enzimas são expressas de maneira célula ou tecido específicos (COHEN; VARKI, 2010).

Durante a transformação maligna, o alterado perfil de glicosilação observado na superfície das células transformadas aumenta a sua interação com os

componentes do microambiente tumoral e modula os processos de adesão célula-célula e célula-matriz, sinalização celular, mobilidade e invasão das células tumorais (REYMOND; D'AGUA; RIDLEY, 2013; STOWELL; JU; CUMMINGS, 2015; CARVALHO et al., 2016).

Entre as alterações no perfil de glicosilação, o aumento global da sialilação, particularmente da α2,3- e α2,6- sialilação, na superfície das células tumorais constitui uma das principais modificações associadas ao câncer e está envolvida em processos como a evasão ao sistema imune, resistência a terapia, disseminação vascular, aumento da invasão e metástase e prognóstico reservado (SCHULTZ; SWINDALL; BELLIS, 2012; BULL et al., 2014; NARDY et al., 2016).

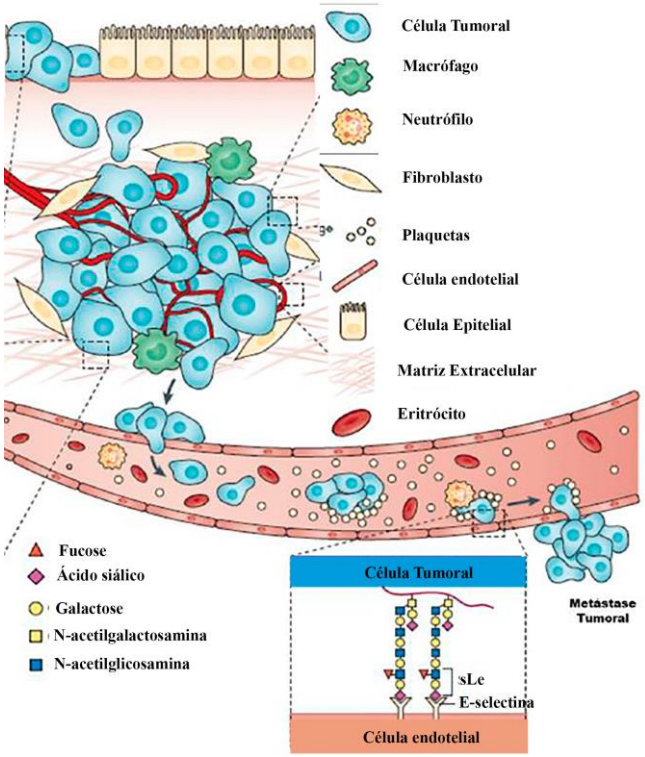
Os antígenos sialilados de Lewis (sLe) são as mais frequentes estruturas α2,3-sialiladas associadas ao câncer e sua expressão é significativamente associada com a invasão linfática, metástase linfonodal, metástase à distância, recidiva, prognóstico reservado e reduzida taxa de sobrevida (LIANG; LIANG; GAO, 2016). A elevada expressão destes antígenos resulta particularmente de alterações na expressão de α2,3- sialiltransferases (ST3GAL1, ST3GAL3, ST3GAL4 e ST3GAL6) e α1,3-/4- fucosiltransferases (FUT3-FUT7) induzidas pela transformação maligna (CAZET et al., 2010; JULIEN et al., 2011; SAKUMA; AOKI; KANNAGI, 2012).

A grande relevância da elevada expressão dos antígenos sLe durante a progressão tumoral é atribuída a sua atuação na promoção da transição epitélio mesenquimal (SAKUMA; AOKI; KANNAGI, 2012) e de metástases, onde favorecem os processos de intravasão, rolamento e extravasão das células tumorais através da ligação as selectinas presentes nas células endoteliais (PINHO; REIS, 2015) (Figura 3). No entanto, apesar do comprovada atuação durante a metástase, estudos também revelaram que a elevada expressão do antígeno sLe^x mediada pela atividade da enzima FUT3 pode modular a sensibilidade a apoptose através da sua ligação com receptores lectinas do tipo C presentes na superfície das células *natural killer (*OHYAMA; TSUBOI; FUKUDA, 1999; OHYAMA et al., 2002; HIGAI et al., 2006).

Além do significativo aumento observado na α2,3-sialilação, o elevado perfil de α2,6-sialilação frequentemente observado na superfície das células tumorais é atribuído ao aumento da expressão das sialiltransferases ST6Gal e ST6GalNAc (revisado em SCHULTZ; SWINDALL; BELLIS, 2012) e significativamente associado

com a ativação de vias de sinalização envolvidas na promoção da motilidade celular (SEALES et al., 2005); e na resistência a apoptose através da modulação da atividade de galectinas (ZHUO et al., 2010; SANTOS et al., 2016).

Figura 3- Participação dos antígenos sialil Lewis na metástase.



Fonte: PINHO; REIS, 2015.

No câncer de mama a relevância do aumento na α2,3-sialilação é geralmente associada a elevada expressão dos antígenos sLe e envolvida com o *status* de expressão hormonal, invasão tumoral, transição epitélio mesenquimal, metástase óssea e monitoramento da progressão metastática (JESCHKE et al., 2005; JULIEN et al., 2011; FUJITA et al., 2011; LIANG; LIANG; GAO, 2016). Por outro lado, além da relevante importância do antígeno STn em diversos eventos da progressão deste tipo tumoral (rever item 3.5), o aumento da α2,6-sialilação regula o tropismo metastático das células tumorais mamárias através da expressão das sialiltransferases ST6GalNAcII (CARCEL-TRULLOLS et al., 2006) e ST6GalNAcV (BOS et al., 2009).

Até então, embora alguns estudos descrevam o papel regulatório da hipóxia sobre a expressão de α2,3- e α2,6- sialiltransferases em alguns tipos tumorais (KOIKE et al., 2004; PEIXOTO et al., 2016), os efeitos do microambiente hipóxico

sobre a regulação da α2,3- e α2,6- sialilação no câncer de mama permanecem desconhecidos.

2.7 ABERRANTE FUCOSILAÇÃO E CÂNCER DE MAMA

Com relevante papel em várias funções biológicas, o aumento de glicanos fucosilados é significativamente observado na superfície das células de vários tipos tumorais e comumente associado com redução da adesão, aumento da proliferação e metástase tumoral (CHRISTIANSEN et al., 2014; PINHO; REIS, 2015; VAJARIA; PATEL, 2017). A fucosilação é considerada um processo não extensível e geralmente é subdividida em fucosilação terminal, particularmente envolvida na síntese dos antígenos de Lewis (Le), e *core* fucosilação, envolvida na *core* fucosilação do trimanosil (CARVALHO et al., 2010; CHRISTIANSEN et al., 2014).

Os glicanos fucosilados são sintetizados pela transferência de unidades fucose a complexos glicanos através de ligações específicas mediadas pelas enzimas fucosiltransferases (FUT). No genoma humano, os genes de codificam as enzimas FUT são divididos em três subfamílias: α-1,2-FUT (FUT1-2), α-1,3-/4-FUT (FUT3 a FUT7 e FUT9 a FUT11) e α-1,6-FUT (FUT8) (NATONI; MACAULEY; O'DWYER, 2016).

Neste contexto, FUT1 e FUT2 estão envolvidas na síntese dos antígenos Le^b e Le^y; FUT3 exibe predominantemente a atividade α-1,4 FUT a qual está envolvida na síntese dos antígenos Le^a, sLe^a e Le^b, mas também possui atividade α-1,3 FUT onde atua na síntese dos antígenos Le^x, sLe^x e Le^y; FUT5, FUT6 e FUT7 sintetizam predominantemente os antígenos Le^x, sLe^x e Le^y, no entanto FUT5 também foi associada a síntese dos antígenos Le^a, Le^b e sLe^a; FUT8 atua na *core* fucosilação do trimanosil; FUT9 sintetiza os antígenos Le^x e Le^y (revisado em VAJARIA; PATEL, 2017); FUT10 sintetiza o antígeno Le^x (KUMAR et al., 2013); e FUT11 sintetiza as antígenos sLe^x e sulfo- sLe^x (GROUX-DEGROOTE et al., 2008). Estas enzimas são expressas em vários tecidos e comumente alteradas no soro e tumores de vários tipos de câncer (MIYOSHI et al., 2016).

No câncer de mama, vários estudos evidenciaram a relação entre a alterada expressão das FUT e diversos eventos da progressão tumoral, entre eles: a elevada expressão de FUT1 e FUT3 foi associada com prognóstico reservado (BREIMAN et

al., 2016); a elevada expressão de FUT4 foi associada com proliferação, metástase e proposta como novo biomarcador para diagnóstico e prognóstico (YAN et al., 2015); a elevada expressão dos antígenos sLe, mediada especialmente pela atividade das FUT3-FUT6, foi associada com metástase, prognóstico reservado e redução da sobrevida (URA et al., 1992; JULIEN et al., 2011; CARRASCAL et al., 2017); o aumento da *core* fucosilação, mediada pela elevada expressão de FUT8, foi associada com o aumento da migração e metástase (TU et al., 2017); e a elevada expressão das FUT10-11 foi observada na doença metastática (CARRASCAL et al., 2017).

Semelhante à sialilação, embora alguns estudos descrevam o papel regulatório da hipóxia sobre a expressão de α1,2- e α1,3-/4- fucosiltransferases em alguns tipos tumorais (KOIKE et al., 2004; BELO et al., 2015), os efeitos do microambiente hipóxico sobre a regulação da α1,2- e α1,3-/4- fucosilação no câncer de mama permanecem desconhecidos.

2.8- LECTINAS: RELEVANTES FERRAMENTAS PARA MONITORAMENTO DA ABERRANTE GLICOSILAÇÃO

Amplamente encontradas em todos os seres vivos, as lectinas são proteínas com especificidade de reconhecimento e ligação a carboidratos livres ou associados a oligossacarídeos e glicoconjugados (HASSAN et al., 2015). As formas endógenas estão presentes no meio intracelular, na superfície das células ou como proteínas secretadas e apresentam papeis fisiológicos primordiais além de estar envolvidas em diversos eventos da progressão tumoral (revisado em NARDY et al., 2016). As lectinas exógenas comumente são de origem vegetal e abundantemente encontradas nas sementes das plantas (BENEDITO et al., 2008; DIAS et al., 2015).

Por causa de sua especificidade a carboidratos, as lectinas exógenas são consideradas como valiosas ferramentas para a identificação e o monitoramento de alterações na glicosilação durante a transformação maligna (HASHIM; JAYAPALAN; LEE, 2017). Diversos pesquisas têm avaliado a utilização das lectinas em respostas inflamatórias e imunológicas (SINGH et al., 2011; DITAMO et al., 2016), na cicatrização de feridas cutâneas (BRUSTEIN et al., 2012; CORIOLANO et al., 2014), na inibição da adesão e crescimento das células tumorais (SILVA et al., 2014;

JEBALI et al., 2014; QUIROGA; BARRIO; AÑÓN, 2015) e sua participação em sistemas de entrega de fármacos anti-tumorais (NEUTSCH et al.,2013).

Além disso, a utilização das lectinas exógenas recebe crescente interesse na pesquisa por biomarcadores no câncer devido a diversos fatores, entre eles o fato das glicoproteínas apresentarem maior estabilidade em relação a proteínas não glicosiladas em contextos patológicos; a existência de múltiplos glicanos numa glicoproteína pode amplificar a detecção de um pequeno número de células transformadas; e o fato da maioria dos biomarcadores atualmente utilizados na oncologia serem glicoproteínas estruturalmente alteradas e aberrantemente expressas durante a transformação maligna (DRAKE et al., 2012; HENRY; HAYES, 2012).

Considerando a relevância das alterações na α2,6-sialilação e na α1,3-/4-/6-fucosilação durante a transformação maligna, as lectinas *Sambucrus nigra* (SNA) e *Aleuria aurantia* (AAL), as quais reconhecem resíduos de ácido siálico na posição α2,6- e resíduos de fucose na posição α1,3-/4-/6- sobre N- ou O-gliganos, respectivamente, são comumente utilizadas na pesquisa por novos biomarcadores em diversos tipos tumorais (KIM et al., 2014; LLOP et al., 2016; SILVA; GOMES; GARCIA, 2017).

No câncer de mama, o padrão de reconhecimento da SNA em diferentes tipos de carcinomas mamários sugeriu o seu uso como sonda prognóstica para carcinomas ductais invasivos (DOS-SANTOS et al., 2014) e foi significativamente expresso em regiões de hipóxia (RÊGO et al., 2014). De forma semelhante, a utilização da AAL verificou o aumento aberrante da α1,3-/4-/6-fucosilação em diferentes estágios do câncer de mama (WI et al., 2016). Além disso, a utilização de ambas lectinas em diferentes linhas tumorais mamárias revelou a presença de ácido siálico e fucose em diversas glicoproteínas (presentes na superfície celular ou secretadas) reconhecidas pelo elevado potencial como novo biomarcador do câncer de mama (DRAKE et al., 2012).

Em consonância com estes resultados, estudos evidenciaram a ampla similaridade do perfil glicoproteico e da glicosilação de superfície entre os modelos *in vitro* e os diversos subtipos moleculares do câncer de mama (TÃO et al., 2008; YEN et al., 2011). Além disso, embora a cultura de células não possa decifrar a heterogeneidade dos glicanos em sua total complexidade (CHRISTIANSEN et al., 2014), a elevada similaridade entre as alterações no DNA, alterações epigenéticas e

o perfil de marcadores fenotípicos observados entre as linhas tumorais mamárias e o diferentes subtipos moleculares do câncer de mama (DAI et al., 2017), tornam o uso desses modelos in vitro importante ferramenta para melhor compreensão da regulação da expressão de glicosiltransferases, glicanos de superfície celular e o impacto da aberrante glicosilação sobre o desenvolvimento e progressão deste tipo câncer.

3 METODOLOGIA

3.1 CÉLULAS TUMORAIS E CULTIVO CELULAR

As células tumorais mamárias T47D (subtipo Luminal A), MCF7 (subtipo Luminal A) e MDA-MB-231(Triplo negativo, subtipo *basal-like*) foram adquiridas junto ao *American Type Culture Colection* e cultivadas em meio RPMI1640 (Gibco, Life Technologies) ou DMEM (Gibco, Life Technologies) suplementado com 10% de soro fetal bovino (SFB) (Gibco, Life Technologies) em estufa de atmosfera úmida a 37ºC com 5% de CO_2. Todos os experimentos foram realizados com confluência entre 60 e 80%. O meio de cultivo foi renovado a cada 48h.

