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PROGRAMA DE PÓS-GRADUAÇÃO EM GENÉTICA

Bruna Victorasso Jardim-Perassi

Avaliação da angiogênese em resposta ao tratamento com melatonina no câncer de mama: Estudo *in vitro* e *in vivo*

Tese apresentada para obtenção do Título de Doutor em Genética

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"A mente que se abre para uma nova ideia jamais voltará ao seu tamanho original"

Albert Einstein

Sumário

Sumário

Lista de abreviaturas e símbolos

Lista de abreviaturas e símbolos

[6(OH)M]	Fórmula química do 6-hidroximelatonina
µg	micrograma
µl	micro-litro
µM	micromol
13-HODE	do inglês *13-Hydroxyoctadecadienoic Acid*
5-HTP	Fórmula química do 5-hidroxitriptofano
AA-NAT	Arilalquilamina N-acetiltransferase
ACTB	do inglês *Beta Actin*
Akt	do inglês *Protein Kinase B*
ANOVA	do inglês *Analysis of Variance*
bFGF	do inglês *Basic Fibroblast Growth Factor*
cAMP	3`, 5-cyclic monophosphate
cDNA	DNA complementar
cm	Centímetros
cm^2	Centímetros quadrados
CO_2	Fórmula química do gás carbônico
$CoCl_2$	Fórmula química do Cloreto de Cobalto
DAB	do inglês 3,3'-diaminobenzidine tetrahydrochloride
DMBA	7,12 dimethylbenz(alpha)_anthracene
DMEM	do inglês *Dulbecco's Modified Eagle Medium*
DMSO	do inglês *Dimethyl sulfoxide*
EGF	do inglês *Epidermal Growth Factor*
EGFR	do inglês *Epidermal Growth Factor Receptor*
FAMERP	Faculdade de Medicina de São José do Rio Preto
FAPESP	Fundação de Amparo à Pesquisa do Estado de São Paulo
g	Grama
GAPDH	do inglês *Glyceraldehyde 3-phosphate dehydrogenase*
G-CSF	do inglês *Colony Stimulating Factor*

HER-2/neu	Receptor do Fator de Crescimento Epidermal 2
HIF	do inglês Hypoxia Inducible Factor
HIOMT	Hidroxi-indol-O-metiltransferase
HRP	do inglês *horseradish peroxidase*
HUVEC	do inglês *Human Umbilical Vein Endothelial Cell*
HYNIC	do inglês *Hydrazine Nicotinamide*
IACUC	do ingles *Institutional Animal Care and User Committee*
IGF-I	do inglês *Insulin-like Growth Factor I*
INCA	Instituto Nacional do Câncer
IP	Intraperitoneal
Kg	Quilogramas
Ki-67	do inglês *antigen identified by monoclonal antibody Ki-67*
LIMC	Laboratório de Investigação Molecular no Câncer
MOD	do inglês *Mean Optical Density*
MAPK	do inglês *Mitogen-Activated Protein Kinase*
mCi	millicurie
mg	Miligrama
mL	Mililitro
mM	Milimolar
mm^2	Milímetros quadrados
mm^3	Milímetros cúbicos
MMPs	Matriz Metaloproteinases
mRNA	RNA mensageiro
MTT	do inglês *3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide*
MVD	do inglês *micro-vessel density*
NAS	N-acetilserotonina
^oC	Graus Celsius
OMS	Organização Mundial de Saúde
PBS	do inglês *Phosphate Buffer Solution*
PDGF	do inglês *Platelet-derived Growth Factor*

PDGF-Ra	do inglês *platelet-derived growth factor receptor, alpha polypeptide*
PGF	do ingles *PlacentalGrowthFactor*
pH	Potencial Hidrogeniônico
PHDs	do inglês *Hydroxylated by Prolyl-4-hydroxlyases*
PI3K	do inglês *phosphatidylinositol 3-kinase*
PKC	do inglês *Protein Kinase C*
pVHL	do inglês *Protein Von Hippel-Lindau*
RANTES	Quimiocina que regula expressão e secreção por Linfócitos T
RE	Receptor de estrógeno
RENCA	do inglês *Mice Bearing Renal Adenocarcinoma*
RNA	Ácido Ribonucléico
ROI	do inglês *Regions of Interest*
RP	Receptor de Progesterona
RZR/ROR	Receptor Z para Retinóide / Receptor Órfão para Retinóide
S.E.M.	Desvio Padrão
SDF-1α	do inglês *Stromal-Cell-Derived Factor-1α*
SPECT	do ingles *Single-Photon Emission Computed Tomography*
Tc-99m	do inglês *Technetium-99m*
Tie-1	Receptor de Tirosina-quinase 1
Tie-2	Receptor de Tirosina-quinase 2
TNM	Sistema de estadiamento clínico, T= tumor, N=linfonodo (do inglês node), M= metástase
TPH1	Enzima Triptofano Hidroxilase 1
u.a	Unidades arbitrárias
VEGF	do inglês *Vascular Endothelial Growth Factor*
VEGFR	do ingles *Vascular Endothelial Growth Factor Receptor*
vWF	do inglês *von Willebrand Factor*

Resumo

RESUMO

O câncer de mama apresenta altas taxas de incidência e mortalidade, sendo a neoplasia mais comum entre as mulheres. O rápido crescimento do tumor resulta em hipóxia no microambiente tumoral, levando a uma cascata de eventos que induzem a angiogênese e conseqüente progressão do câncer. Assim, a identificação de agentes terapêuticos que possam inibir a angiogênese é essencial para o controle da progressão tumoral. Nesse sentido, tem sido demonstrado que a administração exógena da melatonina, um hormônio naturalmente secretado pela glândula pineal, apresenta diversos efeitos oncostáticos em diferentes tipos de câncer. Assim, o objetivo desse estudo foi avaliar a efetividade do tratamento com melatonina sobre a angiogênese do câncer de mama, em estudos *in vitro* e *in vivo*. No estudo *in vitro,* as linhagens de câncer de mama MCF-7 e MDA-MB-231 foram tratadas com melatonina sob condições de hipóxia mimetizadas pelo Cloreto de Cobalto ($CoCl_2$). A viabilidade celular foi verificada pelo ensaio MTT, a expressão do fator de transcrição induzido por hipóxia (HIF-1α) e do fator de crescimento endotelial vascular (VEGF-A) foi avaliada por PCR em tempo real e imunocitoquímica. Além disso, demais proteínas envolvidas na angiogênese foram avaliadas por *Protein Array*. No estudo *in vivo*, as células MDA-MB-231 foram implantadas em camundongos nude atímicos, os quais foram tratados com melatonina (40 mg/kg) por 21 dias. O tumor foi medido semanalmente e a avaliação da angiogênese foi realizada pela técnica de tomografia computadorizada por emissão de fóton único (SPECT), com o radiotraçador Tc-99m-HYNIC-VEGF-c, agente específico para os receptores de VEGF (VEGFR2/VEGF3). Além disso, as proteínas VEGFR2, VEGFR3, fator de fator de von Willebrand (vWF) e marcador de proliferação celular (Ki-67) foram avaliados no tecido tumoral por imuno-histoquímica, e demais proteínas angiogênicas por *Protein Array*. O estudo *in vitro* demonstrou que o tratamento com 1 mM de melatonina em condições de hipóxia (200 μM de $CoCl_2$) levou a diminuição da viabilidade celular, bem como dos níveis de HIF-1α e VEGF-A nas duas linhagens avaliadas ($p < 0.05$). Entre as demais proteínas avaliadas, o tratamento com melatonina sob hipóxia levou a diminuição de VEGF-C,

VEGFR2, VEGFR3, matriz metaloproteinase 9 (MMP-9) e Angiogenina em células MCF-7 ($p < 0.05$). Para linhagem MDA-MB-231, foi observada redução significante para as proteínas VEGFR2, receptor do fator de crescimento epidermal (EGFR) e Angiogenina ($p < 0.05$). No estudo *in vivo* os camundongos tratados com melatonina apresentaram menor crescimento tumoral em relação aos animais controle (144,90 ± 38,38 mm^3 *vs* 282,00 ± 88,53 mm^3; $p < 0,05$). Além disso, um animal apresentou regressão do tumor durante o tratamento com melatonina (Dia 7 = 27,38 mm^3; Dia 14 = 8,79 mm^3; Dia 21 = 4,8 mm^3). Pela técnica de SPECT foi possível detectar menor radioatividade de Tc-99-HYNIC-VEGF-c e consequente expressão reduzida de VEGFR2/3 nos tumores tratados com melatonina (150,46 ± 17,06 % *vs* 183,55 ± 20,92 %) mas o nível de significância estatística não foi alcançado ($p > 0,05$). A diminuição de VEGFR2 nos tumores tratados com melatonina foi confirmada por imuno-histoquímica ($p < 0,05$), assim como a redução da microdensidade vascular (vWF) e da proliferação celular (Ki-67) ($p < 0,05$). Não houve alteração na expressão das demais proteínas avaliadas, no entanto um aumento significante de EGFR e do fator de crescimento semelhante a insulina tipo 1 (IGF-I) foi observado nos tumores tratados com melatonina ($p < 0.05$). Dessa forma, conclui-se que a melatonina apresenta importante ação anti-angiogênica *in vitro* e *in vivo*, evidenciando sua potencial ação terapêutica no câncer de mama.

Palavras-chaves: Neoplasias da Mama; Melatonina; Neovascularização; SPECT

Abstract

ABSTRACT

Breast cancer has high rates of incidence and mortality, and it is the most common cancer among women. The rapid tumor growth results in hypoxia on tumor microenvironment, leading to a cascade of events that induce angiogenesis and subsequent cancer progression. Thus, the identification of therapeutic agents that can inhibit angiogenesis is essential for the control of tumor progression. Exogenous administration of melatonin, a hormone secreted by the pineal gland, has been shown several oncostatics effects on different types of cancers. The aim of this study was to evaluate the effectiveness of melatonin treatment on angiogenesis in breast cancer, in the in vitro and in vivo studies. In the in vitro study, breast cancer cell lines (MCF-7 and MDA-MB-231) were treated with melatonin under cobalt chloride ($CoCl_2$)-induced hypoxic conditions. Cell viability was measured by MTT assay, the expression of hypoxia-inducible factor 1-alpha (HIF-1α) and vascular endothelial growth factor (VEGF-A) was assessed by real-time PCR and immunocytochemistry. Additionally, other proteins involved in angiogenesis were evaluated by the Protein Array. In the in vivo study, the MDA-MB-231 cells were implanted in athymic nude mice, which were treated with melatonin (40 mg/kg) for 21 days. The tumor was measured weekly and evaluation of angiogenesis was performed by single-photon emission computed tomography (SPECT) with Tc-99m-HYNIC-VEGF-c, which is specific for VEGF receptors (VEGFR2/VEGF3). Moreover, VEGFR2, VEGFR3, von Willebrand factor (vWF) and cell proliferation marker (Ki-67) were evaluated in tumor tissue by immunohistochemistry, and other angiogenic proteins by Protein Array. Results from the in vitro study showed that 1 mM of melatonin under hypoxic conditions (200 μM $CoCl_2$) led to decreased cell viability and levels of HIF-1α and VEGF-A in both cell lines ($p < 0.05$). Among other proteins evaluated, melatonin treatment under hypoxia resulted in a decrease of VEGF-C, VEGFR2, VEGFR3, matrix metalloproteinase 9 (MMP-9) and angiogenin in MCF-7 cell line ($p < 0.05$). For MDA-MB-231, a significant reduction was observed for VEGFR2 protein, epidermal growth receptor (EGFR), and angiogenin ($p <0.05$). In vivo study, mice treated with melatonin showed reduced tumor growth

compared to control animals (144.90 ± 38.38 mm3 vs 282.00 ± 88.53 mm3, p < 0.05). Furthermore, one animal showed tumor regression during melatonin treatment (Day 7 = 27.38 mm^3, 8.79 mm^3 Day = 14, mm^3 Day 21 = 4.8). SPECT detect less radioactivity of Tc-99m-HYNIC-VEGF-c and consequent reduced expression of VEGFR2/3 in tumors treated with melatonin (150,46 ± 17,06 % *vs* 183,55 ± 20,92 %) *but statistical significance was not achieved (p > 0.05). The reduction of VEGFR2 in tumors treated with melatonin was confirmed by immunohistochemistry (p <0.05), as well as the reduction of micro-vessel density (vWF) and cell proliferation (ki-67) (p < 0.05). There was no change in the expression of the other proteins evaluated, however a significant increase in EGFR and Insulin-like growth factor (IGF-I) was observed in tumors treated with melatonin (p< 0.05). Taken together, our results showed that melatonin has a important anti-angiogenic effect, suggesting its potential therapeutic action in breast cancer.*

Keywords: *Breast Neoplasms; Melatonin; Angiogenesis; SPECT*

Introdução

I. INTRODUÇÃO

1. Câncer de mama

O câncer de mama apresenta alta incidência, sendo o tipo mais freqüente entre as mulheres. No Brasil, o Instituto Nacional do Câncer (INCA) estimou a ocorrência de 52.680 novos casos para o ano de 2012, e 57.120 para o ano de 2014 (INCA, 2014). A Organização Mundial da Saúde (OMS) estima que, por ano, ocorram mais de 1.050.000 novos casos de câncer de mama em todo o mundo (INCA, 2011).

Alguns fatores de risco incluem menarca precoce, menopausa tardia, nuliparidade, idade avançada, aumento do peso corporal, entre outros (FRANK; CRITCHFIELD, 2002; WU *et al.*, 2007; LERTKHACHONSUK *et al.*, 2013). Países do norte da Europa, Austrália, Nova Zelândia e América do Norte apresentam as taxas de incidência mais elevadas, seguido de taxas intermediárias na América do Sul e menores índices no continente asiático (JEMAL *et al.*, 2011). Historicamente, a menor incidência atribuída à população asiática relaciona-se a idade mais avançada da menarca, baixo peso corporal e estilo de vida da população, que inclui atividade física regular e alimentação rica em vegetais (WU *et al.*, 2006; SIEGEL *et al.*, 2014).

A taxa de mortalidade por câncer de mama também é elevada, sendo a quinta maior causa de morte relacionada ao câncer (INCA, 2011). No Brasil, este tipo tumoral é o que mais causa mortes entre as mulheres desde 1979 (INCA, 2003). Na população mundial, a sobrevida média das pacientes após cinco anos de acompanhamento é de 61 % e apesar do progresso no diagnóstico e tratamento

nos últimos 30 anos, essa doença é ainda responsável por quase meio milhão de mortes por ano (SNOUSSI *et al.*, 2010).

O estabelecimento do prognóstico e do planejamento terapêutico do câncer de mama baseia-se no tipo, grau histológico do tumor e classificação pelo sistema de estadiamento TNM, que abrange a avaliação do tamanho tumoral, presença de metástase em linfonodos regionais e de metástase à distância (PEDERSEN *et al.*, 2004). Além dos fatores clínico-patológicos, o conhecimento das características moleculares dos tumores tem contribuído com a avaliação mais precisa do prognóstico, e com o desenvolvimento e incorporação de novos agentes e estratégias terapêuticas (GONZALEZ-ANGULO *et al.*, 2007; GRALOW *et al.*, 2008; HICKS; KULKARNI, 2008; DUFFY *et al.*, 2011). Atualmente, o *status* do receptor de estrógeno (RE), receptor de progesterona (RP) e do receptor do fator de crescimento epidermal 2 (HER-2/neu) são exemplos de características moleculares dos tumores mamários que foram incorporados à rotina clínica e permitem que se estabeleça um tratamento individualizado (HSIAO *et al.*, 2010).