3.2 ENSAIOS EM CONDIÇÕES DE HIPÓXIA E PRIVAÇÃO DE SORO

Para mimetizar o microambiente hipóxico e privado de soro as células T47D e MCF7 foram incubadas com meio RPMI1640 suplementado com 10% ou 1% de SFB em câmara de hipóxia (Billupus-rothenberg - Modular Incubator Chamber) em atmosfera com 95% de N_2, 5% de CO_2 (20 L/min) e concentração de O_2 monitorada por um detector (PAC 3000, Dräeger), durante 6, 24 e 48h. Desta forma, quatro condições experimentais foram estabelecidas: normóxia (21% O_2/ 74.7% N_2/ 5% CO_2) com 10% de SFB (N10); normóxia com 1% de SFB (N1); hipóxia (1% O_2/ 74.7% N_2/ 5% CO_2) com 10% de SFB (H10) e hipóxia com 1% de SFB (H1).

As células MDA-MB-231 foram incubadas com meio DMEM suplementado com 10% ou 0% de SFB em estufa BINDER C-150 (BINDER GmbH, Tuttlingen) durante 48h, 72h e 6 dias, de modos que quatro condições experimentais foram estabelecidas: normóxia (21% O_2/ 74.7% N_2/ 5% CO_2) com 10% de SFB (N10); normóxia sem SFB (N0); hipóxia (1% O_2/ 74.7% N_2/ 5% CO_2) com 10% de SFB (H10) e hipóxia sem SFB (H0).

3.3 ANÁLISE DA VIABILIDADE CELULAR

O efeito da hipóxia e privação do soro sobre a viabilidade celular foi determinada pelo ensaio de proliferação celular CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega). As células MDA-MB-231, $3x10^3$ células por poço, foram incubadas em meio de cultivo completo em placa de 96 poços durante 24h. Em seguida, após lavagem com PBS, as células foram incubadas em normóxia ou hipóxia em meio de cultivo suplementado ou não com SFB durante 48h. Em

seguida, 20µl de MTS foram adicionados ao meio e após 2h de incubação a absorbância foi lida a 490nm em um leitor de microplacas (BioTek, Winooski). Cada condição experimental foi analisada em triplicata. Foram realizados dois experimentos independentes.

3.4 IMUNOFLUORESCÊNCIA

As células foram fixadas com 4% de paraformaldeído (Alfa Aesar) ou metanol (Fisher Scientific) a temperatura ambiente (TA) durante 20min. A permeabilização foi realizada mediante incubação com 0,5% de Triton X-100-PBS a 4ºC durante10min. O bloqueio dos sítios inespecíficos foi realizado com 20% de soro normal não-imune de cabra (Dako) em PBS-BSA durante 1h a TA. Em seguida foi realizada a incubação com os anticorpos primários durante 14-16h a 4°C. Após lavagens com PBS, a incubação com os anticorpos secundários conjugados com fluorocromo correspondente a origem do anticorpo primário foi realizada durante 1h a TA. A contra-coração nuclear foi realizada com DAPI durante 15min a TA e por fim a montagem foi realizada com VectaShield (Vector Laboratories). A análise foi realizada em microscópio fluorescente (Zeiss Axio Imager Z1 Apotome). Os anticorpos e diluições utilizadas estão listados na Tabela 1.

Tabela 1- Anticorpos e lectinas utilizados no *Western Blot* (WB), *Lectin Blot* (LB) e Imunofluorescência (IF)

Anticorpo/Lectina (Clone)	**Antígeno/Especificidade**	**Diluição IF**	**Diluição WB/LB**	**Fabricante**
FUT3 (SAB1401146)	FUT3	-	1:2000	Sigma-Aldrich
E-Caderina (24E10)	E-cadherin	1:100	-	Cell Signaling Technology
HIF1α (H1 alpha 67-NB)	HIF1α	1:50	1:500	Novus Biologicals
α-Tubulin (DM1A)	α-Tubulin	1:750	-	Sigma-Aldrich
β-actina (I-19)	β-actin	-	1:2000	Santa Cruz Biotechnology

Vimentin (V9)	Vimentin	1:500	-	Dako
Aleuria aurantia lectina (AAL)	Fucα6GlcNAc Fucα3GlcNAc Fucα4GlcNAc	-	1:3000	Vector Labs
Sambrucus Nigra lectina (SNA)	Neu5Acα6Gal/GalNAc	-	1:3000	Vector Labs

3.5 EXTRAÇÃO DO RNA, SÍNTESE DO CDNA E RT-QPCR

Após incubação sobre as condições de estudo, o RNA foi extraído com o reagente Trizol® (Invitrogen) segundo as instruções do fabricante. A concentração e pureza do RNA foram determinadas por espectrofotometria usando NanoDrop ND 1000 Spectrophotometer (Nanodrop Technologies). A integridade do rRNA foi avaliada em eletroforese com gel de agarose 1%. Para síntese do cDNA, apenas amostras com os fragmentos 28S e 18S íntegros e valores de absorbância 260/280 entre 1.8-2.0 foram utilizadas. Três microgramas do mRNA foram convertidos em cDNA usando SuperScript® IV Reverse Transcriptase Kit (Invitrogen) segundo as instruções do fabricante.

A reação de RT-qPCR foi realizada em termociclador ABI 7500 (Applied Biosystems) usando 2µL de cDNA diluído (1:20), 10µM de cada primer, 5µL de SYBR® Green Master Mix (1X) (Thermo Fischer Scientific) e água ultrapura para um volume final de 10µL. Dois experimentos independentes e três replicatas técnicas foram realizados por condição experimental. Controles negativos (sem amostras de cDNA) foram utilizados em cada reação. Os *primers* utilizados estão listados na Tabela 2. Os genes ACTB, HPRT1 e PPIA foram utilizados como genes de referência para normalização da expressão dos genes alvo. O método $2^{-\Delta\Delta CT}$ foi utilizado para análise da expressão relativa.

Tabela 2- *Primers* utilizados para análise de expressão em células do câncer de mama submetidas a condições de hipóxia e privação do soro.

Gene	Sequências dos *primers*	Produto/pb
α1,2-fucosyltransferses FUT1	F:aaagactgaaggagcatatgattgc R: tcaaacctggtcctctctagaacaa	110
FUT2	F:gcggctagcgaagattcaag R: tgatgttgaggctagcactggta	67
α1,3/4-fucosyltransferses FUT3	F:caaaatgccaagggtggaca R:ttggcctcaatcaatcctcct	89
FUT4	F:aagccgttgaggcggttt R:acagttgtgtatgagatttggaagct	88
FUT5	F:aagccgttgaggcggttt R:acagttgtgtatgagatttggaagct	70
FUT6	F:caaagccacatcgcattgaa R:atccccgttgcagaacca	95
FUT7	F:ccgcttctacctgtcctttga R:gcgttgcgccagaatttct	250
FUT8	F: ccatttcaggtttgtttggtag R: attggtcccgcttctcactt	200
FUT10	F:caccgtctttctgcttgtca R:ccttcctttcaaacttcccc	62
FUT11	F:gctttggcaatgtggaagaga R:gccaataatcttgcagccac	69
α2,3-sialyltransferses ST3Gal3	F:ggtggcagtcgcaggattt R:catgcgaacggtctcatagtagtg	76
ST3Gal4	F:cctggtagctttcaaggcaatg R:cctttcgcacccgcttct	74
ST3Gal6	F:cggctgattttagaaagattgctt R:cggctgattttagaaagattgctt	90
α2,6-sialyltransferses ST6GalNAc1	F: tccaagggaacacttgaacca R: gcctcaggacctacagcaat	100
ST6GalNAc2	F: ctttgccctgtacttctcg R: cagcactggaatggagaga	200
ST6GalNAc5	F: ggatcccaatcacccttcag R: tagcaagtgattctggtttcca	200
Housekeeping genes ACTB	F: agaaaatctggcaccacacc R: tagcacagcctggatagcaa	173
GUSB	F: agccagttcctcatcaatgg R: ggtagtggctggtacggaaa	160
HPRT1	F: ggaccccacgaagtgttg R: ggcgatgtcaataggactcc	210
PPIA	F: agacaaggtcccaaagac R: accaccctgacacataaa	118
β2M	F: agcgtactccaaagattcaggtt R: atgatgctgcttacatgtctcgat	206
18S	F: cgccgctagaggtgaaattc R: cattcttggcaaatgctttcg	67

3.6 ANÁLISES *IN SILICO*

Para avaliar a relação entre as alterações transcricionais induzidas pela hipóxia e privação de soro e o nível de expressão intrínseco dos genes alvos, a expressão gênica de linhas celulares do câncer humano e de pacientes com câncer de mama foi extraída da plataforma OncomineTM (www.oncomine.org) (RHODES et al., 2004). Para tal, os valores da expressão gênica de 913 linhas celulares do câncer humano foram extraídos banco de dados Barretina CellLine (BARRETINA et al., 2012) e o valor de expressão de cada gene foi normalizado em valores entre 0 e 100. Em seguida, a mediana de cada gene normalizado foi determinada entre 56 linhas celulares do câncer de mama humano e o intervalo de expressão de cada gene normalizado foi definido utilizando-se o valor da mediana ± 10. Para a análise da expressão gênica em 1989 carcinomas mamários humanos utilizou-se o conjunto de dados Curtis Breast (CURTIS et al., 2012).

3.7 *WESTERN BLOT*

Para análise da expressão proteica, as proteínas celulares totais foram extraídas mediante incubação com tampão de lise (50mM Tris-HCl, pH 8,0, 150Mm NaCl, 1% NP-40 e coquetel inibidor de proteases) por 30min a 4°C e em seguida centrifugadas a 15000 rpm a 4°C por 30min. A dosagem de proteínas foi realizada utilizando-se o método de Bradford, de acordo com as instruções do fabricante (BioRad). Após análise da absorbância, 40-60µg de proteínas foram submetidas a eletroforese em gel de poliacrilamida 12% contendo SDS (SDS-PAGE) em tampão de corrida (25mM Tris base, 192mM Glicina, 0,1% p/v SDS, pH 8,3) a 100V por aproximadamente 3h. Em seguida, os extratos proteicos separados foram transferidos para membrana de nitrocelulose (GE Healthcare Life Sciences) em tampão de transferência (25mM Tris base, 192mM Glicina, 0,1% p/v SDS, 20% v/v Metanol, pH 8,3) a 100V por aproximadamente 90min. O bloqueio dos sítios inespecíficos foi realizado através de incubação da membrana com leite seco desnatado 5% em TBST a TA durante 2h. A incubação com os anticorpos primários diluídos em TBST-BSA 5% foi realizada a 4°C durante 14-16h. Após lavagens, a incubação com os anticorpos secundários conjugados com peroxidase diluídos em TBST-BSA 1% foi realizada a TA durante 2h. A revelação das membranas foi realizada utilizando o substrato quimioluminescente Amersham *ECL Western*

Blotting Detection Reagent (GE Healthcare Life Sciences). Os anticorpos e diluíções utilizadas estão listados na Tabela 1.

3.8 LECTIN BLOT

A análise do glicofenótipo das células MDA-MB-231 foi realizada utilizando-se as lectinas AAL e SNA. Para tal, a eletroforese e transferência foram realizadas como descrito no *item 4.7*. O bloqueio da membrana foi realizado em TBS-0,2% de polivinilpirrolidona a 4°C durante 14-16h. Após o bloqueio foram realizadas lavagens em TBST e em seguida a membrana foi incubada com as lectinas biotiniladas AAL e SNA durante 1h a TA. Posteriormente, após lavagens com TBST, a incubação com o link estreptoavidina-peroxidase foi realizada durante 1h a TA. A revelação das membranas foi realizada utilizando o substrato quimioluminescente Amersham *ECL Western Blotting Detection Reagent* (GE Healthcare). As lectinas e concentrações utilizadas estão listadas na Tabela 1.

3.9 ANÁLISES ESTATÍSTICAS

A análise estatística dos dados foi realizada através do software GraphPad Prism (versão 6.0) utilizando-se os testes *Two Way ANOVA*, *One Way ANOVA* e *T student*. O valor de $p \leq 0,05$ foi considerado estatisticamente significativo. Experimentos independentes foram realizados para validação dos resultados.

4 FUT3 CHARACTERIZATION IN HYPOXIC MICROENVIRONMENT: REGULATION AND POSSIBLE IMPACT IN BREAST CANCER BIOLOGY

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Abbreviations: ACTB- Actin Beta; cDNA- Complementary DNA; ER- Estrogen Receptor; FBS- Fetal bovine Serum; FUT- Fucosyltransferases; FUT3- Fucosyltransferase 3; HER2- Epidermal Growth Receptor-2; HPRT1- Hypoxanthine Phosphoribosyltransferase-1; H10- Hypoxia in RPMI /10%FBS; H1- Hypoxia in RPMI/1%FBS; N10- Normoxia in RPMI /10%FBS; N1- Normoxia in RPMI /1%FBS; PR- Progesterone Receptor; RPMI- Roswell Park Memorial Institute Medium; RT-qPCR- Real-time quantitative PCR; TBS- Tris Buffered Saline; TNF- Tumor Necrosis Factor; TRAIL- TNF-related Apoptosis-inducing Ligand; XIAP- X-linked Inhibitor of Apoptosis Protein.

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Abstract

This study aimed to investigate the effects of hypoxic microenvironment on FUT3 expression regulation in breast cancer cells models. Materials and Methods: FUT3 expression was evaluated in T47D and MCF7 cells. Transcriptional and protein analysis were performed under hypoxia and serum deprivation conditions after 6 and 24 hours; and after 24 and 48 hours, respectively. In T47D cells, experimental conditions induced a significant FUT3 expression decrease at both, transcriptional and protein levels, while in MCF7 cells under the same conditions induced a significant FUT3 expression increase of transcriptional and protein expressions. Conclusions: The results suggest that FUT3 expression regulation in hypoxic microenvironment may be involved on responding and/or adding the intrinsic features of T47D and MCF7 breast cell lines such as the acquisition of advantages regarding apoptosis resistance and metastasis promotion.

Keywords: Breast cancer; FUT3; Hypoxic microenvironment.

Introduction

In tumor microenviroment, cancer cells are under extremely stressful conditions, especially nutrient starvation and hypoxic conditions, because of their uncontrolled growth and proliferation [1]. In response to this scenario, cancer cells activate adaptive mechanisms at different molecular levels to suppress cell death and promote tumor progression [2, 3, 4]. Of note, the hypoxic regulation of glycosylation was suggested to be involved in tumor progression and chemoresistance mechanisms [5, 6].

During malignant transformation the increase of fucosylation, one of the most important glycosylation modifications associated with tumor progression, often results from the fucosyltransferases (FUTs) altered activity [reviewed in 7]. Among α1,3/4-FUTs involved in tumorigenesis, FUT3 expression acts in the epithelial–mesenchymal transition [8, 9], in sialylated Lewis antigens synthesis [8, 10] and in the induction or resistance apoptosis mediated by C-type lectin and TNF-related apoptosis-inducing ligand (TRAIL) receptors [11, 12].

In breast cancer, FUT3 expression is associated with molecular subtype, poor prognostic and survival [13]; is an effector of metastasis in hormone receptor dependent manner [14] and acts in the sialylated Lewis antigens synthesis [15]; indicated as the most suitable gene for circulant breast tumor cells detection [16];

and possibly acts in the apoptosis resistance [17]. However, although its functions in breast cancer biology are known, its role in breast cancer hypoxic microenvironment remain unclear.

In an effort to investigate the hypoxia and serum deprivation effects on FUT3 regulation in breast cancer, this study evaluate its expression at the transcriptional and protein levels, in two breast cancer cell models with differences in their metastatic potential and apoptosis resistance [18, 19].

Materials and Methods

Cell lines and culture conditions

T47D and MCF7 breast cancer cell lines were obtained from the American Type Culture Collection (ATCC, USA) and routinely grown in Roswell Park Memorial Institute medium (RPMI) (Gibco, Life Technologies) supplemented with 10% Fetal Bovine Serum (FBS) at 37°C in 5% CO_2 and 21% O_2.

Hypoxia and serum deprivation assays

To establish the hypoxic and serum deprived microenvironment, T47D and MCF7 cells were cultivated under normoxic conditions and then incubated in RPMI medium supplemented with 10% or 1% FBS in a hypoxia chamber (Billupus-rothenberg - Modular Incubator Chamber) for 24 and 48h. Thus, four conditions were analyzed: normoxia (21% O_2/ 74.7% N_2 / 5% CO_2) in RPMI 10% FBS - control group (N10); normoxia in RPMI 1% FBS- N1 group; hypoxia (1% O_2/ 74.7% N_2/ 5% CO_2) in RPMI 10% FBS- H10 group; and hypoxia in RPMI 1% FBS- H1 group.

*Real-time quantitative reverse transcriptase PCR (*RT-qPCR)

Six and twenty-four hours after incubation under N10, N1, H10 and H1 conditions, total RNA was extracted from T47D and MCF7 cells using TRyzol Reagent (Invitrogen, USA) according to manufacturer's instructions. Complementary DNA was synthetized from 1µg of RNA using the SuperScript® II Reverse Transcriptase Kit (Invitrogen, USA) following the manufacturer's instructions. RT-qPCR was performed with 2µL of diluted cDNA, 10µM of each primer, 5µL SYBR® Green Master Mix (1X) (Thermo Fischer Scientific, USA) and ultrapure water to a final volume of 10µL using the ABI 7500 (Applied Biosystems, USA). The primers used were the following: FUT3 for5'- cctgctggagtcctttgtggcc -3'; rev5'- gcaggcaagtcttctggagggg -3'; ACTB for5'- agaaaatctggcaccacacc -3'; rev5'- agaggcgtacagggatagca -3; and HPRT1 for5'- ggaccccacgaagtgttg -3'; rev5'- ggcgatgtcaataggactcc -3'. Normalization of target

gene abundance was carried out with ACTB/HPRT1. Two independent experiments and three technical replicates per condition were performed.

Immunoblotting

Total cellular proteins were extracted with lysis buffer (50mM Tris-HCl, pH 8,0; NaCl 150 Mm; 1% NP-40 and Protease Inhibitor Cocktail) and centrifuged at 15,000 rpm at 4°C for 30min. Protein quantification was performed by Bradford, 40-60µg were electrophoresed in SDS-PAGE gel and transferred to nitrocellulose membranes (GE Healthcare Life Sciences). Membranes were blocked with 5% milk solution in TBS-T/0.05% (Tris Buffered Saline with Tween 20 to 0.05%) at 25°C for 2h, FUT3 antibody (SAB1401146, Sigma-Aldrich) was incubated overnight at 4°C in TBS-T/0.05%. After washing, secondary antibody incubation was performed at 25°C for 2h followed by TBST/0.05% washes. Western blot signal was detected using Enhanced Chemiluminescent substrate (GE Healthcare Life Sciences).

Statistical analysis

Two-way ANOVA was performed to determinate statistical significance using GraphPad Prism (version 6) software. All error bars represent the standard error of the mean. P≤0.05 values were considered significant (*), p≤0.05 (**), p≤0.001 and (****) p≤0.0001.

Results

FUT3 transcription analysis of breast cancer cells under hypoxia and serum deprivation

Our results evidenced that hypoxia and serum deprivation induce different FUT3 transcriptional regulation in T47D and MCF7 cells after 6 and 24h of exposure. In T47D cells, hypoxia with or without serum deprivation induced a significant downregulation of FUT3 in all incubation times, except in H1 after 6h of exposure where FUT3 expression was similar to the control (Figure 1A). On the other hand, exposure of MCF7 cells to hypoxia with or without serum deprivation promoted significant upregulation of FUT3 in all incubations (Figure 1B).

Hypoxia and serum deprivation induces different regulation of FUT3 expression in T47D and MCF7 cells

The FUT3 expression in T47D and MCF7 cells was studied to address whether the changes found at transcriptomic level were translated into protein. For this purpose, cells were cultured 24 and 48h under different conditions. Transcriptional analysis

showed that FUT3 expression in T47D cells decreased significantly in all conditions analyzed except in H1 after 24h of exposure where the expression was similar to the control (Figure 2A). Hypoxia with or without serum deprivation induced a significant decrease of FUT3 expression after 24h (Figure 2B) in MCF7 cells. Moreover, a significant increase in FUT3 expression was observed under hypoxia with serum deprivation after 48h (Figure 2B).

Discussion

Breast cancer still kills a high number of women worldwide [20]. In this cancer type, the hypoxic microenvironment is associated with poor prognosis [21], regulation of numerous genes related to tumor initiation, progression, metastasis, apoptosis [reviewed in 4] and possibly promoting the aggressiveness and resistance by regulation of glycosylation-related genes [5]. In addition, although the increase of α1,3/4-fucosylation in breast cancer has been correlated to the tumor invasive status [22] and the FUT3 expression has been associated with poor prognostic and survival [13], its function in breast cancer hypoxic and serum deprivation microenvironment is unknown.

Although MCF7 and T47D are breast cancer cell models with the same molecular subtype, Luminal-A (ER positive, PR positive and HER2 negative) [18], studies reveal intrinsic differences related to: (1) metastatic potential- MCF7 cells are derived from a metastatic adenocarcinoma and T47D cells from invasive ductal carcinoma [23]; (2) caspase-3 expression- MCF-7 cells lack caspase-3 expression as a result of a functional deletion mutation while T47D expresses all caspases [24], the presence or absence of Caspase-3 may determine the sensitivity or resistance to apoptosis [25, 26]; and (3) TRAIL-mediated apoptosis resistance - MCF7 cells presented natural resistance to TRAIL- induced apoptosis when compared to T47D cells [24].

In hypoxic microenvironment, the pro-apoptotic TRAIL signaling, recognized by the capacity to selectively induce the death of tumor cells [27], and C-type lectin-mediated apoptosis are severely impaired through X-linked inhibitor of apoptosis protein (XIAP) stabilization and modulation of the pro-apoptotic activity of the CD95 death receptor [28]. In addition, in breast cancer cells the XIAP stabilization, the most potent caspase inhibitor [29] was crucial to block autophagy, necroptosis and apoptosis induced by hypoxic and serum deprivation conditions [2, 3].

FUT3 activity in tumor progression pathways involves the promotion of metastasis through sialylated Lewis antigens synthesis [8, 15, 30] as well as its involvement in

apoptosis regulation mediated by C-type lectin receptors, such as CD94, and TRAIL receptors, such as DR4 and DR5 [11, 12].

Our results revealed that FUT3 expression was significantly regulated by hypoxia and serum deprivation conditions, both at the transcriptional and protein levels. In T47D cells, the FUT3 expression was negatively regulated while in MCF7 was positively regulated. Based on the highest sensitivity of T47D cells to TRAIL-induced apoptosis, as well as in the FUT3 expression relevance for apoptosis regulation and metastatic phenotypes, we suggest that FUT3 expression regulation under hypoxic and serum deprivation conditions may be involved with the acquisition of advantages related to apoptosis resistance and metastasis promotion, according to the intrinsic differences presented by T47D and MCF7 cells.

In this context, we infer that negative regulation of FUT3 induced by hypoxia and serum deprivation conditions in T47D cells may be an adaptive strategy since the expression of this FUT3 could increase sensitivity to C-type lectin or TRAIL receptors-induced apoptosis. This hypothesis is in agreement with Nascimento et al (2015) where the FUT3 negative expression was suggest as a immunoresistance strategy in biopsies of invasive ductal carcinoma. Furthermore, studies showed that when apoptosis is blocked, TRAIL death receptors and CD95 can promote tumor progression by elicit pro-inflammatory signaling pathways [31, 32]. Nevertheless, further researches are need to determine how FUT3 expression can regulate the apoptosis resistance and tumor progression mechanisms in breast cancer hypoxic and serum private microenvironment.

On the other hand, we believe that the natural resistance to TRAIL-induced apoptosis presented by the MCF7 cells allows the significant induction of FUT3 expression under hypoxia and serum deprivation conditions as a possible strategy for the acquisition of metastatic phenotype, since the increased expression of this FUT in breast cancer is directly associated with expression of sialylated Lewis antigens and promotion of metastasis [15]. Moreover, it is well known that hypoxia condition induces the increase in expression of sialylated Lewis antigens [33].

The differences in glycan expression signatures observed between subtypes molecular of breast cancer and normal breast tissue [34, 35] suggest that glycosylation alterations may be used to track disease progression, treatment response and/or the development of chemoresistance and can provide predictive biomarkers for diagnostic, prognostic and therapy. In this scenario, the effects of

hypoxic microenvironment under glycosylation might have therapeutic relevance in breast cancer.

In summary, FUT3 expression in breast cancer cells under hypoxic and serum deprivation stressful environments may be involved in the survival and metastatic regulatory control system, functioning differently by apoptosis inhibition or promoting metastasis, although this possibility should be better elucidated in further researches.

Acknowledgments

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Conflict of interests

The authors declared no conflict of interests.

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FIGURES

Figure 1- FUT3 transcription analysis in breast cancer cells under hypoxia and serum deprivation. (**A**) Quantitative real-time PCR showing levels of expression of FUT3 gene in T47D cells relative to the N10 condition. (**B**) Quantitative real-time PCR showing levels of expression of FUT3 gene in MCF7 cells relative to the N10 condition. The ACTB/HPRT1 housekeeping genes analysis were used to normalize the expression. RNA was collected after 6 and 24h of exposure to normoxia with serum supplementation (21% O_2 10% FBS - N10), hypoxia with serum supplementation (1% O_2 10% FBS - H10) and normoxia and hypoxia with 1% FBS supplementation, N1 and H1, respectively. Graphs represent the average value of two independent experiments with three technical replicates. Significant values are as follows: *p < 0.05, **p < 0.01; ****p < 0.0001 in 6h and #p < 0.05, ##p < 0.01; ####p < 0.0001 in 24h.

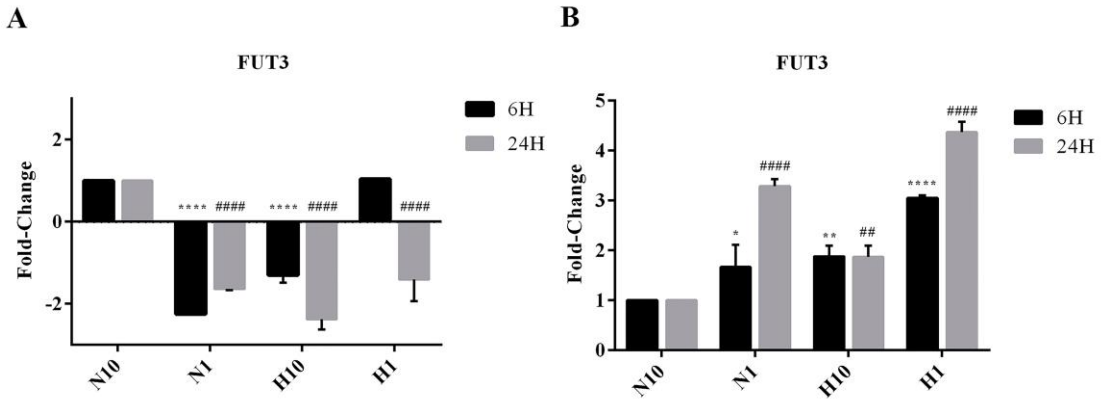
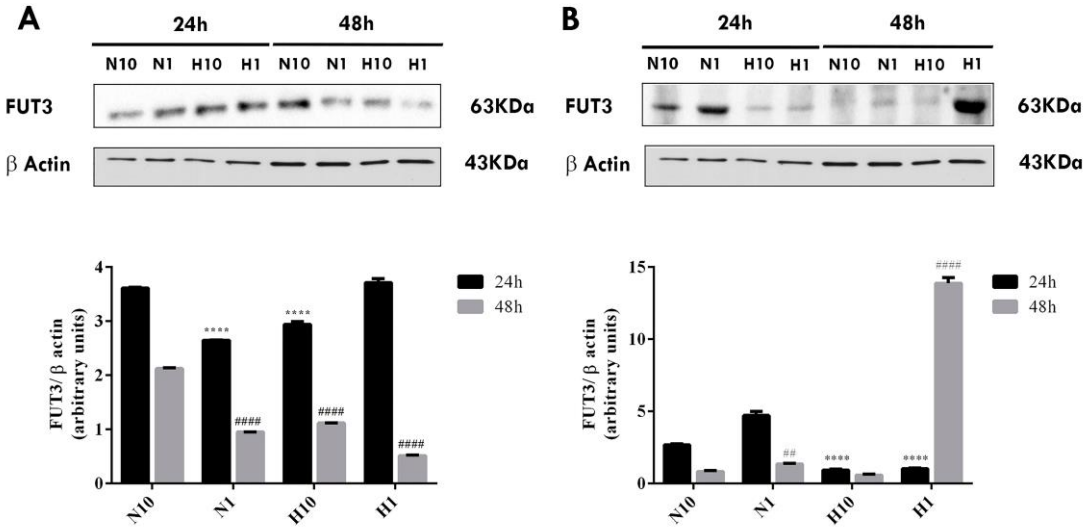


Figure 2- FUT3 protein regulation under hypoxic and serum deprivation conditions. (**A**) Immunoblotting and densitometry of expression of FUT3 protein in T47D cells. (**B**) Immunoblotting and densitometry of expression of FUT3 protein in MCF7 cells. β-actin was used as a loading control. Protein was collected after 24 and 48h of exposure to normoxia with serum supplementation (21% O_2 10% FBS - N10), hypoxia with serum supplementation (1% O_2 10% FBS - H10) and normoxia and hypoxia with 1%-FBS supplementation, N1 and H1, respectively. Graphs represent the average value of two independent experiments with three technical replicates. Significant values are as follows: **p* < 0.05, ***p* < 0.01; *****p* < 0.0001 in 24h and #*p*< 0.05, ##*p*<0.01; ####*p*<0.0001 in 48h.