Pacientes com tumores RE positivos podem ser tratados com antagonistas desse receptor, enquanto o uso do trastuzumabe em pacientes com câncer de mama que apresentam superexpressão da proteína HER-2/neu é um exemplo de sucesso da terapia-alvo com anticorpos monoclonais (FERNANDES *et al.*, 2009; HSIAO *et al.*, 2010) Porém, tumores triplo-receptor-negativos (RE-, RP- e HER-2/neu-) apresentam pior prognóstico, uma vez que não respondem a tratamentos específicos, relacionando-se a ocorrência de metástases e menor sobrevida das pacientes (FERNANDES *et al.*, 2009).

Nesse contexto, atualmente muitas pesquisas exploram os mecanismos moleculares que contribuem com a progressão do tumor, a fim de identificar novos alvos de terapias. Dentre esses mecanismos, o processo de formação de novos vasos sanguíneos no tecido tumoral é essencial para seu crescimento e disseminação metastática e, portanto, cresce o interesse pelo desenvolvimento de agentes terapêuticos que atuem nesse processo (HANAHAN; WEINBERG, 2011; GACCHE; MESHRAM, 2013).

2. Angiogênese

O crescimento do tumor é dependente da angiogênese, processo de formação de novos vasos sanguíneos a partir da proliferação e migração de células endoteliais preexistentes. Além disso, estudos recentes têm demonstrado que outros mecanismos também contribuem com a formação de novos vasos no tumor, como a vasculogênese, até então um processo considerado restrito à fase embrionária (WEIS; CHERESH, 2011; ARBAB, 2012). Ao contrário da angiogênese, a vasculogênese consiste na diferenciação de células progenitoras, os angioblastos, em células endoteliais maduras (Figura 1) (FOLKINS *et al.*, 2009; EL HALLANI *et al.*, 2010; PATENAUDE *et al.*, 2010; WEIS; CHERESH, 2011; ARBAB, 2012).

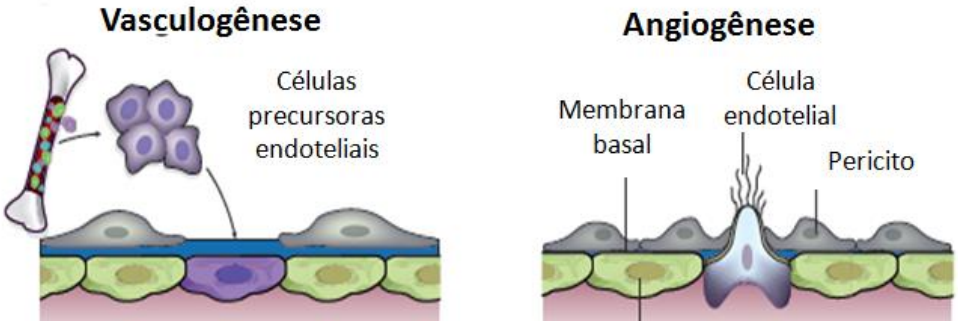

Figura 1. Representação dos diferentes modelos de formação de novos vasos sanguíneos. Adaptado de (CARMELIET; JAIN, 2000).

A formação de novos vasos sanguíneos no tecido tumoral visa o fornecimento de nutrientes e oxigênio permitindo a proliferação das células e consequente crescimento e progressão do tumor (TAKAHASHI; SHIBUYA, 2005; GAVALAS *et al.*, 2013). Além disso, permitem a retirada do gás carbônico (CO_2) e dos resíduos metabólicos, e representam uma importante via de disseminação metastática (ZHANG *et al.*, 2010; LIU; OUYANG, 2013).

O processo angiogênico é regulado por inúmeros fatores pró e anti-angiogênicos, no entanto, durante a carcinogênese estímulos fisiológicos podem levar ao aumento dos fatores pró-angiogênicos e diminuição dos anti-angiogênicos, resultando na ativação do "interruptor angiogênico" (*angiogenic switch*). Assim, as células endoteliais quiescentes passam a responder a estímulos de proliferação e migração para formação de novos vasos (Figura 2) (BERGERS; BENJAMIN, 2003).

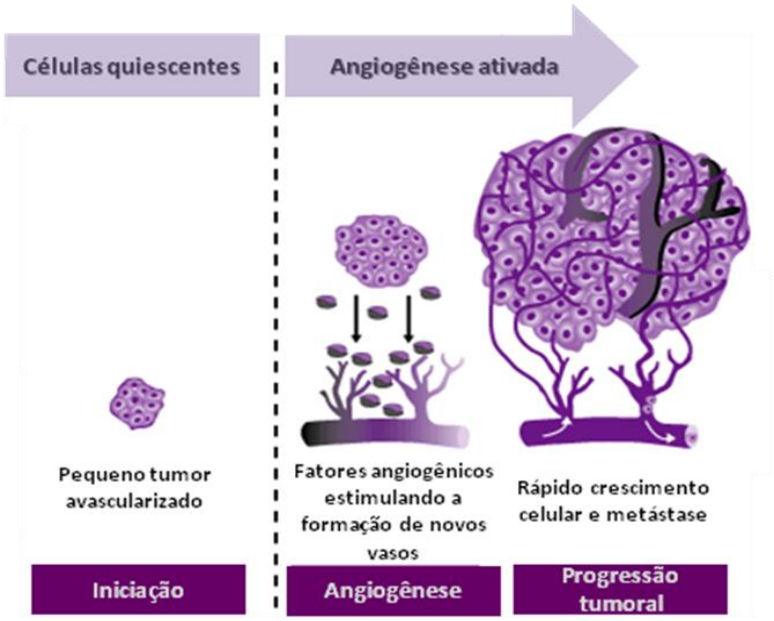

Figura 2: Representação da ativação da angiogênese a partir de células quiescentes. Adaptado de http://www.clinicaloptions.com/

Durante seu crescimento, o tumor pode alcançar aproximadamente 1-2 mm^3 antes que suas demandas metabólicas sejam restritas devido ao limite de difusão de oxigênio e nutrientes no local (CARMELIET; JAIN, 2011). A baixa oxigenação é caracterizada como hipóxia e pode ocorrer devido à proliferação descontrolada das células e rápido crescimento do tumor, além da perfusão inadequada em parte do tecido resultante da estrutura caótica dos novos vasos sanguíneos formados (CARMELIET; JAIN, 2000; HARRIS, 2002; MILANI; HARRIS, 2008; VORDERMARK, 2010).

De acordo com a intensidade, a hipóxia pode resultar em apoptose, ou induzir respostas adaptativas de sobrevivência celular (SADRI; ZHANG, 2013). Assim, ao contrário das células normais, para manter a sobrevivência em situações de hipóxia, as células tumorais são capazes de promover mecanismos adaptativos,

como a indução de fatores envolvidos no processo de angiogênese (KALLERGI *et al.*, 2011).

O fator de transcrição induzido por hipóxia (HIF-1) é essencial na manutenção da homeostase do oxigênio, e responsável por essas respostas adaptativas. O HIF-1 é um fator heterodímero composto de duas subunidades HIF-1α e HIF-1β. Em normóxia o HIF-1β é constitutivamente expresso e o HIF-1α sofre degradação dependente de oxigênio (SEMENZA, 2002). O HIF-1α é hidroxilado e então reconhecido pela proteína supressora de tumor Von-Hippel-Lindau (pVHL) e ubiquitinilado para sofrer degradação proteossomal (DANG; SEMENZA, 1999). Em condições de hipóxia, essa degradação não acontece e então o HIF-1α migra para o núcleo, associando-se a HIF-1β, atuando como fator de transcrição de diversos genes (Figura 3) (SEMENZA, 2002; MIMEAULT; BATRA, 2013; TUNG *et al.*, 2013).

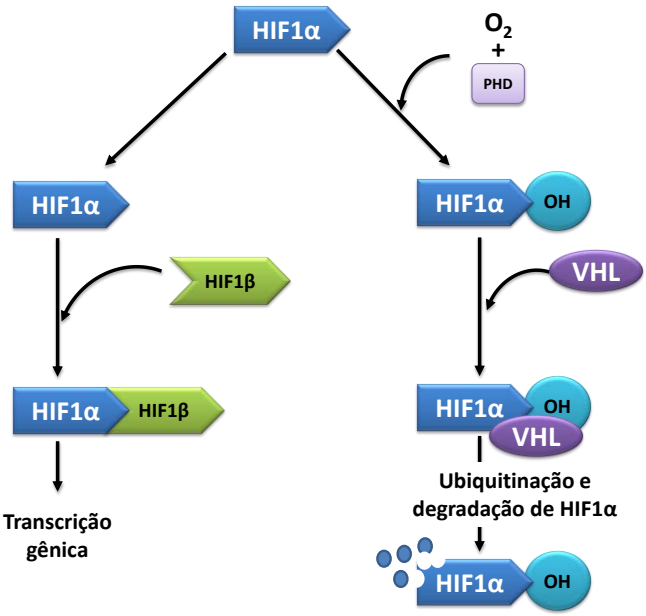

Figura 3. Ativação do HIF-1α em situações de hipóxia e degradação em normóxia.

O HIF-1α pode regular mais de 100 genes envolvidos nos processos da eritropoiese, metabolismo do ferro e da glicose, proliferação celular, apoptose e angiogênese (KE; COSTA, 2006). Em condições de hipóxia, o principal alvo do HIF-1α é o fator pró-angiogênico denominado fator de crescimento endotelial vascular (VEGF) (KE; COSTA, 2006).

O VEGF é um potente mitógeno que atua em diferentes etapas do processo angiogênico, promovendo o aumento da permeabilidade vascular, estimulação da migração, proliferação e invasão de células endoteliais (HOEBEN *et al.*, 2004; DELLI CARPINI *et al.*, 2010; GREENBERG; RUGO, 2010; SHIBUYA, 2011). Esse fator foi primeiramente descrito em células endoteliais e, portanto, denominado "fator de crescimento endotelial vascular", no entanto, o VEGF pode exercer ação mitogênica em outros tipos celulares (FERRARA; DAVIS-SMYTH, 1997). O VEGF é composto por uma família de cinco isoformas denominadas VEGF-A, VEGF-B, VEGF-C, VEGF-D e fator de crescimento placentário (PGF), os quais ligam-se a receptores específicos do tipo tirosina quinase, promovendo uma cascata de eventos intracelulares (BRITO *et al.*, 2011; FINLEY *et al.*, 2013).

Cada isoforma pode ativar um ou mais receptores conhecidos, como VEGFR1 localizado na superfície de células hematopoiéticas, macrófagos e monócitos, VEGFR2 encontrado no endotélio vascular e linfático e o VEGFR3 localizado predominantemente no endotélio linfático (STEFANINI *et al.*, 2008; TANEJA *et al.*, 2010). Receptores de VEGF também são encontrados em células

tumorais, e podem estimular o crescimento celular de maneira autócrina (WEIS; CHERESH, 2011; ARBAB, 2012).

O VEGF–A se liga a dois receptores específicos, o VEGFR1 e o VEGFR2 enquanto o VEGF–B e PGF são reconhecidos apenas pelo receptor VEGFR1. O VEGF-C e VEGF-D se ligam ao VEGFR2 e também são reconhecidos pelo VEGFR3 (Figura 4) (RAHIMI, 2012). A ligação entre o VEGF-A e VEGFR2 é considerada o mais importante passo do processo de angiogênese (WOOLARD *et al.*, 2004; MATSUURA *et al.*, 2009; COULON *et al.*, 2011), enquanto a ligação de VEGF-C com VEGFR3 está envolvida no processo de linfangiogênese (BRITO *et al.*, 2011).

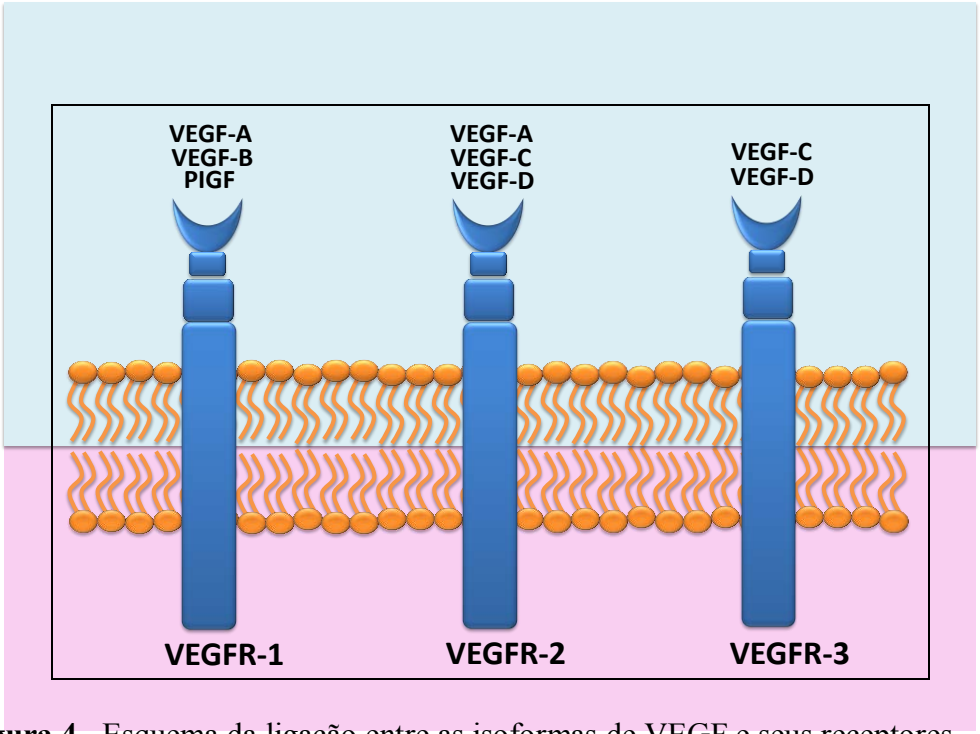

Figura 4. Esquema da ligação entre as isoformas de VEGF e seus receptores.

A angiogênese envolve um número sequencial de etapas e além do VEGF e seus receptores, outros inúmeros fatores participam desse processo, como as Angiopoitinas 1 e 2 e seus receptores Tie-1 e Tie-2, Angiogenina,

Metaloproteinases de matriz (MMPs), fator de crescimento derivado de plaquetas (PDGF), fator de crescimento de fibroblastos básico (bFGF), fator de crescimento epidermal (EGF), fator de crescimento dependente de insulina (IGF-I), citocinas pró-inflamatórias, entre outros (ARBAB, 2012).

Inicialmente, ocorre a ativação de células endoteliais quiescentes por fatores liberados pelas células tumorais em resposta a condições adversas como privação de nutrientes e oxigênio. Alguns fatores como VEGF e Angiopoitina-2 atuam na desestabilização inicial dos vasos pré-existentes e aumento da permeabilidade vascular. Em seguida ocorre a degradação da membrana basal e da matriz extracelular, permitindo a migração de células endoteliais pelo espaço intersticial e eventual liberação de fatores pró-angiogênicos ligados a matriz. Subsequentemente ocorre a migração e proliferação de células endoteliais, bem como a formação do tubo, recrutamento e diferenciação de células de suporte perivascular (pericitos). Nessa etapa inúmeros fatores estão envolvidos como VEGF, Angiogenina, PDGF, bFGF, EGF, entre outros. Ao final, ocorre a maturação e estabilização dos novos vasos formados, com a participação de fatores como a angiopoitina-1 e seu receptor Tie-2 (Figura 5) (CLAPP *et al.*, 2009; COULON *et al.*, 2011; WEIS; CHERESH, 2011).

Essas etapas podem ser inibidas por fatores anti-angiogênicos como a endostatina, ou o fragmento derivado do plasminogênio, denominado de angiostatina, os quais podem induzir a apoptose em células endoteliais ou inibirem a ativação de alguns fatores pró-angiogênicos (COULON *et al.*, 2011).