5 IDENTIFICATION OF APPROPRIATE HOUSEKEEPING GENES FOR QUANTITATIVE RT-QPCR ANALYSIS IN MDA-MB-231 CELLS LINES UNDER HYPOXIC AND SERUM DEPRIVATION CONDITIONS

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Highlights

- Housekeeping genes in hypoxia and serum deprivation conditions were identified.
- ACTB/PPIA was the most stable combination for normalization in MDA-MB-231 cells.

Abstract [1]

Gene expression studies to study cancer cells under hypoxia and serum deprivation conditions show major potential for understanding mechanisms associated with tumor progression as well as treatment resistance. To our knowledge, a validation study for appropriate housekeeping genes in breast cancer cells under hypoxia and serum deprivation conditions was missing. Given the relevance of an accurate normalization, we aimed to identify the appropriate housekeeping genes for studies in MDA-MB-231 cell line cultured under hypoxia and/or serum deprivation. The stability of five commonly used housekeeping genes (ACTB, β2M, GUSB, 18S and PPIA) was assessed after reverse-transcription quantitative real-time PCR using the GeNorm, NormFinder and BestKeeper softwares. GeNorm and NormFinder ranking revealed ACTB, GUSB and PPIA as the most stable genes in MDA-MB-231 cells. Our results supports the use of ACTB/PPIA for MDA-MB-231 cells as the most stable

[1] Abbreviations

ACTB- Actin beta
β2M- β2-microglobulin
Ct- Cycle threshold
CV- Coefficient of variation
DMEM- Dulbecco's modified Eagle's medium
FBS- Fetal bovine serum
GUSB- β-glucuronidase
HKG- Housekeeping genes
H10- Hypoxia in DMEM/10%FBS
H0- Hypoxia/without FBS
N10- Normoxia in DMEM/10%FBS
N0- Normoxia in DMEM/without FBS
PPIA- Peptidylprolyl isomerase A
r- Correlation coefficient
R^2- Correlation coefficient
RT-qPCR- Real-time quantitative PCR
SD- Standard deviation
18S- 18S ribosomal RNA

combination for normalization in hypoxic and serum deprivation assays. Our results emphasized the importance of the selection of the housekeeping genes in breast cancer cells subjected to different physiological stresses, such as hypoxia and serum deprivation.

Keywords: Breast Cancer; Housekeeping Genes; Hypoxia; RT-qPCR; Serum Deprivation.

Introduction

The sensitivity and reproducibility of the RT-qPCR technique to measure transcriptional abundance and to detect differences in gene expression [1, 2] has made it an essential tool in several laboratories dedicated to life sciences. RT-qPCR is frequently employed to understand disease mechanisms and to research for new biomarkers and therapeutic strategies [3]. Therefore, housekeeping genes (HKG), known for their abundant and stable expression, with independence of the model and the experimental conditions, are used to minimize methodological errors and to ensure correct quantification of gene expression [4, 5]. Recent studies proved that there is no universal HKG for RT-qPCR analysis since the stability of HKG can be altered by several factors, such as the model employed and the experimental conditions [6, 7]. Hypoxia and serum deprivation are among the experimental conditions that can lead to modulation of the HKG expression [8, 9, 10, 11].

For decades, the abundance and the stable expression of some structural and metabolism-related genes were the first choice as expression genes normalizers without any stability analysis in the assessed context [6, 7]. In order to appropriately select the best HKG for RT-qPCR analysis, several mathematical approaches have been developed to evaluate candidates' stability and to avoid inaccurate normalizations. Among the most used algorithms, there are: (1) GeNorm, based on the geometric mean to determine the stability (M value) of each candidate. The highest stability is defined by values of M <1.5 [12, 13]; (2) NormFinder, which determines the stability of each candidate and the ideal number of reference genes considering intra- and inter-specific variations between candidate groups [14]. Similar to GeNorm, genes with the lowest M value are considered to be the most stable ones [14, 15]; and (3) BestKeeper, which is based on standard deviation (SD) and coefficient of variation (CV) to determine the correlation coefficient (r) of each

candidate gene. Values of SD [± CP] <1 and SD [± x-fold] <2 associated with an r value closer to 1 indicate the most stable gene [16].

Since hypoxia and serum deprivation are conditions that partially resemble the tumor microenvironment *in vivo* context, we decided to investigate the best HKG combination for transcription analysis under these conditions. For this purpose, we analyze the expression stability of five commonly used HKGs (ACTB, β2M, GUSB, 18S and PPIA) in MDA-MB-231 cells. To our knowledge, this is the first study analyzing the expression stability of genes of reference in MDA-MB-231 cell line cultured under hypoxic and serum deprivation conditions.

Material and Methods

Culture conditions

MDA-MB-231 (Triple negative, Basal subtype) breast cancer cell line was obtained from the American Type Culture Collection (ATCC, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Life Technologies) supplemented with 10% fetal bovine serum, FBS (Gibco, Life Technologies) with medium renewal every 48h. Cultures were maintained at 37°C in a 5% CO_2 humidified atmosphere.

Hypoxic and serum deprivation conditions

MDA-MB-231 cells were incubated for 48h at 37°C under the following conditions: normoxia (21% O_2/74.7% N_2/5% CO_2) in DMEM 10% FBS - control group (N10); normoxia in DMEM/without FBS - N0 group; hypoxia (1% O_2/74.7% N_2/5% CO_2) in DMEM/10% FBS - H10 group; and hypoxia in DMEM/without FBS - H0 group. For hypoxia, a BINDER C-150 incubator (BINDER GmbH, Tuttlingen, Germany) was used.

RNA extraction and cDNA synthesis

Forty-eight hours after incubation under N10, N0, H10 and H0 conditions, total RNA was extracted from MDA-MB-231 cells using TRyzol Reagent (Invitrogen, USA) according to manufacturer's instructions. RNA concentration and purity were determined by NanoDrop ND 1000 Spectrophotometer (Nanodrop Technologies Inc, Delaware, USA). Ribosomal RNA band integrity was evaluated by conventional 1% agarose gel electrophoresis. Synthesis of cDNA was carried out only in samples that showed integrity of 28S and 18S fragments and the absorbance of 260/280 ratio values ranged from 1.8 to 2.0. RNA (3µg) was reverse transcribed with random

primers using the SuperScript® IV Reverse Transcriptase Kit (Invitrogen, USA) following the manufacturer's instructions.

Quantitative Real-Time PCR assay and candidate reference genes

RT-qPCR was performed with 2µL of diluted cDNA, 10µM of each primer, 5µL SYBR® Green Master Mix (1X) (Thermo Fischer Scientific; former Savant, MA, USA) and ultrapure water to a final volume of 10µL using the ABI 7500 (Applied Biosystems, Foster City, CA, USA). Two independent experiments and three technical replicates per condition were performed. Negative controls without templates were added for each reaction. Thermo cycling conditions used were: initial denaturing at 95°C for 10 minutes followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. Melting curve analysis was performed to ensure the specificities of the amplification reactions.

The HKG candidates were selected according to a panel of reference genes used for RT-qPCR normalization in breast cancer, hypoxia and serum deprivation studies [11, 18, 19, 20, 21, 22]. A total of five genes were selected: Actin beta (ACTB), β2-microglobulin (B2M), β-glucuronidase (GUSB), Peptidylprolyl isomerase A (PPIA) and 18S ribosomal RNA (18S). The efficiency value and correlation coefficients (R^2) for each primer were calculated by ABI 7500 system software. The sequences and details of the candidate reference genes are listed in Table 1.

Analysis of gene expression stability

GeNorm (Version v3.5) [23], NormFinder (Version 20) [14] and BestKeeper (Version 1) [16] softwares were used to evaluate expression stability of the five HKG candidates. The equation $RQ = E^{-(minCq-sampleCq)}$, suggested by Spiegelaere et al. [15], was used to calculate and correcte the relative quantities used to GeNorm and Normfinder input. Analysis of best gene combinations was also performed with GeNorm and NormFinder; Bestkeeper does not provide this information.

Table 1-. Primer sequences used for the expression analysis of housekeeping genes.

Symbol	**Gene name**	**Primer sequences (5'→3')**	**Product size/bp**	**R^2**	**E(%)**
ACTB	Actin, beta	F: agaaaatctggcaccacacc R: tagcacagcctggatagcaa	173	0.99	90
β2M	Beta-2-microglobulin	F: agcgtactccaaagattcaggtt R: atgatgctgcttacatgtctcgat	206	1	105

GUSB	Beta-glucuronidase	F: agccagttcctcatcaatgg R: ggtagtggctggtacggaaa	160	1	90
PPIA	Peptidylprolyl isomerase A	F: agacaaggtcccaaagac R: accaccctgacacataaa	118	1	100
18S	18S ribosomal	F: cgccgctagaggtgaaattc R: cattcttggcaaatgctttcg	67	0.99	90

Results

Specificity and amplification efficiencies

RT-qPCR and agarose gel electrophoresis analyses revealed the specificity of each primer through the detection of a single peak in the melting curve and a single band in the gel, respectively. The R^2 and E values of the five HKG candidates ranged from 0.999 – 1.000 and 90% - 105%, respectively (Table 1).

Expression profile of HKG candidates

The variation of mRNA expression levels of each candidate gene was evaluated by mean cycle threshold (Ct) analysis. The 18S was the most abundantly expressed gene, with Ct values ranging from 5.898 to 6.345, while GUSB was the least expressed gene, Ct values ranging from 22.427 to 22.678 (Figure 1).

Expression stability analysis of the HKG candidates

GeNorm ranking revealed that ACTB, PPIA, GUSB were the most stable genes, exhibiting the lowest M values, and 18S and β2M was the least stable genes, with the highest M value (Figure 2A). In addition, GeNorm analysis revealed ACTB/PPIA, with a combined stability value of 0.04, as the best combination for normalization in MDA-MB-231 cells.

Gene stability was also ranked by NormFinder, which calculates the variation of the candidate and the variation between subgroups of each sample. The NormFinder ranking identified PPIA as the most stable gene followed by ACTB and GUSB in MDA-MB-231 cells (Figure 2B). The most suitable combination of genes was ACTB/18S, with a combined stability value of 0.010 under normoxic, hypoxic and serum deprivation conditions.

The SD analysis performed with BestKeeper software showed that the gene with lowest variation value was β2M (SD [± CP] = 0.19; SD [± x-fold] = 1.13) while PPIA exhibited the highest value (SD [± CP] = 0.38; SD [± x-fold] = 1.28). The correlation coefficient (*r*) revelated 18S (*r* = 0.932; *p* = 0.001) followed by GUSB (*r* = 0.877; *p* = 0.001) and ACTB (*r* = 0.872; *p* = 0.001) as the better reference genes (Figure 2C).

To evaluate the correlation of each HKG with BestKeeper index, we selected the correlation coefficient (*r*) due to its greater precision when compared to the standard deviation (SD) [15].

Discussion

In the hypoxic microenvironment, cancer cells activate major adaptive pathways, including a shift towards anaerobic metabolism and a significant reprogramming of the cell transcriptional activity as a survival strategy [24]. Besides, cancer cell culture with serum deprivation, commonly used to establish conditions that mimic tumor milieu, where the lack of blood vessels inefficiently supply cancer cells neither with oxygen nor nutrients [25] was recently associated with altered HKG expression [11]. Given the importance of RT-qPCR analysis for cancer cells characterization [16], we investigated the expression stability of five HKG commonly used in MDA-MB-231 cells cultured in hypoxia and serum deprivation conditions. For this study, we selected the most commonly employed cell line in breast cancer research field, MDA-MB-231, and we applied three different statistical algorithms to evaluate HKG expression. To our knowledge, this is the first study identifying the appropriate set of HKG for reliable expression analysis in MDA-MB-231cells under these circumstances.

Our results showed similar expression profile observed in Ct mean analysis associated with the low stability values (M) identified by GeNorm and NormFinder softwares, and the potential of the ACTB, β2M, GUSB, PPIA and 18S genes as strong candidates for the normalization of gene expression analysis in MDA-MB-231 cells under hypoxic and serum deprivation conditions.

Despite the GeNorm and NormFinder ranking similarities observed, the combined stability analysis differed between softwares. GeNorm revealed that ACTB/PPIA combination were the most recommendable, while NormFinder revealed ACTB/18S combination as the most stable combination in all the evaluated conditions.

The best combination variability found in our results with GeNorm and NormFinder is not surprising since they are based on two different algorithms and this is also supported by other studies [18, 26, 27, 28, 29]. Despite this difference, the first three ranked genes by GeNorm and NormFinder were the same, implying that there are no substantial differences in their stability.

NormFinder, BestKeeper and GeNorm apply different and highly complex mathematical algorithms to determine the stability of reference gene candidates. The average variations of candidate genes, the sensitivity towards co-regulation and the different primer efficiencies are key factors for the softwares analyses [13, 15, 30]. In this context, we can explain the differences between the most stable reference gene presented by BestKeeper analysis in comparison to NormFinder and GeNorm results. Besides, the correction of the relative quantification of each primer efficiency was performed on the input data of the latter two softwares.

Despite of the ACTB/18S combination stability in breast cancer cell lines described by Liu and collaborators [21], the present work showed more similar values between ACTB/PPIA by the ΔΔCt and the subsequent normalization analysis as well as the output of GeNorm and NormFinder algorithms. Thus, we propose the combination ACTB/PPIA as the most stable for MDA-MB-231 cells gene expression studies under hypoxic and serum deprivation conditions.

In relation to the stability analysis, β2M, although being in the limit of tolerance, resulted to be the least stable among the analyzed candidates. Despite being used as a control gene in cell models under hypoxia [20, 26, 31, 32], several studies have also demonstrated its unsuitability as a reference gene under hypoxia and serum deprivation conditions due to its variable expression [11, 21, 22, 29, 33]. Our results, together with those from the literature, do not support the use of β2M gene as a reference gene.

Considered as classical internal controls, ACTB, GUSB and PPIA are commonly used in expression analyses performed by RT-qPCR, including normalization studies in breast cancer tumor and hypoxia [5, 12, 21, 29, 34, 35, 36]. Despite contradictory evidences regarding the use of ACTB as a reference gene under hypoxic and serum deprivation conditions [11, 20], our results are in agreement with previous studies proving its stability in different tumor models subjected to hypoxia [6, 22, 33] and now specifically in MDA-MB-231 cells.

Although the limited accessibility to nutrients may potentially affect the expression of HKG in a similar way to oxygen deprivation [6, 25, 26, 37], the effects of the serum availability on tumor cells is poorly known [11]. In this context, despite the constant use of normalizers genes in several studies [38-42], including hypoxia [10, 29, 43] and serum deprivation conditions [11], this is the first study that identifies PPIA as reference gene for RT-qPCR analysis in MDA-MB-231 cells undergoing both hypoxia and serum deprivation.