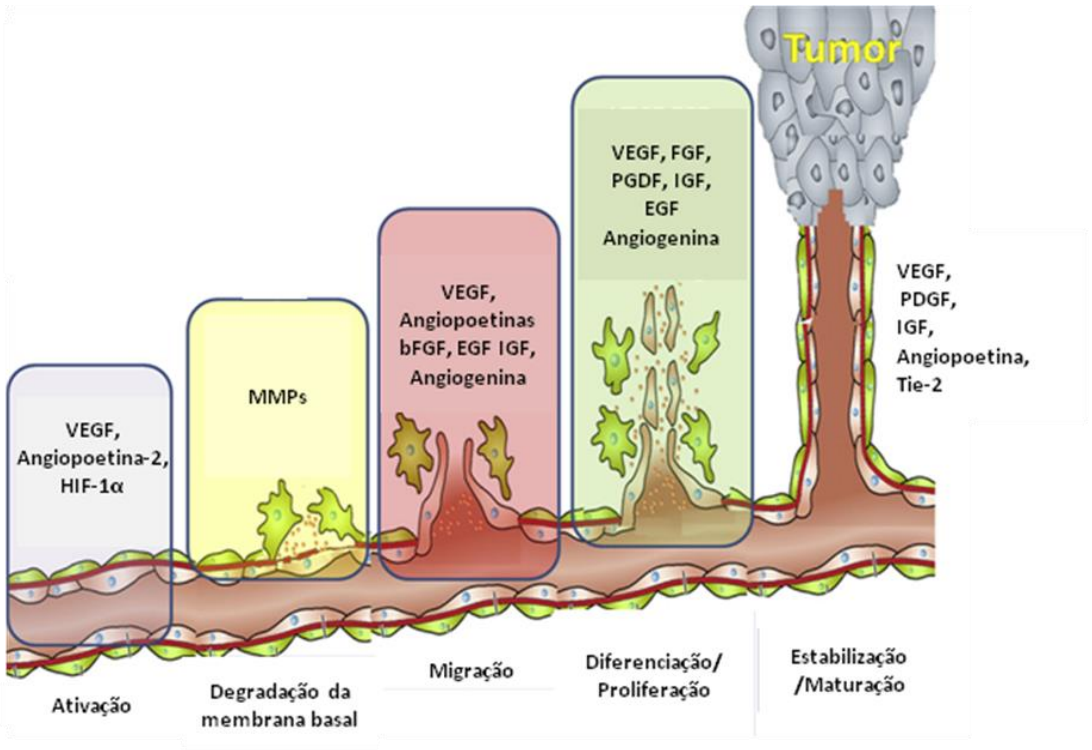

Figura 5. Representação das etapas do processo de angiogênese com a participação de inúmeros fatores pró-angiogênicos (GACCHE; MESHRAM, 2013).

Nesse contexto, dada a variedade de sinais envolvidos no processo angiogênico, diversos fatores podem ser considerados alvos terapêuticos, auxiliando no bloqueio da progressão do câncer (ARBAB, 2012; GACCHE; MESHRAM, 2013).

3. Melatonina

3.1. Síntese e degradação

A melatonina é um hormônio naturalmente produzido e secretado principalmente pela glândula pineal e possui diversas funções fisiológicas, dentre as quais se destaca a cronobiológica. Esse hormônio é considerado um "tradutor-neuroendócrino" do ciclo circadiano, controlando os padrões secretórios de

diversas substâncias, como o cortisol e adrenalina, atuando sobre os ciclos de atividade-repouso e vigília-sono (SOUSA-NETO, 2005; MAGANHIN, 2008). Além disso, a melatonina atua sobre o sistema reprodutor, cardiovascular, sistema imunológico, crescimento e envelhecimento (MAGANHIN, 2008; PANDI-PERUMAL *et al.*, 2013).

Sua produção segue um padrão rítmico, com pico secretório no período noturno, atingindo níveis plasmáticos máximos entre 03:00 e 04:00 horas em humanos, e quase nenhuma produção no período diurno (RAVINDRA *et al.*, 2006; FERREIRA, 2010; ESPINO *et al.*, 2012). É sintetizada a partir da conversão do aminoácido triptofano em 5-hidroxitriptofano (5-HTP) pela enzima triptofano hidroxilase 1 (TPH1). O 5-HTP é descarboxilado pela 5-HTP descarboxilase em serotonina, a qual é acetilada em N-acetilserotonina (NAS) na reação catalisada pela enzima arilalquilamina N-acetiltransferase (AA-NAT). Então, a NAS é convertida em melatonina pela enzima hidroxi-indol-O-metiltransferase (HIOMT) (ESPINO *et al.*, 2012; AL-OMARY, 2013) (Figura 6).

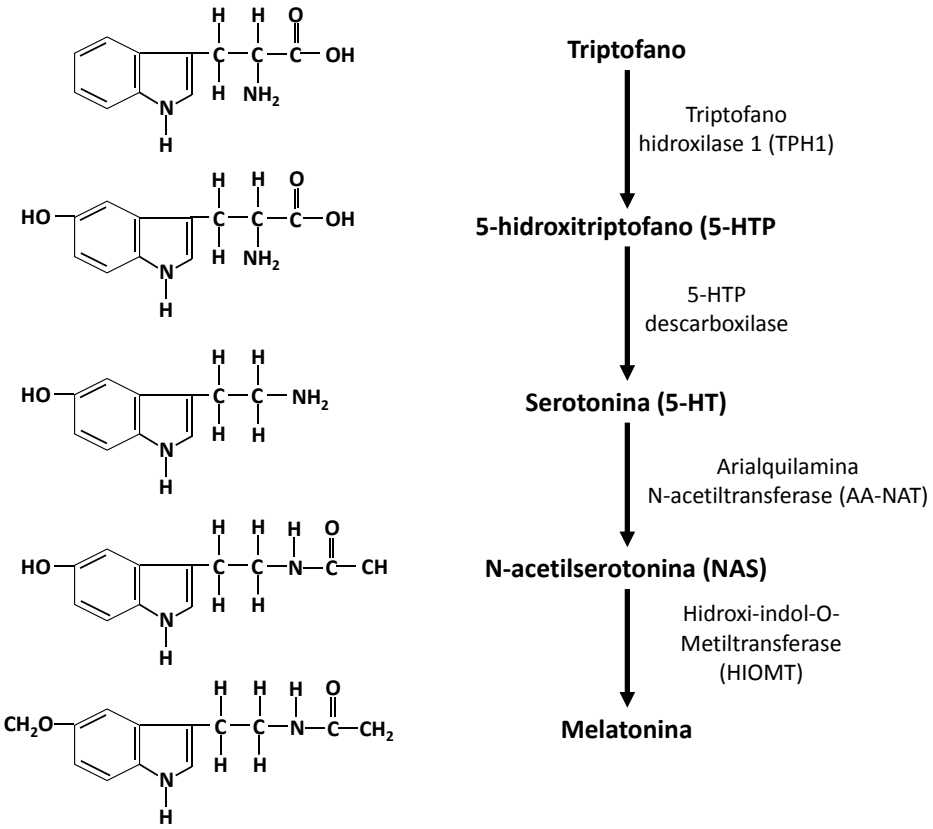

Figura 6. Esquema representativo da via de síntese da melatonina, desde o aminoácido triptofano até o último passo para a formação de melatonina pela enzima HIOMT.

A enzima AA-NAT apresenta um ritmo diário, atingindo concentrações 100 vezes superiores na fase escura, quando comparado à fase clara. Esta variação cíclica da AA-NAT faz com que a redução dos níveis de serotonina na fase escura seja acompanhada pelo aumento das concentrações de NAS e melatonina (HISSA, 2008). A melatonina é secretada durante a noite em todas as espécies de mamíferos, independente de possuírem hábito diurno ou noturno (TUREK; GILLETTE, 2004; KORF; VON GALL, 2006; BILU; KRONFELD-SCHOR, 2013).

Sua degradação ocorre principalmente no fígado, envolvendo a hidroxilação em 6-hidroximelatonina [6(OH)M], seguida dos processos de sulfatação ou glicuronidação, e posterior excreção na urina (REITER, 1991; FACCIOLA *et al.*, 2001; SEMAK *et al.*, 2008).

3.2. Receptores da melatonina

A melatonina pode atuar por meio de receptores de membrana acoplados a proteína G, denominados MT1 e MT2. Esses receptores são encontrados em diversos tecidos, incluindo sistema nervoso central, trato gastrointestinal, pulmão, pele, glândula adrenal, coração, vasos sanguíneos, células do sistema imune, entre outros (DUBOCOVICH; MARKOWSKA, 2005). Um terceiro receptor com menor afinidade pela melatonina é denominado MT3 e sua ativação ainda não possui papel fisiológico definido, no entanto apresenta 95 % de homologia com a enzima quinona redutase II, envolvida na detoxificação de radicais livres (FOSTER *et al.*, 2000).

A melatonina também pode atuar por mecanismos independentes de seus receptores de membrana, exercendo atividade antioxidante diretamente na redução de radicais livres ou pelo aumento de enzimas antioxidantes. Ainda, por ser lipossolúvel, a melatonina pode atravessar diretamente a membrana celular e interagir com proteínas intracelulares, como a calmodulina, e com receptores nucleares RZR/ROR (receptor Z para retinóide / receptor órfão para retinóide). Apesar dessa capacidade de interação ainda ser controversa, a ligação da melatonina com receptores nucleares explica muitas de suas funções, inclusive

relacionadas à alteração de genes envolvidos na proliferação celular e apoptose (SANCHEZ-BARCELO *et al.*, 2003).

3.3. Melatonina e câncer

Além de estar envolvida em muitas funções fisiológicas, a melatonina tem um importante papel em processos patológicos, incluindo o câncer. Os primeiros indícios de que a melatonina poderia ser útil na terapêutica oncológica surgiram em um estudo realizado por Cohen et al. (COHEN *et al.*, 1978). Os autores propuseram que a diminuição da função da glândula pineal poderia aumentar o risco de desenvolvimento do câncer de mama, sugerindo que a ausência da síntese de melatonina poderia induzir a exposição prolongada ao estrógeno resultando no desenvolvimento do tumor mamário.

Em 1981, Tamarkin *et al.*, (1981) demonstraram que o tratamento com melatonina inibiu o desenvolvimento de tumores mamários induzidos pelo carcinógeno 7,12-dimethylbenz(alpha)-anthracene (DMBA) em ratas Sprague-Dawley, e, ao contrário, a remoção da glândula pineal estimulou o desenvolvimento desses tumores. No mesmo ano, Bartsch *et al.*, (1981) demonstraram que pacientes com câncer de mama apresentam diminuição nos níveis plasmáticos de melatonina.

Em seguida, estudos epidemiológicos demonstraram que o trabalho noturno com consequente exposição à luz e bloqueio da síntese de melatonina é um importante fator de risco para o câncer de mama. Sugere-se que qualquer alteração no ciclo circadiano pode contribuir para o desenvolvimento do câncer, e

sendo assim, associa-se o risco elevado da ocorrência desta doença em trabalhadores noturnos, e, de provável idêntica razão, baixo risco em mulheres cegas (STEVENS *et al.*, 2013).

Na década de 90 alguns estudos clínicos foram realizados, demonstrando que a utilização da melatonina, em conjunto com terapias convencionais, apresenta efeitos benefícios em diferentes tipos de tumores avançados intratáveis ou em pacientes com tumores metastático, quer seja aumentando a eficácia do tratamento (LISSONI *et al.*, 1995; LISSONI *et al.*, 1996), ou diminuindo os efeitos colaterais contribuindo com a melhora da qualidade de vida desses pacientes (BARNI *et al.*, 1990; LISSONI *et al.*, 1992).

Desde então, têm sido demonstrado que a melatonina inibe o desenvolvimento e a progressão de diferentes tipos de câncer, e muitos mecanismos de ação estão sendo investigados (VIJAYALAXMI *et al.*, 2002; JUNG; AHMAD, 2006; MARTIN *et al.*, 2006; DI BELLA *et al.*, 2013; OPREA-ILIES *et al.*, 2013).

No câncer de mama, a ação da melatonina é principalmente descrita em tumores REα-positivos, uma vez que pode interagir com a via de sinalização do estrógeno, atuando como um modulador do RE e de enzimas envolvidas na síntese do estrógeno (MEDIAVILLA *et al.*, 2010; PROIETTI *et al.*, 2013). Além disso, entre outros mecanismos, a melatonina pode controlar o ciclo celular, atuando como pró-apoptótica e anti-proliferativa e induzindo a diferenciação celular. Possui importante ação antioxidante, reduzindo os danos oxidativos provocados por radicais livres e prevenindo o efeito colateral de tratamentos citotóxicos. Atua

ainda na inibição da enzima telomerase, na modulação do sistema imune, inibição de proteínas envolvidas na invasão celular e no processo metastático (MEDIAVILLA *et al.*, 2010; DI BELLA *et al.*, 2013; PROIETTI *et al.*, 2013).

Também tem sido demonstrado que a melatonina pode atuar na angiogênese direta ou indiretamente, inibindo a proliferação de células endoteliais e de fatores pró-angiogênicos em diferentes tipos tumorais (LISSONI *et al.*, 2001; CUI *et al.*, 2006; KAUR *et al.*, 2007; DAI *et al.*, 2008; PARK *et al.*, 2009; PARK *et al.*, 2010; ALVAREZ-GARCIA *et al.*, 2013a; b; CARBAJO-PESCADOR *et al.*, 2013).

Objetivos

II. OBJETIVOS

Sendo a angiogênese é um processo essencial para o crescimento do tumor, a identificação de agentes terapêuticos que atuem nesse processo é fundamental para a inibição da progressão tumoral. Nesse sentido, nos últimos anos tem sido demonstrado que a melatonina apresenta grande potencial terapêutico no câncer, e diversos efeitos anti-tumorais estão sendo identificados. Assim, o objetivo geral desse estudo foi avaliar a eficácia do tratamento com melatonina na angiogênese do câncer de mama, em estudos *in vitro* e *in vivo*.

- **Estudo *in vitro*:** Verificar a viabilidade celular, expressão gênica e protéica do HIF-1α e VEGF-A e a expressão de demais proteínas envolvidas no processo angiogênico nas linhagens de câncer de mama MCF-7 (REα-positiva) e MDA-MB-231 (triplo-receptor negativo) após a indução da hipóxia e tratamento com melatonina.
- **Estudo *in vivo:*** Desenvolver o modelo animal de câncer de mama pela implantação das células da linhagem MDA-MB-231 em camundongos nude atímicos e avaliar a eficácia do tratamento com melatonina sobre a proliferação celular e crescimento tumoral. Além disso, avaliar a angiogênese *in vivo* com o radiotraçador Tc-99m-HYNIC-VEGF-c pela Tomografia Computadorizada por Emissão de Fóton único (SPECT) e a expressão de proteínas angiogênicas no tecido tumoral.

Capítulos

III. CAPÍTULOS

Os resultados referentes aos objetivos desta tese serão apresentados a seguir na forma de dois artigos científicos:

Artigo I

Título: Effect of Melatonin on Tumor Growth and Angiogenesis in Xenograft Model of Breast Cancer

Autores: Bruna Victorasso Jardim-Perassi, Ali S. Arbab, Lívia Carvalho Ferreira, Thaiz Ferraz Borin, Nadimpalli R. S. Varma, A. S. M. Iskander, Adarsh Shankar, Meser M. Ali, Debora Aparecida Pires de Campos Zuccari

Periódico: PLoS ONE - publicado em 9 de janeiro de 2014 (doi:10.1371/journal.pone.0085311).

Artigo II

Título: Melatonin regulates angiogenic factors under hypoxia in breast cancer cell lines

Autores: Bruna Victorasso Jardim-Perassi, Mateus Repolês Lourenço, Gabriel Mandarini Doho, Ingrid Helen Grígolo, Gabriela Bottaro Gelaleti, Lívia Carvalho Ferreira, Thaiz Ferraz Borin, Marina Gobbe Moschetta, Debora Aparecida Pires de Campos Zuccari

Artigo I

OPEN ACCESS Freely available online

PLOS ONE

Effect of Melatonin on Tumor Growth and Angiogenesis in Xenograft Model of Breast Cancer

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Abstract

As neovascularization is essential for tumor growth and metastasis, controlling angiogenesis is a promising tactic in limiting cancer progression. Melatonin has been studied for their inhibitory properties on angiogenesis in cancer. We performed an *in vivo* study to evaluate the effects of melatonin treatment on angiogenesis in breast cancer. Cell viability was measured by MTT assay after melatonin treatment in triple-negative breast cancer cells (MDA-MB-231). After, cells were implanted in athymic nude mice and treated with melatonin or vehicle daily, administered intraperitoneally 1 hour before turning the room light off. Volume of the tumors was measured weekly with a digital caliper and at the end of treatments animals underwent single photon emission computed tomography (SPECT) with Technetium-99m tagged vascular endothelial growth factor (VEGF) C to detect *in vivo* angiogenesis. In addition, expression of pro-angiogenic/growth factors in the tumor extracts was evaluated by membrane antibody array and collected tumor tissues were analyzed with histochemical staining. Melatonin *in vitro* treatment (1 mM) decreased cell viability ($p<0.05$). The breast cancer xenografts nude mice treated with melatonin showed reduced tumor size and cell proliferation (Ki-67) compared to control animals after 21 days of treatment ($p<0.05$). Expression of VEGF receptor 2 decreased significantly in the treated animals compared to that of control when determined by immunohistochemistry ($p<0.05$) but the changes were not significant on SPECT ($p>0.05$) images. In addition, there was a decrease of micro-vessel density (Von Willebrand Factor) in melatonin treated mice ($p<0.05$). However, semiquantitative densitometry analysis of membrane array indicated increased expression of epidermal growth factor receptor and insulin-like growth factor 1 in treated tumors compared to vehicle treated tumors ($p<0.05$). In conclusion, melatonin treatment showed effectiveness in reducing tumor growth and cell proliferation, as well as in the inhibition of angiogenesis.

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Introduction

Breast cancer is the most common type of cancer in women [1] with increasing incidence and mortality which is becoming a global public health problem [2,3]. Furthermore, breast cancer has a high mortality rate, mainly due to tumor progression and metastatic spread, which require neovascularization [4–6].

Tumor growth has traditionally been associated with angiogenesis, which is the formation of new blood vessels from the pre-existing vasculature. Angiogenesis is controlled by pro-angiogenic and anti-angiogenic factors in the body. The angiogenesis is regulated by several growth factors such as vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), epidermal growth factor (EGF), Angiogenin, etc [7]. Recent studies have demonstrated that tumor progression can also occur through vasculogenesis. Vasculogenesis is typically followed by classical sprouting angiogenesis, where blood vessels are formed de novo by *in situ* differentiation of the primitive progenitors - i.e. angioblasts - into mature endothelial cells, which was thought to only take place during embryonic development [8,9].

Once a tumor exceeds a few millimeters in diameter, hypoxia triggers a cascade of events to allow angiogenesis and tumor progression [8]. Hypoxia results in expression of VEGF, a potent endothelial cell mitogen [10–13]. VEGF is composed of a family of five isoforms denominated VEGF-A, VEGF-B, VEGF-C, VEGF-D and placental growth factor (PGF). Each of these factors can activate one or more receptors (VEGFR1, VEGFR2 and VEGFR3), promoting angiogenesis through its ability to stimulate the growth, migration and invasion of endothelial cells [14–17].

Hypoxia also up-regulates the expression of stromal-cell-derived factor-1α (SDF-1α), which can recruit pro-angiogenic cells from bone marrow [18,19].Hypoxia can also act in the regulation of RANTES a chemokine of inflammatory cells [20,21]. Other signalling pathways such as basic fibroblast growth factor (bFGF)

and the receptor tyrosine kinase of angiopoietin-1 (Tie-2) can influence angiogenesis by increasing tumor invasion [22,23], and vasculogenesis by mobilizing endothelial progenitor cells [9]. Given the variety of signals involved in the formation of new blood vessels, several proteins can be therapeutic targets. Therefore, it becomes extremely important to identify the most susceptible target to a particular treatment, and to develop effective therapies that involve a combination of several factors [8,9].

Administration of melatonin, a hormone naturally produced and secreted in the pineal gland, appears to play an important role in tumor growth inhibition [24,25] and different mechanisms of action have been proposed [26]. The action of melatonin is especially effective in estrogen receptor (ER)-positive breast cancer by reducing the mitogenic response of cells [27]. Still, melatonin can exert immunomodulatory and antiproliferative effects and act as antioxidants [28–32]. Furthermore, it has demonstrated that the pharmacological concentration of melatonin can inhibit angiogenesis directly or indirectly [33–35]. Recently, some studies have shown that melatonin can decrease the expression of Hypoxia Inducible Factor 1 alpha (HIF-1α) and VEGF in various cancers [5,36–37].

The purposes of this study were to determine: 1) whether melatonin therapy effectively would decrease the size of implanted human triple negative breast cancer in a mouse model, 2) whether there would be changes in the expression of VEGF receptors determined by *in vivo* SPECT scanning in mice treated with melatonin or vehicle, 3) the changes in expression of angiogenic factors in tumors in response to melatonin. The ultimate goal was achieved by determining the therapeutic effectiveness of melatonin.

Materials and Methods

Ethics statement

All animal experiments were performed according to National Institutes of Health guideline and the protocol was approved by Institutional Animal Care and User Committee (IACUC No. 1203) of Henry Ford Health System.

In vitro study

Cell culture. Cell culture. Triple negative human breast cancer cells (MDA-MB-231) (ATCC, Manassas, VA, USA) were cultured in 75 cm^2 culture flasks with Dulbecco's modified Eagle's medium (DMEM) (GIBCO, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (GIBCO, Grand Island, NY, USA), penicillin (100 IU/mL) and streptomycin (100 µg/mL) (GIBCO, Grand Island, NY, USA)until they were 80–90% confluent.

Cell viability by MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Individual well of a 96-well plate was inoculated with 100 µl of medium containing 5×10^4 cells. Cells were incubated in medium with different concentrations of melatonin (0.0001 mM, 0.001 mM, 0.01 mM, 0.1 mM and 1 mM) for 24 h. Melatonin was diluted in ethanol 0.05%. In control cells, equivalent amount of ethanol was added as vehicle. Thereafter, 10 µL of MTT solution (ATCC, Manassas, VA, USA) were added to each well and the plates were incubated at 37°C for an additional 4 h. To solubilize the MTT formazan crystals, the cells were incubated with detergent (ATCC, Manassas, VA, USA) overnight at 37°C. Absorbance was measured at 570 nm by ELISA Plate reader (VICTOR3 – PerkinElmer, Waltham, MA, USA). Medium was used as background and subtracted from the samples. Cell viability (%) was calculated for all groups compared to control sample. All experimental samples were in triplicate.

Animal model

Female athymic mice, 7–8 weeks of age and 25 g in weight (Charles River Laboratory, Inc.) were housed. MDA-MB-231, cultured *in vitro*, was harvested and re-suspended serum free media at a concentration of 6×10^7 cells/ml. The animals received subcutaneous injection of 50 µl of cell suspension (3 million cells) in the right mammary gland or hind flank. These tumor cells are efficient in making xenografted tumors with almost 90% efficiency and show marked vascularity with less central necrosis. Two different implantation sites were chosen to determine whether treatment effects will differ based on implantation sites.

Melatonin administration

Animals were randomly assigned to either the melatonin administration (n = 5) or the control group (vehicle treated, n = 8). Vehicle solution was prepared with 8 ml of phosphate buffered saline (PBS), 1 ml of dimethyl sulfoxide (DMSO) and 1 ml of Cremophor (Sigma, St. Louise, MO, USA). The animals of the control group received 100 µl of vehicle solution by intraperitoneal injection (IP).

Melatonin (Sigma, St. Louise, MO, USA) was diluted in vehicle and the animals from the melatonin group received IP of 100 µl of melatonin treatment (at dose of 40 mg/kg of body weight) for five days a week. Melatonin was administered 1 hr before room lighting was switched off. Administration of melatonin prior to the nocturnal increase in endogenous melatonin may be most effective because tissues are most sensitive to the hormone at this time [38,39].

Melatonin or vehicle administration started on the day of tumor implantation (soon after implantation) and continued for five days a week for 21 days. On the 22nd day, all animals underwent SPECT scanning with Tc-99m-HYNIC-VEGF-c followed by euthanasia and collection of tumors for immunohistochemistry and membrane antibody array to determine the expression of different pro-angiogenic and growth factors in tumor extracts.

Tumor measurement by caliper

Tumor volume was measured by digital caliper (Thermo Fisher Scientific, Rockford, IL, USA) on day 7, 14 and 21 after tumor implantation. The major longitudinal diameter (length) and the major transverse diameter (width) were determined. Tumor volume was calculated based on caliper measurements by the modified ellipsoidal formula [40]: Tumor volume = ½ (length × $width^2$)

SPECT study

VEGF-c. Recombinant rat VEGF-c was purchased from Prospec (Rehovot, Israel). VEGF-C, known as Vascular Endothelial Growth Factor Related Protein (VRP), is a recently discovered member of VEGF growth factor family that is most closely related to VEGF-D. Similar to VEGF-D, VEGF-C has a VEGF homology domain spanning the middle third of the precursor molecule and long N- and C-terminal extensions. Recombinant rat VEGF-c, lacking the N- and C-terminal extensions and containing only the middle VEGF homology domain, forms primarily non-covalently linked dimers. This protein is a ligand for both VEGFR2 and VEGFR3.

Preparation of Hydrazine Nicotinamide (HYNIC). Succinimidyl 6-hydrazinopyridine-3-carboxylate hydrochloride was synthesized and conjugated with rat VEGF-c as previously

described [41]. The conjugate protein was purified with a Centricon C-3 diafilter with a 3,000 molecular weight cutoff. Then, the nicotinyl hydrazine conjugate of VEGF-c was radio labeled with 99m-Tc-pertechnetate in the presence of tricine and stannous chloride as reported by [42–43].

Image acquisition. An appropriate state of anesthesia was obtained using ketamine/xylazine (100/15 mg/kg). One hour after injection of 0.5 mCi Tc-99m-HYNIC-VEGF-c, SPECT images were obtained using a modified PRISM 3000 gamma camera dedicated to animal studies and fitted with multi-pinhole collimators (Bioscan, Washington DC, USA). The following image parameters were used: 360 degree rotation with 36 degree increments, 180 sec per projection, using 256×256 matrices with a field of view of 4×6 cm. Total SPECT image acquisition time was 10 minutes. After the SPECT analysis animals were euthanatized and the tumors was collected for further analysis. The projection images were reconstructed with HiSPECT software (Bioscan, Washington DC, USA).

Image analysis. Multi planar reconstruction and SPECT analysis were performed using ImageJ software (NIH, Bethesda MD, USA). The tumor center was identified using orthogonal views and all sections containing the tumor, either in axial or coronal views, were added. Total activity of Tc-99m-HYNIC-VEGF-c was determined by drawing irregular regions of interests (ROI) around the tumor and on the contralateral muscles to determine the activity in the contralateral side. The percentage of change in the total activity was calculated using the following formula:

(Mean activity in the total tumor volume/mean activity in the contralateral muscles)*100

Protein extraction

Animals used for membrane antibody array analysis were euthanatized with 100mg/kg of pentobarbital administration (intravenous). The radioactive fluids were collected and contained in a shielded area to decay and the tumors were collected and snap frozen. Tissues from the tumors were mechanically pulverized over dry ice and total protein (80–150 mg of the frozen tissue powder per sample) was extracted using Ray Bio 2x Cell Lysis Buffer (RayBiotech, Norcross, GA, USA) according to the manufacturer's instructions. Protein concentration in recovered protein extracts was determined using Micro BCA Protein Assay Kit (Thermo Fisher Scientific, Rockford, IL, USA) using Bovine Serum Albumin as a standard.

Membrane antibody array

Custom RayBio Human Cytokine Array kit (RayBiotech, Norcross, GA, USA) (Table 1) was used to analyze 20 protein expression in mammary tumors. All sample measurements were performed in duplicate, containing positive and negative controls.

Membranes were incubated in 8-well plates with 2 ml of RayBio 1X Blocking Buffer solution for 30 minutes. 500 ug of protein was added to each sample and the membranes were incubated overnight at 4°C. The solution was discarded and the membranes washed three times with 1X RayBio Wash Buffer I, and twice with 1X RayBio Wash Buffer II for 5 minutes each. BiotinConjugated Anti-Cytokines was added and samples were incubated overnight at 4°C. Membranes were washed with Wash Buffer I and II and incubated with 1000X HRP-Conjugated Streptavidin overnight at 4°C. After that, it was washed again with Wash Buffer I and II and incubated for 2 minutes with Detection Buffer. Membranes were exposed in a Multispectral In-Vivo Imaging System (Kodak^{TM} Multispectral system, Carestream Health Inc., NY, USA).

The optical density of the expression of each protein was normalized to positive control and quantified using the ImageJ software (NIH, Bethesda, MD USA).

Immunohistochemistry

Animals were euthanatized, perfused by intracardiac injection of 10 ml PBS followed by 10 ml 3% paraformaldehyde and tumors were removed and fixed in 3% paraformaldehyde containing 3% sucrose. Tumor sections were prepared for paraffin blocking and sectioning. Standard histochemical staining procedures were performed as recommended by the suppliers of primary antibodies. The following antibodies were used to delineate the expression of corresponding antigens: VEGFR2 (Millipore, Billerica, MA, USA), anti-VEGFR3 antibody (Millipore, Billerica, MA, USA), anti- von Willebrand Factor (vWF) (Millipore, Billerica, MA, USA) andanti-Ki67 (Millipore, Billerica, MA, USA).

Evaluation of immunohistochemical staining

At least two sections from each tumor underwent staining for different markers and used for analysis. Multiple fields were examined from each slide, especially demarcated areas with brown staining. There were signs of necrosis in the central part of both melatonin and vehicle treated tumors.

For the analysis of immunohistochemistry slides, five areas were photographed at 40× magnifications (center, bottom, top, left and right regions) and saved in tiff format. VEGFR2 and VEGFR3 were analyzed based on the intensity of the staining by ImageJ software (NIH, Bethesda MD, USA).Each photograph was divided into four quadrants and 20 spots (small circular ROI) were randomly selected (avoiding the nucleus) in each quadrant, analyzing the intensity from 80 spots from each photographed area. The intensity was determined from a total of 400 spots marked on each slide. A negative control section of the corresponding staining was used for measuring background activity.

Evaluation of micro-vessel density (MVD) was detected by immunohistochemical staining with vWF. Each cell stained positive for vWF was considered to be a micro vessel. Five "hot spots" (area with highest vessel concentration) from each slide were identified and vWF positive areas were counted by two independent observers. The total histological area (mm^2) was noted, and MVD was calculated as previously described [44].

Tumors were categorized in relation to cellular proliferation according to the staining of Ki-67.All Ki-67 positive cells were counted from each photographed area. The number of Ki-67 positive cells was normalized to the area of photomicrography.

Statistical analysis

All data are expressed as mean ± standard error of mean (SEM). Comparison between melatonin and vehicle administration groups was done by Student's t-test or ANOVA followed by Bonferroni test with GraphPad Prism 4.0 software (La Jolla, CA, USA). Any p-value of 0.05 was considered significant.