Lastly, the present study recommends the use of ACTB/PPIA as the best combination of genes, but we do not support β2M as a normalizer in hypoxic and serum deprivation conditions. In conclusion, this work highlights the relevance of the correct selection of the HKG before undergoing transcriptomic analyses under different cell culture conditions. Furthermore, this can be applied to all types of samples, including both cells and tissues, where an accurate study of HKG is crucial to draw correct transcriptomic analysis conclusions.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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FIGURES

Figure 1- Mean Ct values of HKG candidates. MDA-MB-231 cells cultured in normoxia with FBS supplementation (21% O_2, 10% FBS - N10), hypoxia with FBS supplementation (1% O_2, 10% FBS - H10) and normoxia and hypoxia without FBS supplementation, N0 and H0, respectively, for 48h. Data are expressed as the mean ± standard deviation. Ct, threshold cycle.

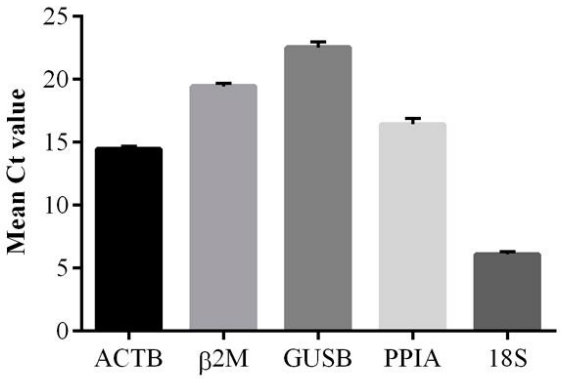
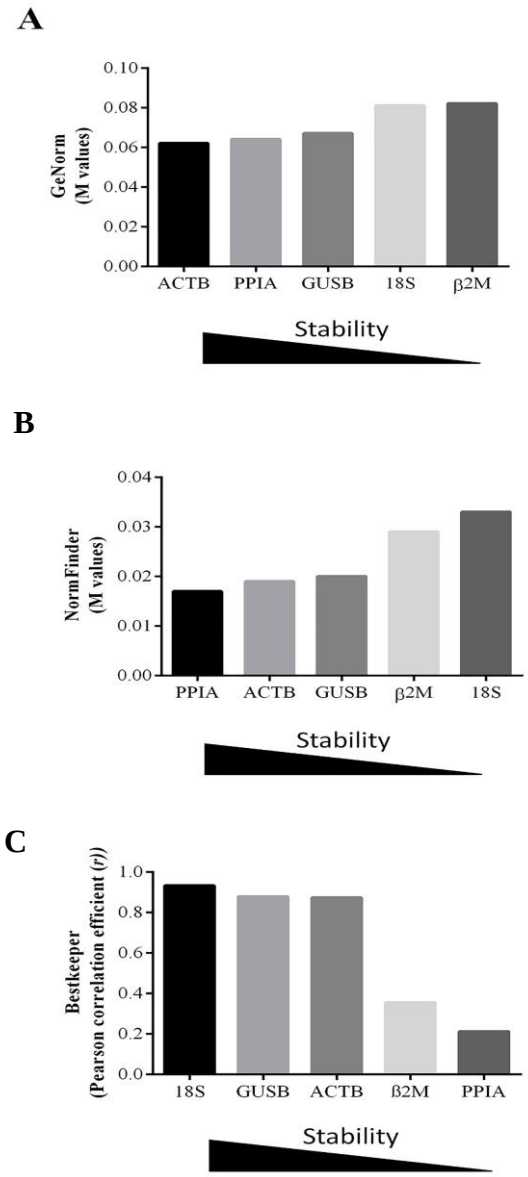


Figure 2- Expression stability values of HKG candidates analyzed by GeNorm, NormFinder and BestKeeper softwares. (A) GeNorm average expression stability measures (M) of HKG candidates in MDA-MB-cells**. (B)** NormFinder ranking of HKG candidates and their expression stability in MDA-MB-231 cells. **(C)** Correlation coefficient (*r*) values of the HKG candidates analyzed by BestKeeper software in MDA-MB-231 cells.



6 HYPOXIA AND SERUM DEPRIVATION INDUCES GLYCAN ALTERATIONS IN MDA-MB-231 CELLS

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ABSTRACT

Triple negative breast cancer (TNBC) is a major global public health problem. The lack of targeted therapy and the elevated mortality evidence the need for better knowledge of the tumor biology. Hypoxia and aberrant glycosylation are associated to advanced stages of malignancy, tumor progression, and treatment resistance. In TNBC, the role of hypoxia in the regulation of glycosylation remains largely unknown. In addition, nutrient deprivation regulates the invasive phenotype and favors TNBC cell survival. The effects of hypoxia and serum deprivation on the expression of glycosyltransferases and glycan profile were evaluated in MDA-MB-231 cell line. Our results showed that the overexpression of HIF-1α was accompanied by the acquisition of epithelial-mesenchimal transition features. Significant upregulation of fucosyl- and sialyltransferases involved in the synthesis of tumor associated carbohydrate antigens was observed together with changes in fucosylation and sialylation detected by AAL and SNA lectin blots. Bioinformatic analysis further indicated a mechanism by which HIF-1α can regulate ST3Gal6 expression and the relationship within the intrinsic characteristics of TNBC tumors. In conclusion, our results evidenced the involvement of hypoxia and serum deprivation in glycosylation profile regulation of TNBC cells triggering breast cancer aggressive features and suggesting glycosylation as a potential diagnostic and therapeutic target.

Keywords: Glycosylation; Glycosyltransferases; Hypoxia; Serum deprivation, Triple Negative Breast Cancer.

INTRODUCTION

Breast cancer is the second most common cancer worldwide, accounting for 25% of all female cancer incidences (Iarc Cruk, 2014), and is responsible for the highest number of women pathology-related deaths. Differences in phenotype and expression of certain proteins in breast cancer subtypes reflect the heterogeneity of these tumors resulting in important consequences on disease progression, treatment response and patient outcome (Perou et al., 2000; Chikarmane et al., 2015). The triple negative breast cancer (TNBC) subtype is the most aggressive form of breast cancer, occurring frequently in young women and presenting the poorest overall survival. TNBC accounts for 12% of all breast cancers diagnosed and due to the lack of expression of the key receptors, estrogen, progesterone and human epidermal growth factor receptor 2, targeted therapies are currently unavailable (Foulkes; Smith; Reis-Filho, 2010; Howlader et al., 2014).

Present in 25 to 40% of metastatic breast cancers, intratumoral hypoxia is associated with epithelial-mesenchimal transition (EMT), invasion, metastasis, resistance to chemotherapy and radiotherapy, recurrence and consequently, poor prognosis (Lundgren et al., 2007; Semenza, 2012; Wang et al., 2014). Furthermore, hypoxia together with nutrient deprivation, a common characteristic of locally advanced tumors, favor aggressive cancer phenotypes by influencing specific signaling pathways regulating cell proliferation and survival, adaptive immune responses, cell metabolism, and eventually metastasis (Badr et al., 2015; Jung et al., 2015; Marchiq and Pouyssegur, 2016). In TNBC cells, hypoxia response is often triggered by HIF-1α accumulation and nuclear translocation leading to the aforementioned phenotypes (Wang et al., 2014). Thus, the analysis of the effects of hypoxia and serum deprivation may be a promising for the discovery of new therapeutic targets for TNBC.

Glycosylation changes are a universal feature of malignant transformation and tumor progression (Pinho; Reis, 2015). In TNBC subtype, expression profile of several glycosyltransferases was correlated with clinical and survival data (Ashkani and Naidoo, 2016), and alterations in *N*- and *O*-glycosylation patterns have been suggested as markers for prognostic and treatment stratification of breast cancer patients (Mild-Langosh et al., 2014; Potapenko et al., 2015). Recently, Greville and collaborators suggested that the induction of a metabolic shift could alter breast cancer glycosylation through increased availability of sugar nucleotides, as well as

epigenetic and HIF-1α mediated regulation of glycogenes (Greville et al., 2016). Thus, identifying a possible link between hypoxia and the regulation of the glycosylation.

Moreover, alterations in glycosylation studied under serum deprivation, an approach commonly used to mimic the insufficient nutrients supply observed in several solid tumors, was identified as a cancer cell survival strategy (Britain, Dorsett and Bellis, 2017). Despite several studies reporting different roles of serum deprivation, such as regulation of mRNA transcription (Nutt and Lunec, 1996; Mahmoodi et al., 2015), induction of invasive phenotype (Reshkin et al., 2000; Ye et al., 2013), evasion of to apoptosis (Jung et al., 2015) or chemoresistance (Yakisich et al., 2017), the effects on the regulation of the glycosylation have been hardly explored.

Considering the pivotal role of hypoxia in cancer glycosylation (Koike et al., 2004; Belo et al., 2015; Peixoto et al., 2016) as well as the effects of serum deprivation in TNBC progression (Ye et al., 2013; Jung et al., 2015), we decided to investigate the alterations in the main fucosyl- and sialyltransferases involved in tumor associated carbohydrate antigens biosynthesis after submitting the TNBC cell line MDA-MB-231 to hypoxia and serum deprivation. Moreover, we evaluated *in silico* the expression of these glycosyltransferases in TNBC tumors. Finally, we investigated a possible mechanism involved in the regulation of these fucosyl- and sialyltransferases.

RESULTS

Hypoxia and serum deprivation induce morphological changes and epithelial-mesenchymal transition of breast cancer cells

In order to validate our hypoxic system, we evaluated the activation of the hypoxia marker HIF-1α in MDA-MB-231 breast cancer cell line when cells were subjected to hypoxia. Cells were exposed to either hypoxia (1% O_2) or normoxia (21% O_2) for 48h. Our analysis showed a striking increase of HIF-1α after 48h of exposure to hypoxia in relation to normoxia. This increase was more marked when cells were subjected to both hypoxia and serum deprivation (Supplementary Figure 1A). In addition to a general increase, the transcription factor HIF-1α translocated from the cytoplasm into the nucleus as a response to hyopoxia (Supplementary Figure 1B).

In addition, a morphological characterization of cells subjected to hypoxia and serum deprivation was performed. Immunostaining of α-tubulin evidenced that hypoxia/serum deprivation induced morphologic changes. In normoxia with serum supplementation, the cell morphology of MDA-MB-231 was as previously described

(Han et al., 2015). However, under hypoxia/serum deprivation conditions, cells presented larger intercellular spaces and acquired a more elongated shape (Figure 1A).

The morphology alterations observed together with the literature describing the role of hypoxia in promoting epithelial-mesenchymal transition (EMT) (Tan et al., 2018), we further characterize our cell model with EMT markers. The expression of the eptihelial marker E-cadherin, an important cell-cell adhesion protein, showed a striking decrease under hypoxic and serum deprivation conditions (Figure 1B). Concomitantly, the mesenchymal marker vimentin was abundantly expressed in MDA-MB-231 cells under hypoxic conditions (Figure 1C). Cells subjected to serum deprivation and hypoxia (H0) showed the most defined EMT, altogether confirming the transition to a more aggressive phenotype of the cells subjected to stress conditions. Despite the aforementioned morphological alterations, no major alterations in cell viability were observed under any of the studied conditions (Figure 1D).

Fuscosyltransferase transcription analysis of breast cancer cells under hypoxia and serum deprivation

In order to evaluate the glycosyltransferase status of the cells subjected to hypoxia and serum deprivation, we performed a broad transcription analysis of the main fucosyltransferases, including the ones involved in the formation of tumor associated carbohydrate antigens. Our results evidenced that after 48h of exposure to hypoxia and serum deprivation, significant changes of *FUT1*, *FUT2*, *FUT3*, *FUT5*, *FUT6*, *FUT7* and *FUT11* gene expression occurred in MDA-MB-231 cells (Figure 2). Most of the alterations were upregulations with hypoxia and serum deprivation presenting the strongest changes, suggesting a cumulative effect. An exception were *FUT4* and *FUT10*, in which hypoxia led to an expressional upregulation but a downregulation in serum deprivation. The fucosyltransferase *FUT8*, responsible for *core* fucosylation, did not show significant changes.

Sialyltransferase transcription analysis of breast cancer cells under hypoxia and serum deprivation

Similar to fucosyltransferases, hypoxia with and without serum deprivation induced alterations in the expression of sialyltransferases. In MDA-MB-231 cells, hypoxia and serum deprivation induced a significant upregulation of *ST3Gal3*, *ST3Gal4*, *ST3Gal6*, *ST6GalNAc1* silalyltransferases (Figure 3). Whereas, *ST6GalNAc5* shows consistent

downregulation in hypoxia, *ST6GalNAc2* exhibits a complex expression profile, being downregulated under serum deprivation with normoxia but upregulated under serum deprivation with hypoxia.

Our data demonstrates that the expression signature of fucosyl- and sialyltransferases was extensively affected by hypoxia and serum deprivation conditions.

Bioinformatic analysis of transcriptomic data

The previously described expressional changes of fucosyltransferases and sialyltransferases are particularly interesting when the intrinsic expression status of the respective genes of MDA-MB-231 is taken into account. For this purpose, the relative expression levels of fucosyl- and sialyltransferases of MDA-MB-231 were compared with 54 other breast cancer cell lines transcriptionaly analyzed by Barretina et al. (Barretina et al., 2012). First, the TNBC cell lines were grouped and compared to the remaining breast cancer cell lines to identify the pattern expression of the selected glycosyltransferases. Then, the glycosyltransferase genes that were cell line intrinsically down- or overexpressed were identified (Figure 4A). This data complements the alterations observed under hypoxic or serum deprivation conditions. In this regards, *FUT5* and *ST3Gal6* were innately overexpressed glycosyltransferases of MDA-MB-231. The cumulative and highly significant further upregulation of *FUT5* and *ST3Gal6* in hypoxia and under serum deprivation foreshadows therefore a strong effect on the cellular glycosylation. Interestingly, the high expression levels of *FUT5* and *ST3Gal6* of the TNBC cell line MDA-MB-231 are a typical feature of TNBC tumors of patients (Figure 4B).