Results

MTT assay

We performed the MTT assay with different concentrations of melatonin to assess whether melatonin acts on the *in vitro* viability of MDA-MB-231 cells. As shown in Figure 1, treatment with 0.0001 mM to 0.1 mM of melatonin did not affect the cell viability after 24 hours ($p>0.05$). Only a pharmacological concentration of 1 mM of melatonin significantly decreased the cell viability

Table 1. Custom designed protein arrays kit, which consist of 20 different cytokines/factors.

	A	B	C	D	E	F	G	H
1	Positive	Positive	Negative	Negative	Angiogenin	Angiostatin	Angiopoietin-1	Angiopoietin-2
2	Positive	Positive	Negative	Negative	Angiogenin	Angiostatin	Angiopoietin-1	Angiopoietin-2
3	G-CSF	PDGF-Ra	PDGF-AA	RANTES	bFGF	EGF	EGF R	IGF-I
4	G-CSF	PDGF-Ra	PDGF-AA	RANTES	bFGF	EGF	EGF R	IGF-I
5	MMP-9	SDF-1 α	Tie-1	Tie-2	VEGF-A	VEGF-C	VEGFR2	VEGFR3
6	MMP-9	SDF-1 α	Tie-1	Tie-2	VEGF-A	VEGF-C	VEGFR2	VEGFR3
7	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Positive
8	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Positive

G-CSF = colony stimulating factor; PDGF-Ra = platelet-derived growth factor receptor, alpha polypeptide; PDGF-AA = platelet-derived growth factor alpha polypeptide; bFGF = fibroblast growth factor (basic); EGF = epidermal growth factor; EGF R = epidermal growth factor receptor; IGF-I = insulin-like growth factor 1 (somatomedin C); MMP-9 = matrix metallopeptidase 9; SDF-1 α = Stromal cell-derived factor 1 alpha; Tie-1 = receptor tyrosine kinase of angiopoietin-1, tyrosine kinase with immunoglobulin-like and EGF-like domains 1; Tie-2 = receptor tyrosine kinase of angiopoietin-1; VEGF-A = vascular endothelial growth factor A;VEGF-C = vascular endothelial growth factor C; VEGFR2 = Vascular endothelial growth factor receptor 2; VEGFR3 = Vascular endothelial growth factor receptor 3. All antibodies are prepared in duplicate.

doi:10.1371/journal.pone.0085311.t001

compared to control cells (*$p<0.05$ vs control) and compared to all concentrations of melatonin evaluated (# $p<0.05$) (Figure 1).

Tumor size

To evaluate whether melatonin treatment reduces the breast tumor growth *in vivo*, we implanted MDA-MB-231 cells in athymic nude mice and treated then with melatonin (40 mg/kg) or vehicle for 21 days.

None of the treated mice showed any loss of weight and lethargy during the treatment for 21 days. On the contrary, the treated animals showed excessive movement but no irritability or aggressive behavior.

The site of implantation of tumor cells (mammary gland or flank) did not alter the rate of tumor growth in the vehicle treated animals ($p = 0.88$) or in the melatonin-treated animals ($p = 0.76$), showing that there was no significant difference in tumor development and response to melatonin treatment between the two models used. Thus, the two models were grouped together into one group for each treatment (melatonin or vehicle) for subsequent analyses.

Treated animals showed significantly smaller tumors after 21 days ($p<0.05$; **Figure 2**). The mean tumor volume of control and treated animals were 282.0 ± 88.5 mm^3 and 144.9 ± 38.4 mm^3, respectively. The mean tumor volume in control animals increased significantly from day 14 (118.9 ± 40.2 mm^3) today 21 (282.0 ± 88.5 mm^3), while this was not observed in the treated group ($p<0.05$, **Figure 2**). Furthermore, there was tumor regression in an animal treated with melatonin (Day 7 = 27.38 mm^3; Day 14 = 8.79 mm^3, Day 21 = 4.8 mm^3) (**Figure 2B**). No similar pattern was seen in any of the control mice (**Figure 2C, 2D**).

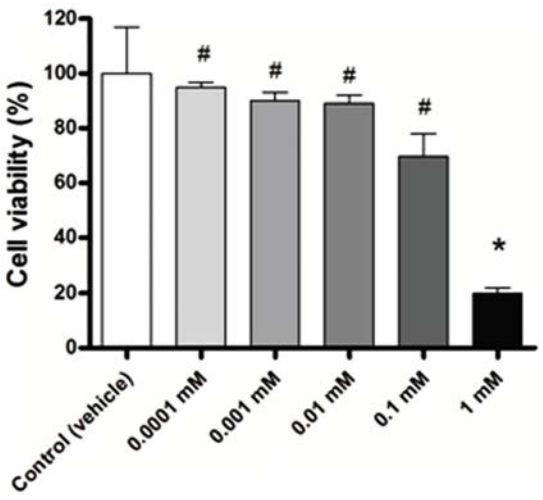

Figure 1. Inhibitory effect of melatonin on viability of the MDA-MB-231 cell line. The MDA-MB-231 cells were treated with five concentrations of melatonin for 24 h and cell viability was measured by MTT assay. Data are shown as mean ± S.D. *$p<0.05$, 1 mM of melatonin vs. Control; #$p<0.05$, 1 mM of melatonin vs other melatonin's concentrations.

doi:10.1371/journal.pone.0085311.g001

In vivo expression of VEGFRs

To determine whether there were any changes in the expression of VEGFRs in the melatonin and vehicle administrated tumors, animals underwent SPECT scanning with Tc-99m tagged VEGF-c. Animals treated with melatonin showed lower activity of Tc-99-HYNIC-VEGF-c in the tumors compared to vehicle treated animals (**Figure 3A, 3B**).

Semi-quantitative analysis of total radioactivity normalized to contralateral muscles showed that the intensity of radioactivity in the control animals was $183.6\pm20.9\%$, while the intensity of radioactivity in animals treated with melatonin was $150.5\pm17.1\%$. Although there was difference in the radioactivity in the tumors between the groups, statistically significant difference was not achieved ($p>0.05$; **Figure 3C**).

Membrane antibody array

Semi-quantitative densitometry analysis of membranes showed that melatonin did not alter the pattern of expression of the majority of evaluated angiogenic proteins ($p>0.05$; **Figure 4**). However, melatonin increased the expression of epidermal growth factor receptor (EGFR) and insulin-like growth factor 1 (IGF-I) proteins ($p<0.05$; **Figure 4**).

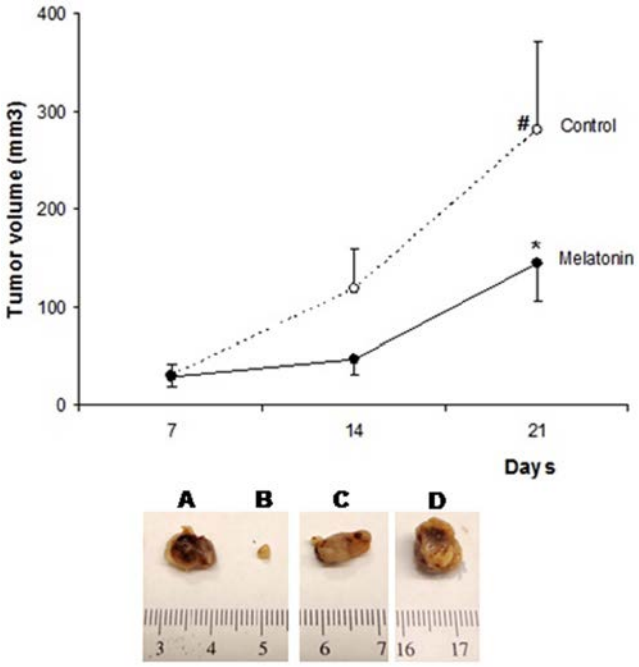

Figure 2. Antitumor effects of melatonin on mammary tumor growth. Melatonin reduced the tumor growth in breast cancer nude mice. Each point in the curves represents the mean ± SD (control n = 8; melatonin n = 5). The melatonin inhibited tumor growth, *$p<0.05$ vs Control. # Significant increase in tumor volume on control group at 14 and 21 after tumor implantation and initiation of treatment with vehicle ($p<0.05$). Detail: Representative samples of mammary tumors developed by MDA-MB-231 cells implantation on the right flank of mice. A, B. Melatonin treated mammary tumors, B. Mammary tumor which regressed with melatonin treatment. C, D. Vehicle treated mammary tumors.
doi:10.1371/journal.pone.0085311.g002

Immunohistochemistry

The expression of VEGFR2, VEGFR3, vWF and Ki-67 was evaluated for the tumors after 21 days of treatment with melatonin or vehicle by immunohistochemistry.

Lower expression of VEGFR2 was observed in the melatonin treated tumors compared to vehicle treated tumors ($p<0.05$; **Figure 5**). Furthermore, melatonin treated tumors exhibited lower expression of VEGFR3 compared with vehicle treated tumors, but statistically significant difference was not achieved ($p>0.05$; **Figure 6**).

Tumor neovascularization was assessed by quantification of MVD in mammary tumors by vWF immunohistochemistry. Melatonin treatment resulted in a decrease of MVD compared to the vehicle treated tumors ($p<0.05$; **Figure 7**).

Numerous Ki-67 positive cells were observed in the vehicle treated tumors, whereas none to very few positive cells were observed in the melatonin treated tumors ($p<0.05$; **Figure 8**).

Discussion

Results from this study showed that melatonin at pharmacologic concentration (1 mM) was able to reduce ER-negative breast cancer cell viability *in vitro*. The direct effects of melatonin on mammary cancer have been studied *in vitro*, basically using the ER-positive human breast cancer cell line (MCF-7) as a model [39]. In physiological concentrations (1 nM corresponding to peak nighttime and 10 pM corresponding to day time serum values in humans), melatonin suppresses the growth of ER-positive (MCF-7, T47D, ZR 75-1) and some ER-negative (MDA-MB-468) human breast cancer cell lines *in vitro* [45]. In addition, melatonin (10 uM – 1 mM) inhibited, to a varying extent, the proliferation of estrogen-responsive cell lines (MCF-7, T47D, ZR-75-1) more effectively than estrogen negative breast cancer cells such as BT-20, MDA-MB-231, MDA-MB-364, Hs587t, T47Dco, suggesting that the antiproliferative effects of melatonin are mediated through the estrogen-response pathway [39,46].

However, agreeing with our results about ER-negative cells, Leman et al.[47] showed that melatonin at pharmacological level (1 mM) decreases the proliferation of MDA-MB-231 and MCF-7 cells significantly. Proliferation of MDA-MB-435 cells, which are highly metastatic and ER-negative, was not significantly affected by melatonin [47]. Jung et al. [48] showed that melatonin showed weak cytotoxicity only at pharmacologically high concentrations of 8 mM and 16 mM in MDA-MB-231 cells. Melatonin induced apoptosis and inhibited the proliferation via the inhibition of anti-apoptotic genes such as BCL-xL, Mcl-1, cyclin D1, cyclin E, p-STAT3, p-mTOR, and p-AKT at high concentration (12 mM) in ER-negative MDA-MB-231 cells [48].

Melatonin has been reported to bind and activate two distinct receptor types, membrane-bound G protein-coupled receptors, MT1 and MT2, and the nuclear orphan RZR/RORa receptors, members of the steroid/thyroid hormone receptor superfamily [49]. MT1 and MT2 are expressed in human cells in various organs and in neoplastic cells, including breast cancer cells [50].

Through the mediation of a subunit of G protein, MT1 receptors inhibit the activity of adenylcyclase, and thereby decrease the production of adenosine 3′, 5-cyclic monophosphate (cAMP). This relationship makes it possible to control the activity of selected protein kinases (PKC, PKA, MAPK) and to influence the levels of transcription factor phosphorylation, that is, cAMP response element-binding, as well as the expression of specific genes, which code proteins involved in the proliferation, angiogenesis, cell differentiation and migration processes [50].

Both MCF-7 and MDA-MB-231 human breast cancer cell lines express low levels of the MT1 receptor [46]; However, overexpression of the MT1 receptor enhanced the growth-inhibitory and gene-modulatory effects of melatonin in ER-positive but not ER-negative human breast cancer cells [49].

Recent studies have demonstrated higher levels of MT1A mRNA in the MCF-7 cell line compared to the MDA-MB-231 cell line [50,51]. Jablonska et al. [50] showed that there is lower expression of MT1 receptors in breast cancer phenotype of triple negative (ER-, PR -, HER2 -) compared to ER-positive cases and the lower expression of MT1 is correlated with poor prognosis.

However, through activation of the MT1 receptor, melatonin can suppress the development of cancer via a broad spectrum of mechanisms with and without involvement of ER [45]. Oprea-Ilies et al. [52] showed that the MT1 positivity was associated with a lower stage and a smaller tumor size at time of diagnosis in triple-negative breast tumors patients.

Furthermore, melatonin can exert antitumoral properties by a set of complex mechanisms of action, not necessary involving the receptor pathway [26]. Melatonin may act directly, independently of its receptors or via them, making it difficult to understand the action of melatonin at the cellular level [26,50].

Since melatonin decreased ER-negative breast cancer cell viability *in vitro*, we implanted cells in athymic nude mice. Our results showed that melatonin treatment (40 mg/kg) reduced the tumor growth with concomitant decreasing of cell proliferation (Ki-67).

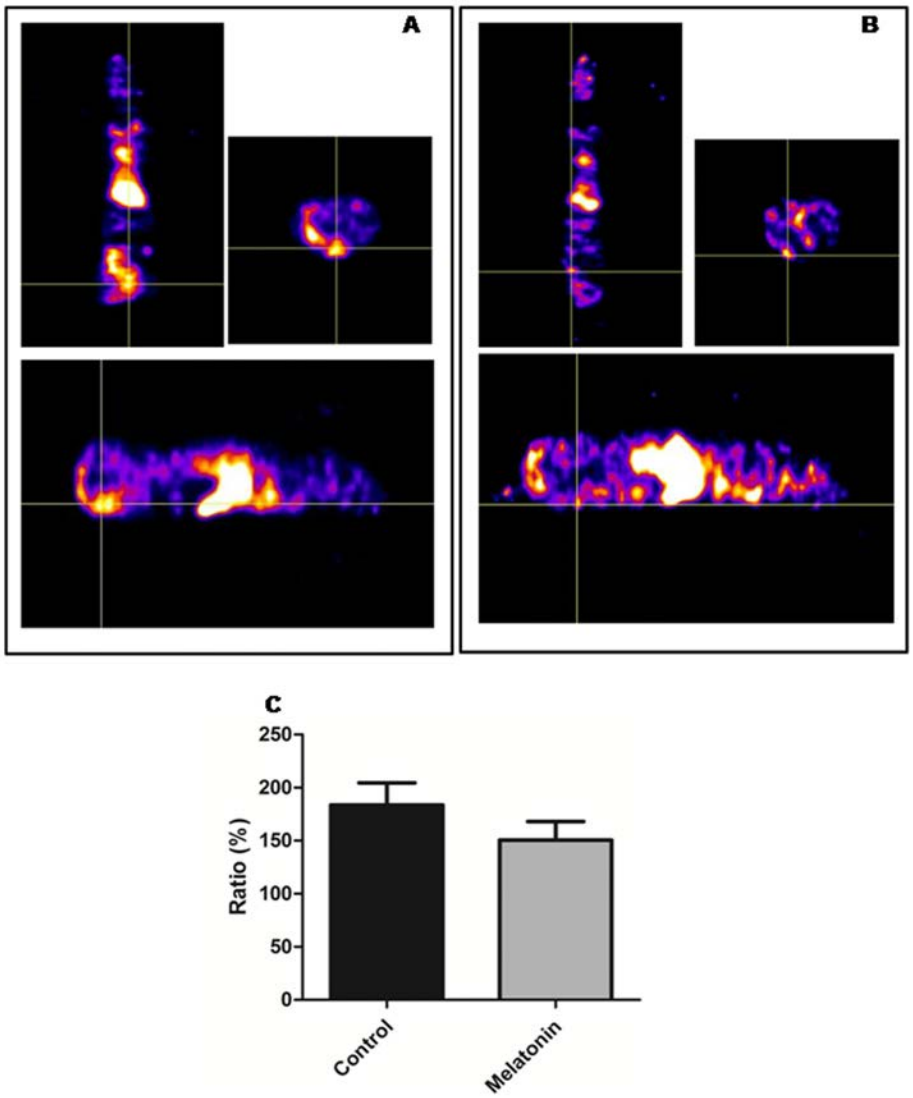

Figure 3. SPECT analysis of *in vivo* accumulation of Tc-99m-HYNIC-VEGF-c. VEGF-c (which targets both VEGFR2 and VEGFR3) was tagged with HYNIC chelators and then labeled with Tc-99m and injected intravenously in melatonin and vehicle treated mice. One hour after injection, SPECT images were obtained using dedicated animal scanner. Vehicle treated mice showed increased accumulation of Tc-99m-HYNIC-VEGF-c in the mammary tumor (A, Intersection of lines indicate the tumor, with a volume of 865.69 mm^3 at the 21th day) compared to that of melatonin treated mammary tumors (B, Intersection of lines indicate the tumor, with a volume of 130.69 mm^3 at the 21th day) C. Semi-quantitative analysis of total radioactivity normalized to contralateral muscles showing the intensity of radioactivity in the vehicle and melatonin treated animals.
doi:10.1371/journal.pone.0085311.g003

There are no studies about melatonin treatment in ER-negative breast cancer *in vivo*, but investigators showed the effectiveness of melatonin in treating rats with breast cancer induced by a carcinogen DMBA [32]. In a study by Cos et al. [53] nude mice bearing ER-positive breast cancer (MCF-7) (inoculated directly into the inguinal mammary fat pad of the mouse) treated with melatonin also showed smaller tumor size compared to control animals. Rao et al. [54] showed that melatonin at 50 mg/kg and 200 mg/kg reduced the incidence of mammary cancer of TG.NK (c-neu) transgenic mice and reduced the number of tumors per mouse and tumor weights as compared with the control group. Although effective, the high-dose of 200 mg/kg caused significantly lower body weight with high mortality. On the contrary, in our study none of the treated mice showed any adverse effect, loss of weight and lethargy during the treatment with 40 mg/kg for 21 days. Liu et al. [55] performing a study with mice with gastric tumor (foregastric murine carcinoma cell line subcutaneously inoculated under the right axilla) showed that melatonin treatment in doses of 25 mg/kg, 50 mg/kg and 100 mg/kg reduced tumor volume when correlated with control mice.

It is accepted that melatonin exerts an anti-tumor effect and multiple mechanisms have been suggested for the biological effects of melatonin but are not yet fully established [56]. It has been shown that melatonin has direct anticancer mechanisms in several types of cancer, as pro-apoptotic, anti-proliferative, anti cell-differentiation and anti-angiogenic actions. Melatonin also has indirect anticancer mechanisms such as antioxidative effects and immune system regulation [26,57]. Little is known about the anti-angiogenic effect of melatonin. Some authors have demonstrated that a pharmacological concentration of melatonin may inhibit angiogenesis directly or indirectly, by inhibiting the proliferation of

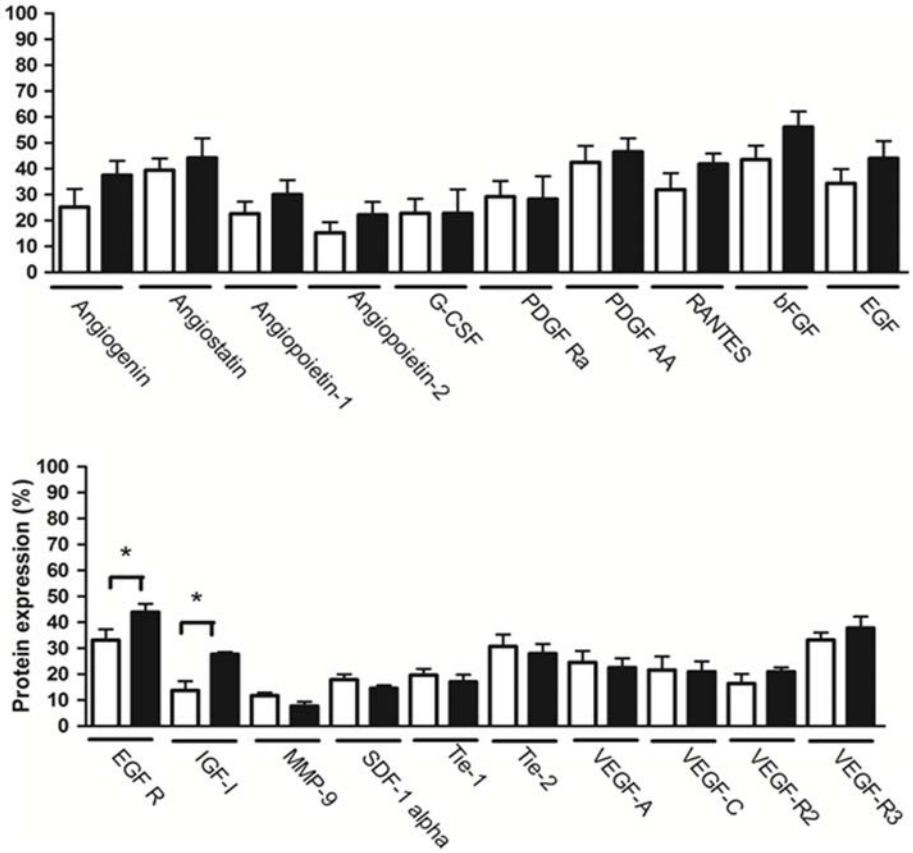

Figure 4. Comparison between proteins expression in mammary tumor in animals treated with melatonin or vehicle. White column = vehicle treatment; Black column = melatonin treatment. Data are shown as mean ± S.D. *$p<0.05$, vs. Control.
doi:10.1371/journal.pone.0085311.g004

vascular endothelial cells [33] and acting in the inhibition of pro-angiogenic factors [34–37].

In this study, we evaluated the action of melatonin on angiogenesis in ER-negative breast cancer *in vivo*. Our findings were able to show that melatonin treatment was effective in inhibiting ER-negative mammary tumor angiogenesis, indicated by the reduction of VEGFR2 expression and MVD. Despite the semi-quantitative analysis of Tc-99m-HYNIC-VEGF-c showing lower VEGFR2 expression in the melatonin treated tumors, a statistically significant difference was not achieved. The non-significant differences of Tc-99m-HYNIC-VEGF-c activity between control and treated tumors could be due to the reduction of tumor size in treated groups and the relative expression of VEGFR2 remained unchanged since our analysis used contralateral ROI identical to tumor size. However, a decrease of VEGFR2 in melatonin treated mice was confirmed by immunohistochemistry.

It is noteworthy that the efficacy of anti-angiogenic therapies can be evaluated by nuclear medicine imaging techniques using the tagging of radioactive isotopes to the specific proteins. Previously, Tc-99m was successfully tagged with different proteins and peptides using the new generation chelator, HYNIC (Hydrazine Nicotinamide) [43]. VEGF receptors have been used as imaging agents mediators of angiogenesis, since they are highly expressed in the vascular endothelium, and when injected into the bloodstream, are internalized by binding to VEGF, allowing the accumulation of contrast agents, such as Tc-99m [43,58]. VEGFR2 is the principal receptor transmitting VEGF signals and is overexpressed in the tumor vasculature compared with normal vasculature [59]. One common mechanism for increased survival and growth of cancer cells is the deregulation of tyrosine kinase receptors like VEGFR2 [60].

In this study, mammary tumors showed necrosis in the central part, both in vehicle and melatonin treated animals, which can be a result of insufficient and defective vasculature in a highly proliferative tumor mass. Hypoxia in tumor tissues is a leading cause of angiogenesis [61]. Low O_2 tension of a growing tumor allows stabilization of HIF-1α, leading to increased VEGF-A transcription, which binds to VEGFR2. Activation of intracellular signaling of VEGFR2 stimulates an angiogenic response, leading to cell proliferation, migration, permeability, survival and ultimately resulting in tumor growth [62].

There are no studies evaluating the expression of VEGFRs in response to treatment with melatonin. We also evaluated 20 angiogenic proteins in breast tumor tissue and showed that melatonin treatment did not alter the pattern of expression of the majority of the proteins evaluated. Previous reports have described a decrease in the production of VEGF protein induced by melatonin in human pancreatic carcinoma cells (PANC-1) and human alveolar adenocarcinoma cells (A549) [36,56].

Melatonin suppresses tumor angiogenesis by inhibiting HIF-1α expression and stabilization in prostate cancer [37] and HCT116 colon cancer cells under hypoxic condition *in vitro* [5]. We evaluated the expression of HIF-1α in tumor tissue of animals treated with melatonin or vehicle, but there was no statistically significant difference ($p>0.05$, data not shown).

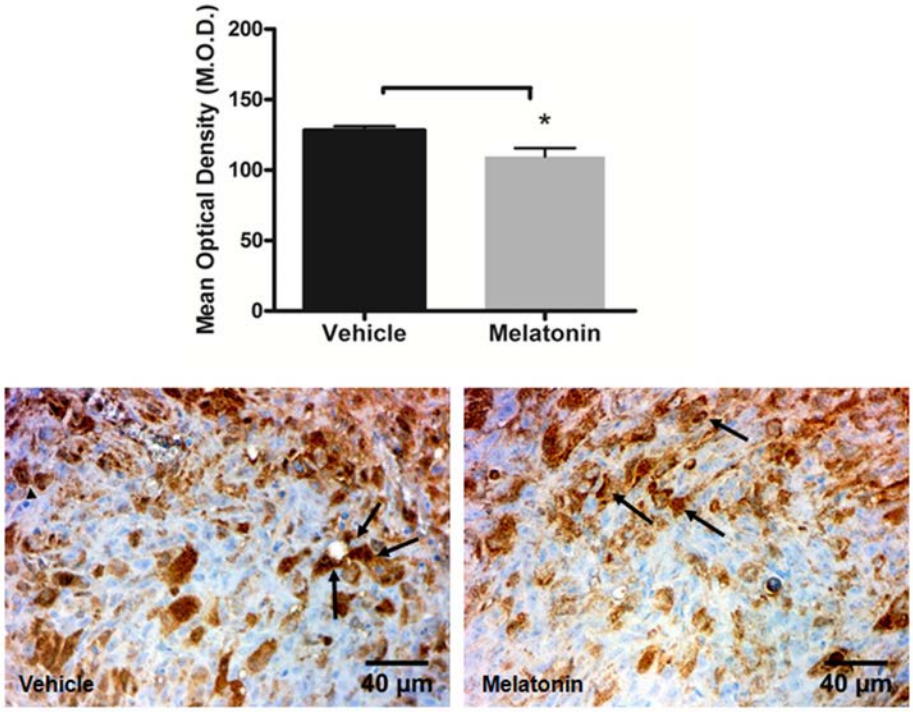

Figure 5. Immunohistochemistry staining with VEGFR2 (arrows) in vehicle treated and melatonin treated tumors. Images were taken with 40× magnification. A significant decrease was observed at the tumor in melatonin treated tumors compared to vehicle treated tumors (*$p < 0.05$). Error bars: ± standard error.
doi:10.1371/journal.pone.0085311.g005

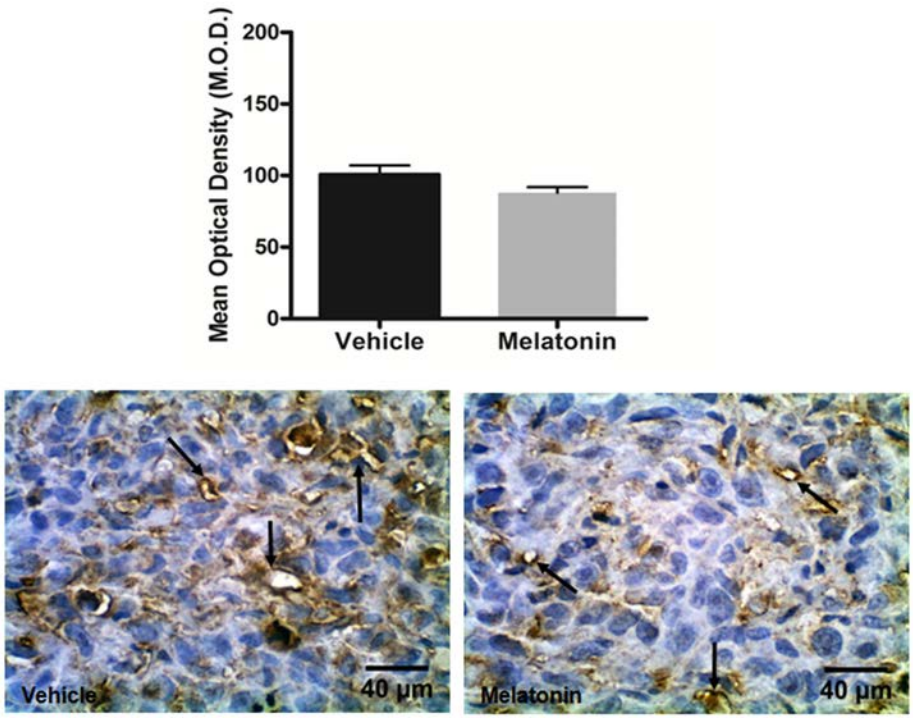

Figure 6. Immunohistochemistry staining with VEGFR3 (arrows) in vehicle treated and melatonin treated tumors. Images were taken with 40× magnification. Melatonin do not decreased significantly the expression of VEGFR3 ($p > 0.05$). Error bars: ± standard error.
doi:10.1371/journal.pone.0085311.g006

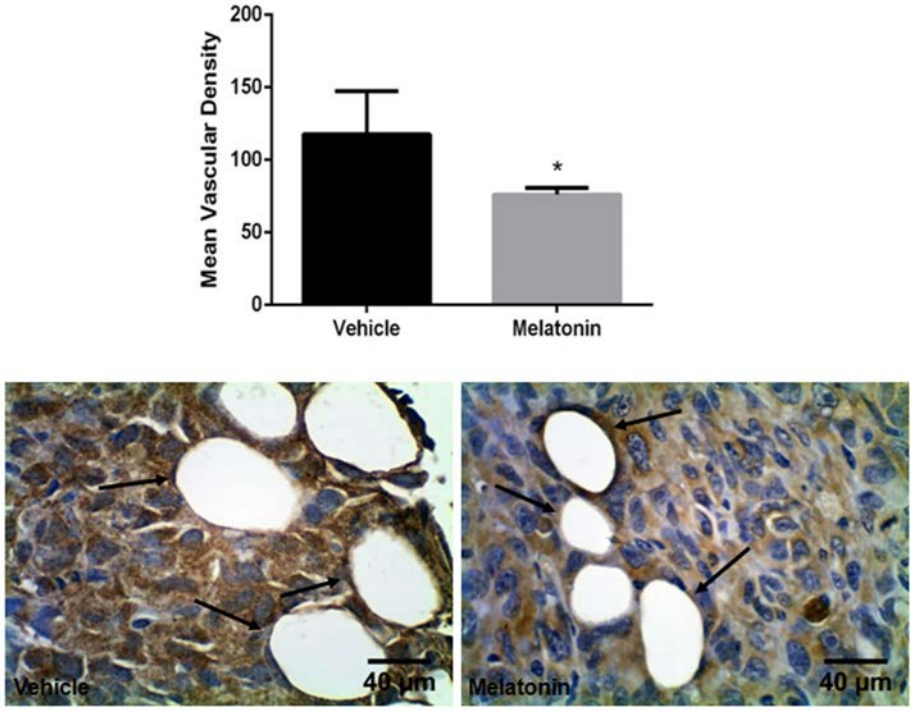

Figure 7. Immunohistochemistry staining with vWF in vehicle treated and melatonin treated tumors. Images were taken with 40× magnification. Quantitative estimation of micro-vessel density (MVD) by counting positive vessels (arrows) revealed a decrease in MVD after melatonin treatment compared to the vehicle treated tumor (*$p<0.05$). Error bars: ± standard error.
doi:10.1371/journal.pone.0085311.g007