Hypoxia and serum deprivation induces altered glycosylation of breast cancer cells

The glycoprofile of MDA-MB-231 cells was studied to address whether the changes found at transcriptomic level were translated into different glycosylation of proteins. For this purpose, cells were cultured under the different conditions for 72h. Analysis with *Sambucus Nigra* Agglutinin (SNA) confirmed a significant increase of α2,6 protein sialylation in both hypoxic conditions (H10 and H0) in agreement with the strong increase observed in *ST6GalNAc1* and *ST6GalNAc2* expression by RT-qPCR (Figure 5A). Evaluation with the *Auleria Aurantia* Lectin (AAL) showed that hypoxia and serum deprivation did not promote alterations in total amount of protein fucosylation after 72h (data not shown). Considering that glycosylation is a post-

translational modification and that changes in the glycan expression require the translation of the glycosyltransferases, we hypothezised that 72h of the experimental condition could be a limiting time for the glycosylation machinery to display these differences. For this reason, we evaluated fucosylation after subjecting the cells to hypoxia and serum deprivation for 6 days. Indeed, differences were displayed by AAL especially stricking in serum deprived conditions, which is in accordance with the transcription expression displayed by *FUT5* and *FUT6* (Figure 5B). Altogether these results show that the complex regulation of fucosyl- and sialyltransferase expression induced by hypoxia and serum deprivation modulate the cellular glycosylation and might thereby contribute to a more aggressive phenotype of TNBC cells.

Hif-1α transcription factor interacts with glycosylatransferase promoters

To understand the mechanism by which hypoxia modulates the expression of glycosyltransferases, we evaluated the transcriptional activation of the sialyltransferase ST3Gal6. The Chromatin Immunoprecipitation Sequencing (ChIP-seq) information from the GTRD database revealed the presence of at least 6 DNA-binding motifs for HIF-1α in the promoter region of *ST3GAL6* gene. This region also correlated with the presence of the transcription promoting histone marker H3K4me3 for the opening of the chromatin and the activation of gene transcription (polymerase II) (Figure 6).

Discussion

We present here a systematic study on transcriptional changes of fucosyl- and sialyltransferases under hypoxic and serum deprived conditions in the TNBC cell line MDA-MB-231. We further demonstrate that the induction of the HIF-1α cascade leads not only to the expression of EMT markers but also to marked changes in cellular glycosylation.

HIF-1α is known to be involved in the aggressive phenotype of TNBCs, with high expression levels associating with worse prognosis and increased metastatic potential. (Jeon et al., 2013; Wang et al., 2014). We present here that the increase in total HIF-1α and its translocation to the nucleus appears to be a corner stone for the transcriptional alteration of fucosyl- and sialyltransferases ultimately resulting in a change in cellular glycosylation. Among the observed changes in glycosyltransferase expression, the upregulation of *ST3GAL6* was the most noteable. The observed significant upregulation of ST3GAL6 in MDA-MB-231 under hypoxic and serum

deprived conditions stood out as the cell line showed already comperatively high basal expression levels of this gene. We described here that the promoter region of *ST3GAL6* has 6 HIF-1α binding sites, suggesting ST3GAL6 as a major responsive gene in hypoxia and stress response in TNBC. This is further underlined by the significant higher expression of ST3GAL6 in TNBC tumors compared to the other breast cancer subtypes.

Furthermore, the role of HIF-1α in metabolic reprograming was considered essential for the dynamic transition of breast cancer tumorigenic states (Kuo et al., 2016) and possibly involved in the breast cancer aggressiveness and resistance by epigenetic regulation of glycosylation-related genes (Greville et al., 2016). In addition, several studies have shown that the morphological changes occurring during EMT are accompanied by a metabolic shift towards glucose metabolism reprogramming and aberrant glycosylation (Li and Li, 2015; Lucena et al., 2016; Carvalho et al., 2018).

Although it has been long known that poor availability of nutrients widely exists in breast cancer due to insufficient blood supply (Vaupel and Hockel, 2000), the effects of serum deprivation in the glycosylation of breast cancer cells are poorly understood. In this context, the present work sets the basis for the comprehension of the impact of hypoxia and serum deprivation in glycosylation of TNBC cell model. It is important to highlight that the conjunction of hypoxia and serum deprivation is a culture condition mimics the *in vivo* tumor microenvironment where nutrient deprivation and hypoxia co-exists (Jung et al., 2015; Wu et al., 2015).

In breast cancer, the enhanced glucose uptake and glycolysis were observed during EMT (Kondaveet et al., 2015), and a hypoxia-induced EMT model showed that the glycosyltransferase expression regulation was involved in the cell migration and expression of EMT markers regulation (Tan et al., 2018). Our results showed for the first time, that hypoxia together with serum deprivation in breast cancer cells led to morphological alterations in the cytoskeleton organization. Moreover, these changes were accompanied with reduced expression of E-cadherin concomitant with an increased expression of vimentin. Similar alterations were observed in previous studies that showed EMT activation and increased invasion and spread under hypoxia and serum deprivation conditions (Wang et al., 2014; Jung et al., 2015; Peixoto et al., 2016). Further investigation will be needed to fully understand the mechanism by which the metabolic reprogramming may be involved in the EMT transition induced by hypoxia and serum deprivation in TNBC.

We ruled out that the differences observed in RT-qPCR results could be attibutted to a cell death effect, since no major alterations in cell viability were observed under any of the studied conditions. The importance of the Warburg effect for TNBC cells (Choi et al., 2013; Kim et al., 2013), leads us to speculate that the upregulation of fucosyl- and sialyltransferases in MDA-MB-231 cells by hypoxia and serum deprivation may be involved in the cell adaptation to metabolic stress conditions. In agreement with this hypothesis, Jones and collaborators reported an association between altered expression of ST6Gal1 sialyltransferase and increased mRNA levels of glucose transporters under hypoxia in ovarian and pancreatic cancer cells (Jones et al., 2018).

The relevance of the glycosyltransferase gene signature for cancer classification and survival was previously reported (Ashkani and Naidoo, 2016). We observed a significant induction of the FUT1/2 expression under hypoxia together with serum deprivation in MDA-MB-231 cells. Although little is known about the regulation of α1,2-fucosylation, factors such as cancer cell type, metastatic capacity and hypoxia have been associated to the expression regulation of FUT1 and FUT2 (Mejías et al., 2007; Zi et al., 2013; Bello et al., 2015).

In addition, we revealed altered expression profiles of α1,3/4-fucosyltransferases and α2,3-sialyltransferases under the applied stress conditions. The role of α1,3/4-fucosylation and α2,3-sialylation are commonly linked in tumor progression and metastasis through the expression of sialyl Lewis antigens, which are related to evasion of immne cell recognition and to cell extravasation (Higai et al., 2006; Cazet et al., 2010; Julien et al., 2011; Monzavi-karbassi et al., 2013; Shirure et al., 2015; Natoni et al., 2016; Carrascal et al., 2017). In relation to increased α2,6-sialylation, our results with the SNA lectin revealed that hypoxia and serum deprivation induces α2,6-sialylation in TNBC cells. These increases were concomitant with the expressional upregulation of the α2,6-sialyltransferases *ST6GALNAC1* and *ST6GALNAC2*. The aberrant increase of α2,6-sialylation is described to promote tumor cell survival and metastasis (Zhuo and Bellis, 2011; Schultz; Swindall; Bellis, 2012). Remarkably, Britain and collaborators demonstrated a protective effect of the increased α2,6-sialylation to serum withdrawal (Britain, Dorsett and Bellis, 2017).

The sialyltransferase ST6GalNAc1 shows very low expression levels in healthy mammary glands as well as in most carcinoma cell line models of the breast (Julien et al., 2001). However, STn, the glycan product of ST6GalNAc1, is observed in

around 30% of all breast carcinomas and more frequently in estrogen-receptor negative tumors (Sewell et al., 2006; Julien et al., 2012). We confirm here the baseline expression of ST6GalNAc1 of MDA-MB-231 under normal conditions and showed a more than hundred-fold increase of ST6GalNAc1 under hypoxia and serum deprivation. This suggests that these adverse conditions may be a regulatory mechanism underlying STn expression in TNBC.

The majority of cancer biomarkers that are currently used in the clinical settings are glycoproteins, although their detection is mainly based in the protein backbone detection (Henry and Hayes, 2012; Reis et al., 2010). Therefore, considering the glycosylation alterations displayed between cancer patients and healthy individuals drew great interest for the discovery of biomarkers based on the glycan detection to lead higher specificity and sensitivity. In most cancers, fucosylation and sialylation expression are found to be significantly modified, showing potential as predictive markers of poor outcome in breast cancer patients (Kyselova et al 2008; Alley and Novotny, 2010; Aloia et al., 2015). Overall, we expect that future studies will incorporate the contribution of hypoxia, nutrient deprivation and glycosylation into further understanding of the cancer biology and the consequences for the clinical prognosis and prediction of treatment response, specially relevant for the TNBC which lacks specific target for personalised treatment.

In summary, the current study evidenced the involvement of hypoxia and serum deprivation in the regulation of fucosyl- and sialyltransferases concomitant with morphological changes and the induction of EMT. These results support that further analysis on the effect of hypoxic microenvironment and/or nutrient deprivation and their translation into specific glycan-related antigens, such as tumor associated carbohydrate antigens, are required.

Material and Methods

Cell culture

MDA-MB-231 (Triple negative, basal-like subtype) breast cancer cell line was obtained from the American Type Culture Collection (ATCC, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Life Technologies, Waltham, MA, USA) supplemented with 10% fetal bovine serum, FBS (Gibco, Life Technologies) with medium renewal every 48h. Cells were maintained at 37°C in a 5% CO_2 humidified atmosphere. Cultured cells were routinely tested for mycoplasma contamination by PCR amplification for mycoplasma pulmonis UABCTIP,

mycoplasma penetrans HF-2 and mycoplasma synoviae 53. MDA-MB-231 identity was confirmed by STR profiling.

Hypoxic and serum deprivation conditions

After three generations, MDA-MB-231 cells were incubated for the corresponding time-points at 37°C under the following conditions: normoxia (21% O_2/94.7% N_2/5% CO_2) in DMEM 10% FBS - control group (N10); normoxia (21% O_2/94.7% N_2/5% CO_2) in DMEM/without FBS - N0 group; hypoxia (1% O_2/94.7% N_2/5% CO_2) in DMEM/10% FBS - H10 group; and hypoxia (1% O_2/94.7% N_2/5% CO_2) in DMEM/without FBS - H0 group. For hypoxia, a BINDER C-150 incubator (BINDER GmbH, Tuttlingen, Germany) was used.

Cell viability assay

The effect on cell viability produced by hypoxia and FBS deprivation was determined by cell proliferation assay (CellTiter 96® AQueous One Solution Cell Proliferation Assay, Promega, Madison, WI, USA). Briefly, three thousand cells per well were seeded in 96-well plates for 24h. Then, cells were PBS washed and subjected to normoxia or hypoxia containing DMEM or DMEM supplemented with 10% FBS. Cell viability was assessed after 48h by adding 20 µL of MTS to the medium, incubated for 2h and read at 490 nm in an automated microplate reader (BioTek, Winooski, VT, USA). Three replicates for each condition were used. Two independent experiments were conducted.

RNA isolation, cDNA synthesis and real-time PCR analysis

Forty-eight hours after incubation under N10, N0, H10 and H0 conditions, total RNA was extracted using TRyzol Reagent (Invitrogen, USA). Three µg of RNA were reverse transcribed with random primers using the SuperScript® IV Reverse Transcriptase Kit (Invitrogen, Carlsbad, CA, USA). RT-qPCR was performed with diluted cDNA (20-fold), 10 µM of each primer, 5 µL SYBR® Green Master Mix (1X) (Thermo Fischer Scientific; former Savant, MA, USA) and ultrapure water to a final volume of 10 µL using the ABI 7500 (Applied Biosystems, Foster City, CA, USA). The primers used are listed in Supplementary Table. Normalization of target gene abundance was performed with ACTB/PPIA (MDA-MB-231), according to the expression stability analysis (Albuquerque A, submitted). Two independent experiments and three technical replicates per condition were performed.

Immunofluorescence

Cells were grown on coverlips under the different experimental conditions. Then, cells were fixed with 4% paraformaldehyde (Alfa Aesar, Haverhill, MA, USA) or methanol (Fisher Scientific, Waltham, MA, USA) at RT for 20 min and permeabilized with 0.5% triton X-100 in PBS at 4ºC for 10 min, and blocked in 20% normal goat non-immune serum (Dako, Agilent, Santa Clara, CA, USA) in PBS, 10%BSA. Then, primary antibodies incubation was performed overnight at 4°C, washed with PBS and incubated with the corresponding fluorescently-labelled secondary antibody for 1 h at RT. After washing, cell nuclei were stained with 1 g/mL of 4',6-diamidino-2-phenylindole (DAPI) for 15 min at RT. Coverslips were mounted in VectaShield (Vector Laboratories, Burlingame, CA, USA) and visualized under a fluorescent microscope (Zeiss Axio Imager Z1 Apotome). The antibodies and concentrations used are listed in Table 1.

***In silico* transcription analysis**

The log2 median centered gene expression data of human cancer cell lines and breast cancer patients were extracted from the OncomineTM platform (www.oncomine.org) (Rhodes et al., 2004).The gene expression profile analysis of the cell lines was performed as described before (Duarte et al., 2017). Briefly, gene expression values of 913 human cancer cell lines of the Barretina CellLine data set (Barretina et al., 2012) were extracted and each gene's expression value was normalized into values between 0 and 100. The median for each normalized gene probe was determined among the 54 human breast cancer cell lines and among the 25 triple negative breast cancer cell lines. We defined the normal expression range for each gene transcript as the median ±10. The Curtis Breast dataset comprising 1989 human breast carcinomas was used for the gene expression analysis of primary tumors of breast cancer patients (Curtis et al., 2012).

Immunoblotting

Total cellular proteins were extracted and quantified using the DC protein assay (BioRad, Hercules, CA, USA). Equal amounts of cellular protein lysates were electrophoresed on SDS-PAGE gel and transfered to nitrocellulose membranes (GE Healthcare Life Sciences, Chicago, IL, USA). The membranes were blocked in 5% non-fat milk in TBS 0.1%Tween 20 (TBST) before incubation with primary antibodies, or in 2% polyvinylpyrrolidone (PVP) (Sigma Aldrich, Saint Luis, USA) in TBS prior to incubation with biotinylated lectins. After washing, the membranes were incubated with secondary antibodies or streptavidin conjugated with horseradish peroxidase.

ECL-plus (GE Healthcare) was used as developer. The antibodies and lectins specifications and the concentrations used are listed in Table 1. SNA specificity was tested by comparing the lectin reactivity of a neuraminidase-digested (Neuraminidase from *Clostridium perfringens*, Sigma) sample with their non-treated control (Supplemntary Figure 2)

Analysis of enriched transcription factor binding sites

The promoter region of *ST3GAL6* gene was identified using the Ensembl regulatory elements (content of CpG methylation sites, polymerase II activation, presence of activated histone H3K4me3 and the inhibitory histone marker H3k27me2) (www.ensembl.org). Prediction of HIF1α::ARNT binding sites was performed using the software GTRD v18.01 (Yevshin et al., 2017). GTRD provided the most complete collection of uniformly processed ChIP-Seq data to identify transcription factor binding sites for human. The GTRD aggregated ChIP-Seq data from GEO was reprocessed with an unified pipeline using four different peak calling tools (*macs, gem, pics, sissrs*). The ChIP-seq signals identified for HIF1α::ARNT on the *ST3GAL6* promoter region were aligned with CpG methylation sites, Polymerase II, H3K4me3 and H3K27me2 to assist the definition of the activation or repression of the *ST3GAL6* expression. The motif discovery for the ChIP-Seq peaks identified in the *ST3GAL6* promoter was assessed using known DNA-binding motifs for HIF1α and HIF1β (ARNT) described in the JASPAR database (Khan et al., 2018). The motifs are specified using position weight matrices that assign weights to each nucleotide in each position of the DNA binding motif for a transcription factor or a group of them.

Statistical analysis

Student's t-test and One-way ANOVA were performed to evaluate the statistical difference using GraphPad Prism (version 6) software. $P \leq 0.05$ values were considered significant (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) and $p \leq 0.0001$ (****).

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Table 1- Antibodies and lectins used for Western blot (WB) and Immunofluorescence

Antibody Clone/ Lectin	Antigen	Working Dilution		Blocking agent	Supplier
		IF	WB	WB	
E-Cadherin (24E10)	E-cadherin	1:100	-	-	Cell Signaling Technology
HIF1α (H1 alpha 67-NB)	HIF1α	1:50	1:500	5% non-fat milk in TBST	Novus Biologicals
α-Tubulin (DM1A)	α-Tubulin	1:750	-	-	Sigma-Aldrich
β-actin (I-19)	β-actin	-	1:2000	5% non-fat milk in TBST	Santa Cruz Biotechnology
Vimentin (V9)	Vimentin	1:500	-	-	Dako
Aleuria aurantia lectin (AAL)	Fucα6GlcNAc Fucα3GlcNAc Fucα4GlcNAc	-	1:3000	2% PVP in TBS	Vector Labs
Sambrucus Nigra lectin (SNA)	Neu5Acα6Gal/GalNAc	-	1:3000	2% PVP in TBS	Vector Labs

FIGURES

Figure 1- Morphological changes and EMT activation of TNBC cells induced by hypoxia and serum deprivation (**A**) Effect of hypoxia and serum deprivation on MDA-MB-231 cells morphology and cytoskeleton organization (α-tubulin staining) after 48h of exposure to normoxia with serum supplementation (21% O_2,10% FBS - N10), hypoxia with serum supplementation (1% O_2 , 10% FBS - H10) and normoxia and hypoxia without FBS supplementation, N0 and H0, respectively. Effect of hypoxia and serum deprivation on E-cadherin (**B**) and vimentin (**C**) expression after 48h of exposure to N10, N0, H10 and H0 conditions. Scale bar represents 50 µm. (**D**) Cell viability of MDA-MB-231 breast cancer lines determined by MTS assay after 48h of exposure to N10, H10, N0 and H0.

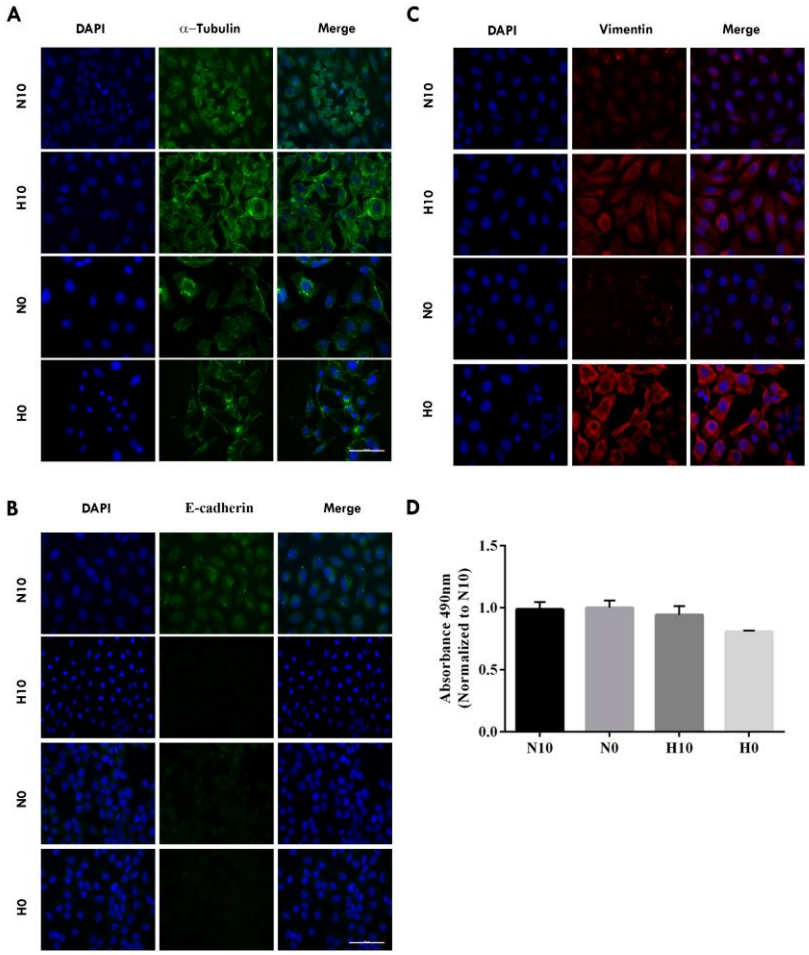


Figure 2- Fucosyltransferase transcription analysis in TNBC cells under hypoxia and serum deprivation. Quantitative real-time PCR showing levels of expression of FUT genes in MDA-MB-231 cells relative to the N10 condition and normalized to the expression of ACTB/PPIA housekeeping genes. RNA was collected after 48h of exposure to normoxia with serum supplementation (21% O_2 10% FBS - N10), hypoxia with serum supplementation (1% O_2,10% FBS - H10) and normoxia and hypoxia without FBS supplementation, N0 and H0, respectevely. Graphs represent the average value of two independent experiments with three technical replicates. Significant values are as follows: *p < 0.05, **p < 0.01; ***p < 0.001 (Student's *T*-test).

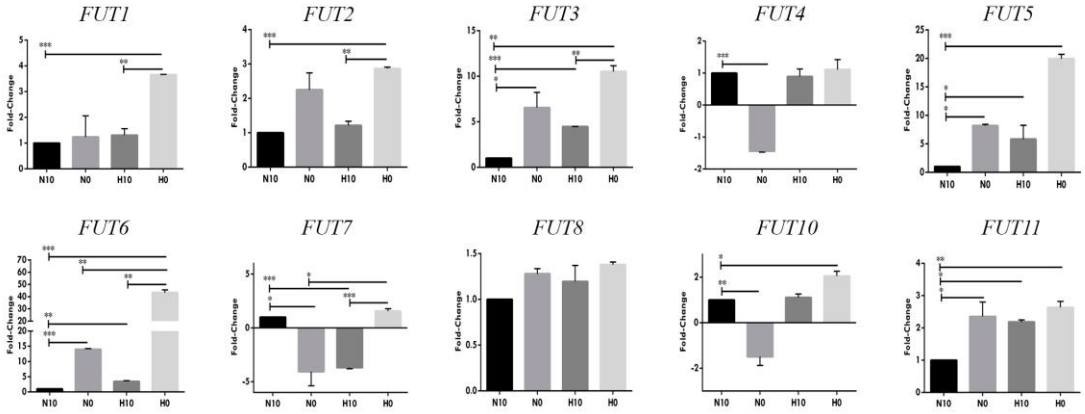


Figure 3- Sialyltransferase transcription analysis in TNBC cells under hypoxia and serum deprivation. Quantitative real-time PCR showing levels of expression of ST genes in MDA-MB-231 cells relative to the N10 condition and normalized to the expression of ACTB/PPIA housekeeping genes. RNA was collected after 48h of exposure to normoxia with serum supplementation (21% O_2 10% FBS - N10), hypoxia with serum supplementation (1% O_2,10% FBS - H10) and normoxia and hypoxia without FBS supplementation, N0 and H0, respectevely. Graphs represent the average value of two independent experiments with three technical replicates. Significant values are as follows: *p < 0.05, **p < 0.01; ***p < 0.001 (Student's T-test).

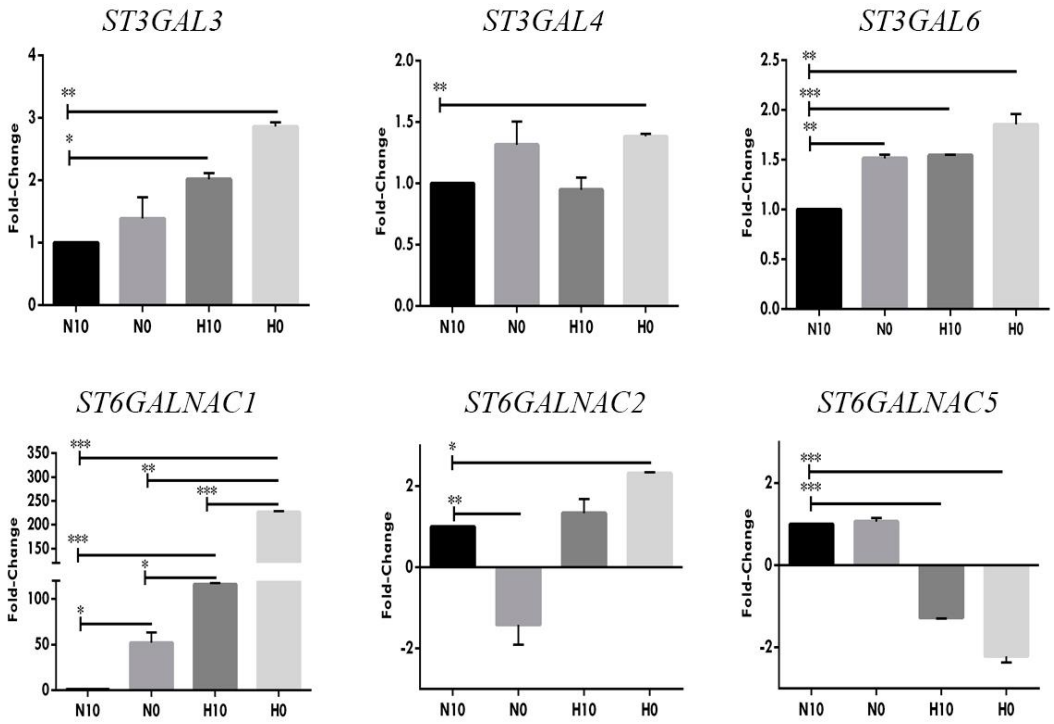


Figure 4- *In silico* analysis of fucosyltransferase and sialyltransferase expression in TNBC cell lines and human breast carcinomas. (**A**) Intrinsic fucosyltransferase and sialyltransferase gene expression of MDA-MB-231 compared to 54 other breast cancer cell lines. The raw data on transcription levels were extracted from the Barretina CellLine data deposited in the OncomineTM database. Based on normalized transcription values of all 54 breast cancer cell lines that were included in the dataset, a range of average expression values (median ±10) for breast cancer cell lines was defined for each gene. (B) Gene expression analysis of FUT5 and ST3Gal6 in overall human breast carcinomas and and TNBC subtypes. The raw data is derived from Curtis Breast data deposited in the OncomineTM database. All breast carcinomas, n=1989; TNBC, n=331. ***p<0.0001 (Student's T-test)

A

Gene	Range (All breast w/o TNBC)		Range (TNBC)		MDA-MB-231
Gene Name	**From**	**To**	**From**	**To**	
FUT1	20.91	40.91	18.14	38.14	16.12
FUT2	4.58	24.58	7.39	27.39	17.37
FUT3	3.30	23.30	5.09	25.09	12.77
FUT4	17.75	37.75	15.35	35.35	35.82
FUT5	26.40	46.40	24.83	44.83	51.00
FUT6	26.84	46.84	22.68	42.68	25.54
FUT7	1.46	21.46	1.63	21.63	16.15
FUT8	31.98	51.98	25.74	45.74	58.30
FUT10	19.18	39.18	18.85	38.85	31.80
ST3GAL3	14.94	34.94	14.48	34.48	29.11
ST3GAL4	25.51	45.51	25.15	45.15	44.45
ST3GAL6	-2.49	17.51	1.12	21.12	39.50
ST6GALNAC1	-2.82	17.18	-2.50	17.50	14.11

B

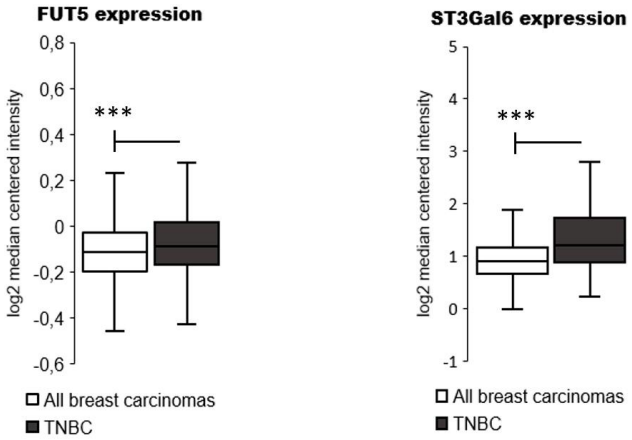


Figure 5- Hypoxia and serum deprivation induces aberrant glycosylation of TNBC cells. Sialylation and fucosylation profile MDA-MB-231 breast cancer cell line determined by SNA (**A**) and AAL (**B**) lectin blots of the cells subjected to different cell culture conditions: normoxia with serum supplementation (21% O_2,10% FBS - N10), hypoxia with serum supplementation (1% O_2 , 10% FBS - H10) and normoxia and hypoxia without FBS supplementation, N0 and H0, respectively. The corresponding β-actin immunoblots are also shown.