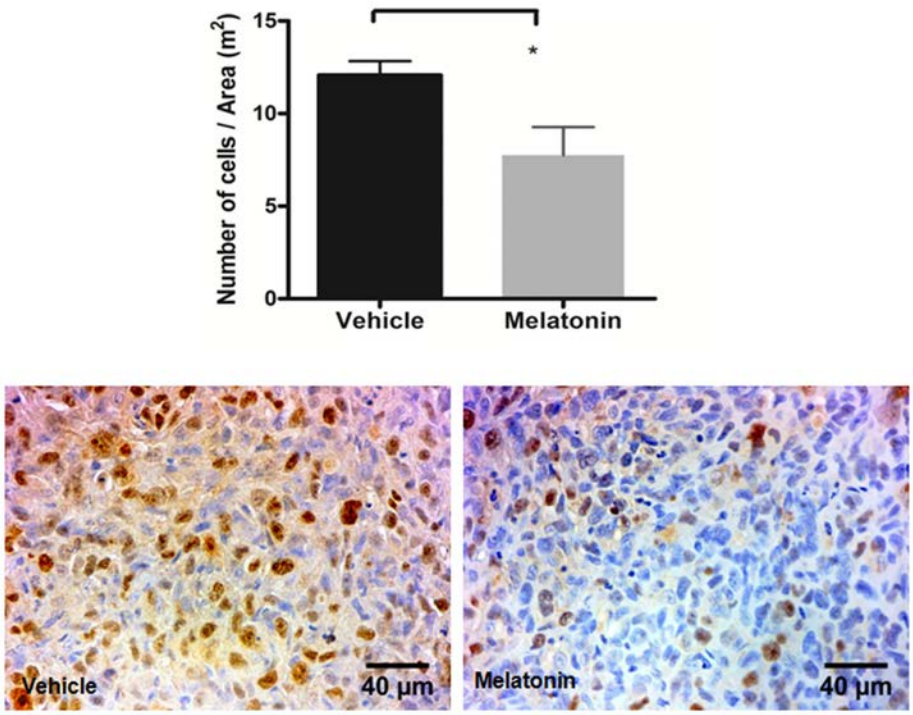

Figure 8. Immunohistochemistry staining with Ki-67 in vehicle treated and melatonin treated tumors. Images were taken with 40× magnification. There was a decreased cell proliferation in tumors treated with melatonin (*$p<0.05$). Error bars: ± standard error.
doi:10.1371/journal.pone.0085311.g008

Melatonin suppressed the VEGF gene expression through the inhibition of the accumulation of HIF-1a only under a hypoxia environment and had no obvious effect under normoxic conditions [63] and in physiological concentrations [56].

Regarding breast cancer, Alvarez-García et al. [64] performed an *in vitro* study and showed that melatonin may play a role in the paracrine interactions between malignant epithelial cells and proximal endothelial cells through a downregulatory action on VEGF expression in MCF-7, which decreases the levels of VEGF around endothelial cells. Lower levels of VEGF could be important in reducing the number of estrogen-producing cells proximal to malignant cells.

Kim et al. [61] showed that melatonin treatment suppressed tumor angiogenesis and diminished the formation of the capillaries inside the tumor, reducing the tumor growth in mice bearing renal adenocarcinoma (RENCA). In addition, it has also been reported in rats injected with melatonin while exposed to hypoxia, that serum VEGF levels decreased, which suggests that melatonin can suppress tumor cellular hypoxic adaptation by inhibiting angiogenesis [35]. It is notable that a decrease in the expression of HIF-1 and its target gene VEGF can happen by antioxidant action of melatonin, removing intracellular reactive oxygen species.

These studies indicate that the regulation of VEGF by melatonin is highly complex. Many factors are involved in the regulation of VEGF synthesis, but the relation between melatonin and VEGF has been unclear until now. Perhaps, melatonin receptors (membrane and nuclear), and its well-known immunomodulatory role and other additional transcriptional factors are involved in the actions of melatonin [56].

In addition, semi-quantitative densitometry analysis of membranes showed that melatonin treatment increased the expression of EGFR and IGF-I proteins in ER-negative breast cancer. Margheri et al. [65] demonstrated that the anticancer effects of melatonin on breast cancer cell line MCF-7 were enhanced by its combination with retinoic acid and somastatin. The combined treatment resulted in a decrease in cell viability, which was concomitant with inhibition of a signalling pathway mediated by EGF. The oncostatic action of melatonin in ER-positive breast cancer cells MCF-7, probably occurs via its binding to the MT1 receptor and the alteration of key kinase pathways, for example IGF-I-PI3K/Akt, which were shown to be involved in the estrogen response pathway of the tumor cell [50].

IGF-I is a growth factor that stimulates the growth of breast cancer. Rao et al. [54] showed that melatonin (50 mg/kg, 100 mg/kg, 200 mg/kg) did not cause significant changes in serum IGF-1 levels in mammary cancer of TG.NK (c-neu) mice. Lissoni et al. [66] developed a clinical phase II, in which it was demonstrated that, in patients with metastatic breast cancer, tamoxifen therapy in conjunction with melatonin resulted in the decrease of serum levels of IGF-I.

In this study we proved that melatonin reduces tumor angiogenesis in an ER-negative breast cancer model, but the detailed mechanism of how melatonin acts on angiogenesis in various cancers should be investigated in depth in further studies. Taken together, our results showed that melatonin inhibits tumor growth, cell proliferation and blocks tumor angiogenesis in breast cancer and the anti-tumor action of melatonin. Furthermore, melatonin treatment has not caused systemic toxicity to achieve the therapeutic activities, suggesting it as a potential therapeutic agent to breast cancer.

Author Contributions

Conceived and designed the experiments: BVJP ASA DAPCZ. Performed the experiments: BVJP ASA LCF TFB NRSV ASMI AS MMA. Analyzed the data: BVJP ASA LCF TFB NRSV AS. Contributed reagents/materials/analysis tools: MMA ASA DAPCZ. Wrote the paper: BVJP ASA DAPCZ.

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ArtigoII

Melatonin regulates angiogenic factors under hypoxia in breast cancer cell lines

Running Title: Melatonin action in breast cancer cell lines

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Abstract: Angiogenesis is the process of new blood vessel formation, regulated by a number of pro- and anti-angiogenic factors and usually begins in response to hypoxia. Exogenous administration of melatonin has shown numerous anti-tumor effects and appears to inhibit tumor angiogenesis. However, many factors involved in the anti-angiogenic effect of melatonin are still under investigation. Here, we evaluate the effects of melatonin on cell viability and expression of angiogenic factors in MCF-7 and MDA-MB-231 breast cancer cells under hypoxic conditions. Cell viability was investigated by MTT and gene and protein expression of the hypoxia-inducible factor-1α (HIF-1α) and vascular endothelial growth factor (VEGF-A) were verified by qPCR and immunocytochemistry after melatonin treatment (1 mM) under hypoxic conditions. Additionally, a protein array with 20 different cytokines/factors was performed on tumor cell lysates. The results showed that 1 mM of melatonin reduced the viability of MCF-7 and MDA-MB-231 cells ($p < .05$). This treatment also decreased both gene and protein expression of HIF-1α and VEGF-A under hypoxic conditions ($p < .05$). Among the proteins evaluated by protein array, melatonin treatment during hypoxia reduced VEGF-C, VEGFR receptors (VEGFR2 and VEGFR3), matrix metalloproteinase 9 (MMP9) and Angiogenin in MCF-7 cells. In MDA-MB-231 cells, a significant decrease was observed in VEGFR2, epidermal growth factor receptor (EGFR) and Angiogenin ($p < .05$). Taken together, these results showed that melatonin acts in the regulation of angiogenic factors in breast tumor cells and suggests an anti-angiogenic activity, particularly under hypoxic conditions.

KEYWORDS: Angiogenesis, Breast Neoplasms, Cell hypoxia, Cell line, Gene expression, Melatonin, Vascular Endothelial Growth Factors

INTRODUCTION

Angiogenesis is the formation of new blood vessels by proliferation and migration of preexisting endothelial cells [1,2]. Increased formation of blood vessels within a tumor has been associated with the development of metastasis, poor prognosis and decreased survival [3,4]. Thus, dysregulation of angiogenesis is a hallmark of cancer [5-7].

Hypoxia is one of the best characterized angiogenic triggers and usually initiates the stabilization of hypoxia-inducible factor-1α (HIF-1α), followed by the induction of several genes involved in tumor aggression that includes pro-angiogenic factors [8-11]. It is well established that HIF-1α is the most important regulator of vascular endothelial growth factor A (VEGF-A), although it is also controlled by some growth factors and oncogenes [12].

In addition to the VEGF family, other molecules have been implicated as either direct or indirect positive regulators of angiogenesis, including basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), platelet-derived growth factor (PDGF), matrix metalloproteinases (MMPs), and angiopoietins [13-21]. These growth factors can stimulate angiogenesis in a paracrine or autocrine manner, giving an advantage to tumor cells and promoting subsequent tumor progression [1,2,22].

Hence, due to the dependence of tumor progression on angiogenesis, blocking this process could be a strategy to arrest tumor growth. Considering the variety of signals involved in the formation of new blood vessels, several proteins may be considered potential therapeutic targets. Moreover, suppressing one

pathway could promote another. Thus, it is extremely important to develop effective therapies that involve a combination of several factors [1,2].

Exogenous administration of melatonin, a hormone produced by the pineal gland, has demonstrated to inhibit tumor development both *in vitro* and *in vivo* [23]. Mechanisms by which melatonin exerts its anti-tumor effects have been suggested, including immuno-modulatory, anti-proliferative and anti-oxidant pathways [24-26]. Recently, several investigations have demonstrated that melatonin suppresses the angiogenic process either directly or indirectly [3,27-33]. However, many factors involved in melatonin's anti-angiogenic effects are still under investigation.

In the present study, we investigated the expression of HIF-1α and VEGF-A, as well as other angiogenic factors, in breast cancer cell lines after melatonin treatment under hypoxic conditions.

MATERIALS AND METHODS

Cells culture

This study was performed using the human breast cancer cell lines MCF-7 (estrogen receptor α (ERα)-positive) and MDA-MB-231 (ERα-negative) (ATCC, Manassas, VA, USA). Both cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) with high glucose (4.5 g/L) (Cultilab, Campinas, SP, Brazil) and supplemented with 10% fetal bovine serum (FBS) (Cultilab, Campinas, SP, Brazil) and 1% antibiotic solution containing penicillin and streptomycin (Sigma,

St. Louise, MO, USA). Cells were cultured at 37°C in a humidified chamber with 5 % CO_2.

MTT assay of cell viability

Cell viability was measured using the MTT assay 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Vibrant MTT Cell Proliferation Assay Kit - Invitrogen Life Technologies – Eugene, OR, USA). Individual wells of a 96-well plate were seeded with 100 µL of medium containing 0.05 x 10^6 cells. Cells were incubated in medium with different concentrations of melatonin (0.0001 mM, 0.001 mM, 0.01 mM, 0.1 mM and 1 mM). Also, cells were incubated in medium with 100 µM and 200 µM of hypoxia mimetic cobalt chloride ($CoCl_2$), and with an additional treatment of 1 mM melatonin for 24 hour. Melatonin (Sigma, St. Louise, MO, USA) was diluted in ethanol, and an equivalent amount of ethanol alone (vehicle) was added to control cells. $CoCl_2$ (Sigma, St. Louise, MO, USA) was diluted in water.

Next, 10 µL of MTT solution were added to each well, and the plates were incubated at 37°C for an additional 4 hours. To solubilize the MTT formazan crystals, the cells were incubated with SDS-HCL solution (10 mL of 0.01 M HCl to 1 mg of SDS) for 4 hours at 37ºC. An ELISA plate reader (Thermo Fisher Scientific, Rockford, IL, USA) was used to measure absorbance at 570 nm. Media alone was used to measure background, which was then subtracted from the samples. Cell viability (%) was calculated for all groups compared to control samples.

Gene expression of HIF-1A and VEGFA by Real-Time PCR

Real-time PCR was performed to evaluate the gene expression of HIF1A and VEGFA under hypoxic conditions in the presence of melatonin (1 mM). The cells were plated on plates with 6-wells (10-cm^2 each) and separated into 5 groups for each cell line, with three wells for each treatment: 1) Control group; 2) Hypoxic group treated with 100 μM $CoCl_2$; 3) Hypoxic group treated with 200 μM $CoCl_2$; 4) Group treated with 100 μM $CoCl_2$ and 1 mM melatonin; and 5) Group treated with 200 μM $CoCl_2$ and 1 mM melatonin. The treatments were performed for 24 hours.

Total RNA was extracted from the cells with Trizol reagent (Invitrogen Life Technologies – Eugene, OR, USA), as recommended by the manufacturer. The RNA concentration of each sample was determined with a NanoDrop2000 (Thermo Fisher Scientific, Rockford, IL, USA), and RNA integrity was confirmed on a 1% agarose gel. The RNA from each sample was reverse-transcribed to complementary DNA (cDNA) using a High Capacity cDNA kit (Applied Biosystems, Foster City, CA, USA).

First, the standard curve was calculated, and analyses for the differential expression of HIF1A or VEGFA and endogenous controls genes GAPDH and ACTB were performed using SystemStepOne Plus (Applied Biosystems, Foster City, CA, USA) and TaqMan Universal Master Mix (Applied Biosystems, Foster City, CA, USA). The assays used were HIF1A (Hs00153153_m1), VEGFA

(Hs00900055_m1), GAPDH (Hs99999905_m1) and ACTB (Hs99999903_m1) (Applied Biosystems, Foster City, CA, USA).

Each reaction consisted of 10 µL of Master Mix, 1 µL of TaqMan, 8 µL of DEPC water and 1 µL of cDNA (100 ng/mL). The qPCR conditions were 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute.

The expression of each gene of interest was calculated relative to the normalized average expression of genes used as endogenous control (ΔΔCt) [34]. The samples were tested in triplicate, and all experiments included the negative control.

Immunocytochemistry staining of anti-HIF-1α and anti-VEGF antibodies

Cells were separated into the following groups: 1) Control group treated with vehicle, 2) Hypoxic group treated with 200 µM $CoCl_2$, and 3) Group treated with 200 µM $CoCl_2$ and 1 mM melatonin. The treatments were performed for 24 hours.

The cells were incubated with 1 mL of 4% paraformaldehyde and washed with phosphate buffered saline (PBS). Endogenous peroxidase activity was blocked with 3% hydrogen peroxidase for 30 minutes and antigen retrieval was carried out in Pan Steam (ARNO, São Paulo, Brazil) at 95°C with citrate buffer (pH 6.0) for 30 minutes. Slides were then covered with blocking solution (Biocare Medical, Concord, CA, USA) for 5 minutes and incubated with anti-HIF-1α (H1α67:sc-53546, Santa Cruz Biotechnology, Dallas, TX, USA) and anti-VEGF-

A (A-20:sc-152, Santa Cruz Biotechnology, Dallas, TX, USA) primary antibodies overnight at 4ºC. The slides were washed with PBS and incubated with secondary antibody for 20 minutes and tertiary antibody for 10 minutes (kit STARR TREK Universal HRP Detection - Biocare Medical, Concord, CA, USA).