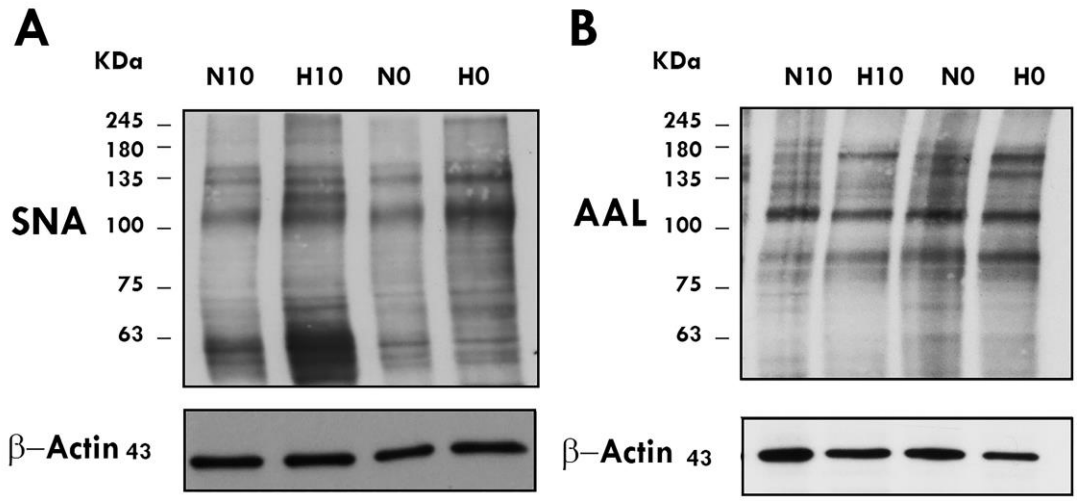
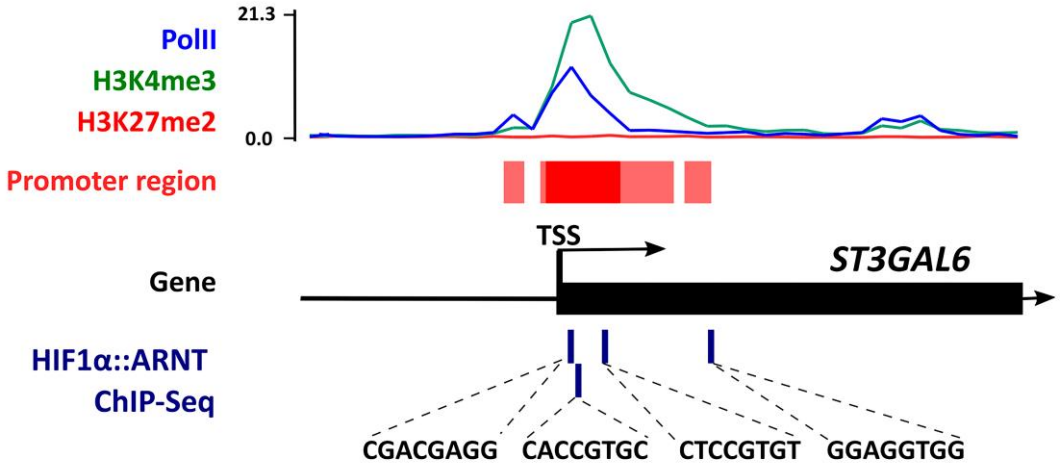


Figure 6- Hif-1α transcription factor interacts with glycosylatransferase promoters. Transcription factor binding sites in promoters of the differentially expressed *ST3GAL6* gene were analysed using known DNA-binding motifs described in the JASPAR database (Khan et al., 2018), released 2018. ChIP-seq experiment information for HIF1a was collected and analysed from GTRD database (v18.01) (Yevshin et al., 2017), released 2017. ChIP-seq binding signal (blue bars) for HIF1a::ARNT and NFkB1 are present in the promoter region of *ST3GAL6* and *FUT11* genes, respectively. Presence of the transcription factors in both genes is correlated with opening of chromatin (H3K4me3) and activation of gene transcription (Pol II), indicating a possible regulatory mechanism for those genes under hypoxic conditions. Note: Information for H3K4me2, H3K27me3, Pol II and the higher content of GCs in the promoter region (dark red) were extracted from Emsembl 92 release 2018 (www.ensembl.org). TSS, Transcription start site.



Supplementary Figure 1- (A) Western blot analysis of HIF-1α in MDA-MB-231 breast cancer cell line after 48h of exposure to different cell culture conditions. Normoxia with serum supplementation (21% O_2 , 10% FBS - N10), hypoxia with serum supplementation (1% O_2, 10% FBS - H10) and normoxia and hypoxia without FBS supplementation, N0 and H0, respectively. The corresponding β-actin immunoblots are also shown. (**B**) HIF-1α immunofluorescence in MDA-MB-231 cells exposed to N10 and H10. Scale bar represents 50 μm.

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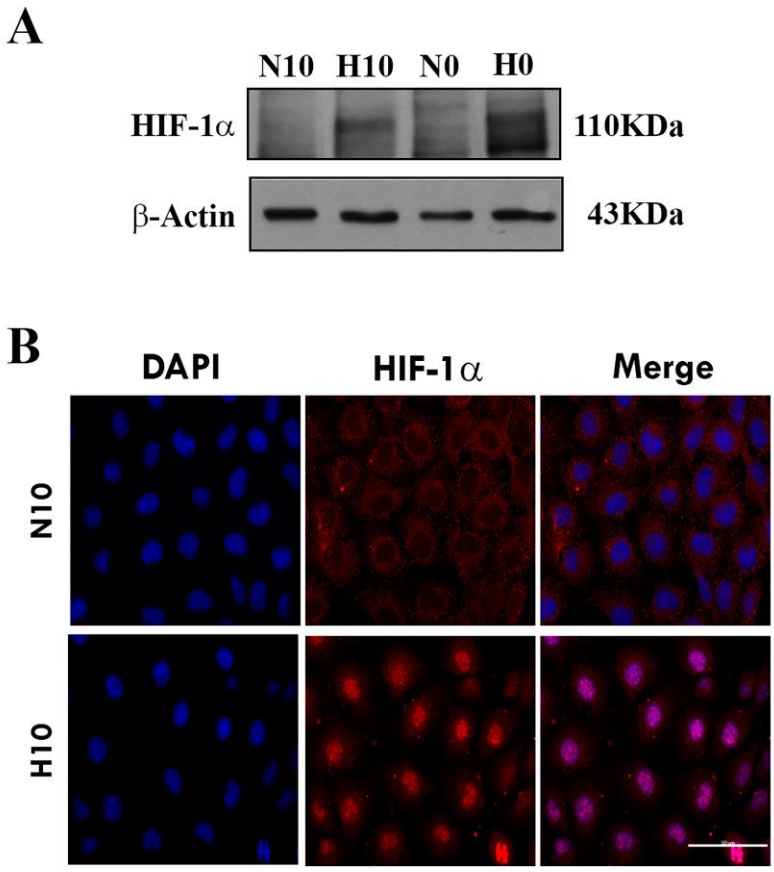
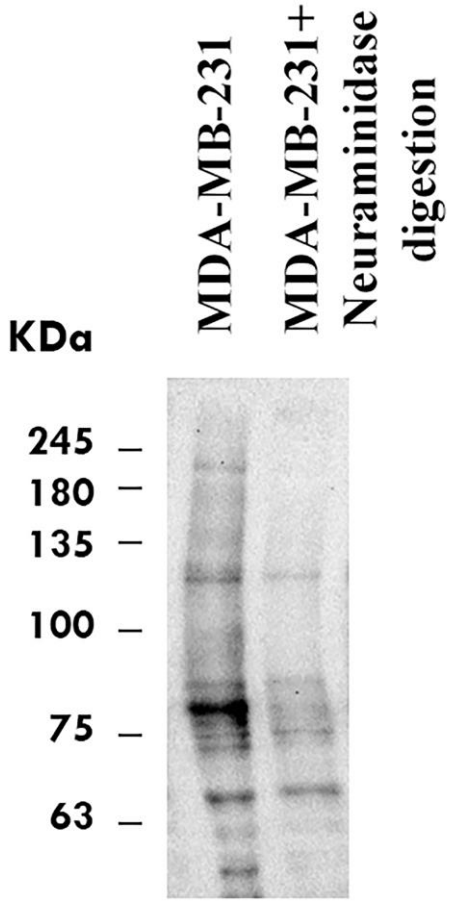


Supplementary Figure 2- SNA lectin specificity in MDA-MB-231 cells. The N10 (21% O2, 10% FBS) condition lysate was subjected to neuraminidase digestion and run in parallel to equal amounts of control N10 sample and subjected to SNA detection**.**



Supplementary Table- Primer sequences for gene expression analysis in MDA-MB-231 cells under normoxic, hypoxic and/or serum deprivation conditions.

Gene	Primer sequences	Product size/bp
α1,2-fucosyltransferses		
FUT1	F:aaagactgaaggagcatatgattgc R: tcaaacctggtcctctctagaacaa	110
FUT2	F:gcggctagcgaagattcaag R: tgatgttgaggctagcactggta	67
α1,3/4-fucosyltransferses		
FUT3	F:caaaatgccaagggtggaca R:ttggcctcaatcaatcctcct	89
FUT4	F:aagccgttgaggcggttt R:acagttgtgtatgagatttggaagct	88
FUT5	F:aagccgttgaggcggttt R:acagttgtgtatgagatttggaagct	70
FUT6	F:caaagccacatcgcattgaa R:atccccgttgcagaacca	95
FUT7	F:ccgcttctacctgtcctttga R:gcgttgcgccagaatttct	250
FUT8	F: ccatttcaggtttgtttggtag R: attggtcccgcttctcactt	200
FUT10	F:caccgtctttctgcttgtca R:ccttcctttcaaacttcccc	62
FUT11	F:gctttggcaatgtggaagaga R:gccaataatcttgcagccac	69
α2,3-sialyltransferses		
ST3Gal3	F:ggtggcagtcgcaggattt R:catgcgaacggtctcatagtagtg	76
ST3Gal4	F:cctggtagctttcaaggcaatg R:cctttcgcacccgcttct	74
ST3Gal6	F:cggctgattttagaaagattgctt R:cggctgattttagaaagattgctt	90
α2,6-sialyltransferses		
ST6GalNAc1	F: tccaagggaacacttgaacca R: gcctcaggacctacagcaat	100
ST6GalNAc2	F: ctttgccctgtacttctcg R: cagcactggaatggagaga	200
ST6GalNAc5	F: ggatcccaatcacccttcag R: tagcaagtgattctggtttcca	200
Housekeeping genes		
ACTB	F: agaaaatctggcaccacacc R: tagcacagcctggatagcaa	173
GUSB	F: agccagttcctcatcaatgg R: ggtagtggctggtacggaaa	160
PPIA	F: agacaaggtcccaaagac R: accaccctgacacataaa	118

7 CONCLUSÕES

A expressão da FUT3 foi significativamente regulada pela hipóxia e privação de soro. Nas células T47D sua expressão foi negativamente regulada a nível transcricional e proteico, fato que pode estar associado a resistência a apoptose. Ao passo que nas células MCF7 a significativa expressão observada a nível proteico e transcricional pode estar envolvida com a aquisição e/ou adição de capacidades metastáticas.

A análise da estabilidade da expressão revelou o ACTB, GUSB e PPIA como os mais estáveis de *housekeeping genes* para análises de expressão em células MDA-MB-231 submetidas a condições de hipóxia e privação de soro. A análise de estabilidade combinada associada a análise de expressão relativa revelou os genes ACTB/PPIA como a mais estável combinação para análises de expressão em células MDA-MB-231 submetidas a condições de hipóxia e privação de soro.

A significativa expressão proteica do HIF-1α bem como a localização nuclear observada após exposição a condições de hipóxia indicaram a ativação da sinalização HIF-1. As condições de estudo avaliadas não promoveram alterações na viabilidade celular. A hipóxia e a privação de soro induziram alterações morfológicas relacionadas a aquisição de características mesenquimais e habilidades migratórias nas células MDA-MB-231.

A hipóxia e a privação de soro induziram redução na expressão da E-caderina nas células MDA-MB-231. Por outro lado, a elevada expressão da vimentina observada indicou a ativação da transição epitélio-mesenquimal nas células MDA-MB-231 sob condições de hipóxia e privação de soro.

A expressão das FUTs envolvidas na síntese de antígenos carboidratos associados ao tumor foi significativamente regulada pela hipóxia e privação de soro nas células MDA-MB-231, exceto pela significativa redução da expressão da FUT8.

Semelhante as FUTs, a hipóxia e a privação de soro induziram significativas alterações na expressão das sialiltransferases envolvidas na síntese de antígenos carboidratos associados ao tumor. Houve significativa indução da expressão das sialiltransferases ST3Gal3-6 e ST6GalNAc1 enquanto que significativa redução da expressão da ST6GalNAc5 foi observada em hipóxia e a expressão da ST6GalNAc2

foi reduzida sob privação de soro e elevada sob hipóxia associada à privação de soro.

Análises *in silico* revelaram que as alterações na expressão das glicosiltransferases observadas sob hipóxia e privação de soro estão associadas as características intrínsecas de tumores do subtipo basal-like ao promover a significativa expressão da FUT5 e da ST3Gal6 nas células MDA-MB-231.

O glicofenótipo das células MDA-MB-231 foi diferentemente regulado sob condições de hipóxia e a privação de soro. Após 72h de exposição não houve alterações na α1,3-/4-/6- fucosilação ao passo que a α2,6- sialilação foi elevada sob condições de hipóxia e privação de soro. Após seis dias de exposição houve alterações na α1,3-/4-/6- fucosilação e o perfil de α2,6- sialilação foi semelhante àquele observado após 72h de exposição.

Assim, podemos concluir que o microambiente hipóxico e privado de soro tem grande impacto na regulação das vias de glicosilação envolvidas na síntese de antígenos carboidratos associados ao tumor; na regulação do aberrante glicofenótipo, especialmente na fucosilação e sialilação das células tumorais; e na regulação da transição epitélio mesenquimal, da resistência a apoptose e da promoção de fenótipos mais agressivos em modelos tumorais mamários dos subtipos luminal e *basal-like.*

8 PERSPECTIVAS

Diante dos resultados obtidos, estudos complementares são cabíveis para melhor caracterizar melhor o impacto do microambiente hipóxico e/ou privado de soro sobre aspectos glicobiológicos do câncer de mama, entre os quais estão:

- ✓ Investigar o papel da FUT3 na promoção da resistência a apoptose ou aquisição de capacidades metastáticas através da análise de vias apoptóticas e da realização de ensaios funcionais utilizando diferentes modelos tumorais mamários sob condições de hipóxia e privação de soro;
- ✓ Avaliar os efeitos da hipóxia e privação de soro sobre a expressão de glicosiltransferases e a regulação do glicofenótipo de várias linhas tumorais mamárias pertencentes ao subtipos luminal, com superexpressão de HER2 e triplo negativo;
- ✓ Avaliar a expressão de antígenos carboidratos associados ao tumor cuja síntese resulte da atividade das alteradas fucosiltransferases e sialiltransferases observadas neste trabalho;
- ✓ Realizar ensaios funcionais que permitam avaliar o impacto da hipóxia e privação de soro sobre o potencial metastático de modelos tumorais mamários com diferentes subtipos moleculares;
- ✓ Investigar a aquisição de fenótipo resistente após incubação de modelos tumorais mamários com quimioterápicos e inibidores da fucosilação e sialilação sob condições de hipóxia e privação de soro;
- ✓ Caracterizar a expressão de glicosiltransferases, antígenos carboidratos associados ao tumor e marcadores de hipóxia em amostras clinicas de pacientes diagnosticados com câncer de mama;
- ✓ Correlacionar a expressão de glicosiltransferases, antígenos carboidratos associados ao tumor e marcadores de hipóxia com os dados clinico-histopatológicos de pacientes diagnosticados com câncer de mama.

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