Samples were developed with 3,3'-diaminobenzidine tetrahydrochloride (DAB) chromogenic substrate (Biocare Medical, Concord, CA, USA) and counterstained with hematoxylin. The slides were embedded in Entellan (Merck, Whitehouse Station, NJ, USA). All experiments were accompanied by a positive control for the antibody and a negative control without primary antibody.

To quantify the protein expression, various fields were examined on each slide, especially the areas delimited with brown staining. The slides were photographed, and proteins were quantified by ImageJ software in the objective lens 40 x of Nikon Eclipse E200 microscope. For each slide, 3 photos were taken, scoring 20 points in each, totaling 60 points for the calculation of the average relative intensity of immunoreactivity. The values were obtained as Arbitrary Units (AU), and the Mean Optical Density (MOD) revealed the intensity of staining in specifically immunoreactive areas.

Evaluation of angiogenic factors by Human Cytokine Antibody Array

Cells were plated at a density of 10 x 10^6 on 75-cm^2 plates, which were divided into the following groups: 1) Control group treated with vehicle, 2) Hypoxic group treated with 200 μM $CoCl_2$, and 3) Group treated with 200 μM $CoCl_2$ and 1 mM melatonin. The treatments were performed for 24 hours.

The cells were centrifuged at 4000 x*g* for 5 minutes and total protein was extracted and quantified from the pellet. The cells were washed twice by adding 1 mL of PBS and centrifuging them at 4000 x*g* for 5 minutes at 4ºC. The pellets were incubated in lysis solution with protease inhibitor for 30 minutes. The solution was transferred to a new tube and centrifuged at 13000 x*g* for 10 minutes at 4ºC. The supernatant was transferred to a new tube, and the total protein concentration was quantified with the Micro BCA Protein Assay Kit (Pierce, Rockford, IL, USA), using serum albumin as standard. Absorbance was measured at 570 nm with an ELISA Plate reader (Thermo Fisher Scientific, Rockford, IL, USA). The protein concentration of each sample was calculated as µg/µl in relation to the concentration of the standard.

The RayBio Human Cytokine Array kit (RayBiotech, Norcross, GA, USA) was used to analyze protein expression in breast cancer cell lines [33]. All sample measurements were performed in duplicate, and positive and negative controls were included (Table 1).

Membranes were incubated in 8-well plates with 2 mL of RayBio 1X Blocking Buffer solution for 30 minutes. Total protein (250 µg) was added to each membrane, which were incubated overnight at 4°C. The solution was discarded, and the membranes were washed three times with 1X RayBio wash buffer I and twice with 1X RayBio wash buffer II for 5 minutes per wash. Biotin-conjugated anti-cytokine antibodies were added, and the samples were incubated overnight at 4°C. The membranes were washed with wash buffers I and II and incubated with 1000X HRP-conjugated streptavidin overnight at 4°C. The membranes were then

washed again with buffers I and II and incubated for 2 minutes with detection buffer. Membranes were exposed in Fusion Capt Advance (Vilber, Eberhardzell, Germany).

The optical density of the signal from each protein was normalized to positive controls and quantified using ImageJ software.

Statistical analysis

All data are expressed as the mean ± standard error of the mean (SEM). The results were previously submitted to descriptive analysis to determine statistical normality. Comparison between groups was performed by Student's t-test or ANOVA followed by the Bonferroni test with GraphPad Prism 5.0 (La Jolla, CA, USA) and StatsDirect. Any p-value of 0.05 or less was considered significant.

RESULTS

Reduction of cell viability with melatonin

To investigate the effect of different concentrations of melatonin on viability of breast cancer cells, the MTT assay was performed after incubation with melatonin for 24 hours.

Under normoxic conditions, we found that melatonin concentrations from 0.001 mM to 1 mM reduced the viability of MCF-7 cells ($p<.05$; Figure 1).

However, only the higher concentration (1 mM) was able to reduce the viability of MDA-MB-231 cell line, as previously shown.[17]

As the 1 Mm concentration decreased the viability of both cell lines, we also tested whether this concentration would be able to decrease the cell viability with addition of 100 μM and 200 μM of $CoCl_2$. The results showed that $CoCl_2$ did not affect the cell viability of both cell lines. Additionally, 1 mM melatonin reduced the viability of cells treated with both $CoCl_2$ concentrations, which was statistically significant with the concentration of 200 μM $CoCl_2$ in both cell lines ($p<.05$; Figure 2). From these results, we were able to conclude that 1 mM of melatonin was appropriate for the following experiments.

Differential gene expression of HIF1A and VEGFA

To verify whether melatonin and $CoCl_2$ were able to alter the gene expression of HIF1A and VEGFA, we performed Real-time PCR after treatment with 100 μM and 200 μM of $CoCl_2$, alone or with 1 mM of melatonin for 24 hours.

The gene expression results showed that concentrations of 100 μM and 200 μM $CoCl_2$ increased HIF1A and VEGFA gene expression in both cells lines ($p < .05$; Figures 3 and 4). Moreover, 200 μM $CoCl_2$ led a more dramatic increase; therefore, this concentration was used in subsequent experiments.

The treatment with 1 mM of melatonin counteracted the effects of $CoCl_2$, leading to a reduction in gene expression of HIF1A under hypoxic conditions in MCF-7 and MDA-MB-231 cell lines ($p<.05$; Figures 3). Furthermore, as

expected, melatonin was also able to reduce the gene expression of VEGFA in both cell lines ($p<.05$; Figure 4).

Differential protein expression

Protein expression of HIF-1α and VEGF-A revealed by immunocytochemistry

To complement the analysis of gene expression, we evaluated the protein expression of HIF-1α and VEGF-A by immunocytochemistry in MCF-7 and MDA-MB-231 cell lines, after treatment with 200 μM CoCl2 alone, or in addition with 1 mM of melatonin for 24 hours. The results were obtained in Arbitrary Units (AU) and demonstrated the value of the Mean Optical Density (MOD) ± standard error.

According to the gene expression data, MOD analysis showed that there was an increase in HIF-1α protein staining with 200 μM $CoCl_2$ in MCF-7 and MDA-MB-231 cells when compared to the control groups. Furthermore, in both cell lines under hypoxia and treated with melatonin, there was a decrease in immunostaining of HIF-1α ($p <.05$; Figures 5).

As far as immunostaining of VEGF is concerned, there was a statistically significant increase in the group under hypoxia in MCF-7 and MDA-MB-231 cells and a reduction after melatonin treatment ($p <.05$; Figures 6).

Expression of angiogenic factors measured by protein array

To assess whether melatonin was able to change other 20 growth factors involved in angiogenesis, we performed the protein array in MCF-7 and MDA-MB-231 cell lines after treatment with vehicle (control group); and 200 μM CoCl2 alone (hypoxia group) or in addition with 1 mM melatonin (hypoxia plus melatonin group).

In MCF-7 cells, semi-quantitative analysis showed a statistically significant increase in both VEGF-C and VEGFR2 proteins in hypoxic group, which were reduced after melatonin treatment ($p<.05$, Figure 7). Additionally, while no increase was observed in protein expression for VEGFR3, MMP-9 and Angiogenin in hypoxic group when compared to the control group, there was a significant reduction in the expression of these proteins in the group treated with melatonin when compared to the hypoxia group ($p < .05$; Figure 7).

In MDA-MB-231 cells, there was a reduction of VEGF-A, VEGFR2, EGFR and Angiogenin proteins in melatonin treatment group under hypoxia when compared to the hypoxia group ($p<.05$, Figure 8). Moreover, under hypoxic conditions it was observed an increase for EGFR protein when compared to the control group ($p < .05$; Figure 8).

Protein expression of colony stimulating factor (GCSF) was not detected in MCF-7 and MDA-MB-231 cells, and other proteins evaluated showed no significant changes upon treatment ($p>.05$).

DISCUSSION

In this study, we found that melatonin treatment reduced the viability of ERα-positive (MCF-7) and ERα-negative (MDA-MB-231) cells, as well as the expression of some angiogenic factors under hypoxic conditions.

Under normoxic conditions, concentrations of 0.001 mM, 0.01, 0.1 mM and 1 mM of melatonin were able to decrease the viability of ERα-positive (MCF-7) cell line, while only the higher concentration (1mM) decreased the viability of ERα-negative (MDA-MB-231) cells. Melatonin has important anti-estrogenic activity, reducing the mitogenic response in ERα-positive breast tumors, and so, it has been shown that these cells respond to lower melatonin concentrations [35], while concentrations less than 1 mM of melatonin does not act in MDA-MB-231 cell line [36].

Several studies demonstrated that 1 mM or higher concentrations of melatonin (8 mM to 16 mM) play an important antitumor activity [37-40]. In the literature, concentrations higher than 1 μM are described as pharmacological while supra-physiological concentrations ranging from 1 nM to 1 μM and physiological concentrations include those lower than 1 nM [41,42]. Endogenous substances, when used for therapeutic purposes are usually administered in much higher concentrations than those corresponding to the physiological, to achieve the pharmacological effects instead of physiological optimizing their clinical use [40]. Additionally, melatonin levels are three fold higher in tumor and breast adipose tissue compared to serum levels [43], which could explain the necessity of the high concentrations of melatonin to achieve its action in some tissues. Based on especially cell culture experiments, oncology is an interesting field for the

application of melatonin, however, more clinical studies are necessary before options and possibilities can be shifted to clinic indications [44].

It is known that the maximum averages of melatonin physiological levels attained in the plasma of adults are of the order from 60 to 70 pg/ml [45,46]. About the exogenous administration of melatonin, studies showed that its bioavailability highly variable. Various dosages ranging, different routes of administration and formulations have been studied, however, the optimal dose to elicit a pharmacological effect, formulation for any applications and its absolute bioavailability remain to be clarified [47-49].

A study of Waldhauser *et al*. [50] evaluated melatonin levels in plasma and urine of young males who received one capsule of 80 mg of melatonin and showed that the peak of melatonin serum levels was from 350 to 10 thousand times higher than the physiological nightly peak. In patients with cancer, the melatonin dose administered in clinical trials ranged from 20 to 40 mg/day. These studies have shown that melatonin, in addition to the conventional therapy, showed beneficial effects in different types of advanced tumors or untreatable patients with metastatic tumors, either increasing the effectiveness of treatment [51,52], or acting as a support treatment [53,54].

Since then, *in vitro* and *in vivo* studies show that melatonin, either in physiologic concentrations in the body or administered in pharmacologic concentrations, has a wide preventive and therapeutic activity in cancer, and several mechanisms of action have been proposed [55,56].

Melatonin has membrane receptors coupled to the G protein, known as MT1 and MT2. These receptors have Kd values for melatonin in the pM and nM range, respectively, whereas much higher concentrations of melatonin were required for anti-proliferative and proapoptotic effects (mM) [39]. Furthermore, melatonin can also act by independent mechanisms of its membrane receptors, hindering the full knowledge of the intracellular actions of melatonin, and shows that it has several other mechanisms of action [26, 56-58].

It is known that maintaining the oxygen balance is key for cell survival, and according to the degree of hypoxia in the tumor microenvironment, tumor cell can undergo apoptosis or induce adaptive mechanisms that allow survival under adverse conditions. In this context, HIF-1α has an essential role in cellular homeostatic responses to hypoxic microenvironments, including the regulation of genes involved in energy metabolism, angiogenesis, proliferation and apoptosis, which can result ultimately in resistance to apoptosis and treatment [59].

Under normoxia, the HIF-1α protein undergoes an oxygen-dependent degradation [60], while under hypoxic conditions, HIF-1α is stabilized and its degradation is blocked [61,62]. As with hypoxic conditions, at normoxic conditions $CoCl_2$ is able to stabilize HIF-1α [3], as well as increase HIF-1α mRNA [63,64]. Thus, to mimic hypoxic conditions in MCF-7 e MDA-MB-231, we used $CoCl_2$ at concentrations of 100 μM and 200 μM $CoCl_2$ for 24 hours.

As expected, our results showed that concentrations of 100 μM and 200 μM $CoCl_2$ increased both gene and protein expression of HIF-1α and VEGF-A in MCF-7 e MDA-MB-231 cells. In addition, 100 μM and 200 μM of CoCl2 did not

change the viability of both cell lines, which could suggest that the increase of HIF-1α contributed with cell survival.

Similarly, Liu *et al*., [59] showed that there was no significant difference in cell survival when hepatocellular carcinoma HepG2 cells were treated with 50, 100 and 200 μmol/l of $CoCl_2$. Moreover, Chang *et al*., [65] showed that $CoCl_2$ increased the migration, cell proliferation and expression of HIF-1α in hepatocellular carcinoma cell line HepG2.

SHG44 cells were treated with different concentrations of $CoCl_2$ and there was a dose and time-dependent decrease. Doses above 200 μmol/L led to decrease the cell viability. Furthermore, as expected, hypoxic conditions increased the HIF1A expression [66]. Another study showed that there was a gradual decrease in cell proliferation of hepatoma cells exposed from 100 μmol/L to 800 μmol/L of $CoCl_2$, and minor changes were observed in the cells viability at $CoCl_2$ concentrations between 100 μmol/L and 200 μmol/L. The authors performed MTT assay and BrdU incorporation assay and showed that HIF-1α silencing inhibited the proliferation of hepatoma cells [67].

Under hypoxic conditions, treatment with 1 mM melatonin reduced cell viability, as well as the gene and protein expression of HIF-1α and VEGF-A. Especially, the effects of melatonin and hypoxia on HIF-1α were showed in other studies with several types of tumor. According to Gonçalves *et al*., [68], 1 mM melatonin inhibited the gene and protein expression of HIF-1α and VEGF under hypoxia in human oral cancer cell line SCC9. Zhang *et al*., [69] showed that melatonin at 1 mM concentration, but not at 1 nM concentration, blocked HIF-1α

protein expression via its antioxidant activity against ROS produced by glioblastoma cells lines in response to hypoxia. Furthermore, 1 mM melatonin inhibited the target genes of HIF-1α, VEGF and MMP-2 and suppressed cell migration. Cho *et al*., [70] showed that 1 mM of melatonin inhibited the protein stability of HIF-1α in human prostate cancer cell line (PC-3) under hypoxia conditions. Also, Kim *et al*. [71] showed that melatonin treatment diminished the expression of HIF-1α protein, reducing the tumor growth and angiogenesis in mice bearing renal adenocarcinoma (RENCA).

In addition, other studies have shown that higher concentrations of melatonin reduces the protein levels of HIF-1α and, therefore, the levels of VEGF-A mRNA and protein under hypoxic conditions, in several tumor cell lines, as human pancreatic carcinoma cells (PANC-1), human alveolar adenocarcinoma cells (A549) [3], prostate cancer cells (DU145, PC-3 and LNCaP) [30], human colon cancer cells (HCT116) [61], and HepG2 liver cancer cells [64]. Moreover, Alvarez-Garcia *et al*. [32] showed that 1 mM melatonin reduced the gene expression of VEGF in MCF-7 cells.

It is well established that tumor cell-derived VEGF-A and VEGF-C serve as key growth factors in the promotion of tumor progression by binding to VEGF receptors (VEGFRs) [72]. The VEGF-A/VEGFR2 paracrine interaction between tumor cells and endothelial cells is one of the most important systems for tumor-associated angiogenesis [9,72,73]. Similarly, the VEGF-C/VEGFR3 paracrine interaction between tumor cells and lymphatic endothelial cells is one of the most important systems for tumor-associated lymphangiogenesis [5,72].

While VEGF receptors are known to be present on the surface of endothelial cells, they and other angiogenic growth factors receptors are also functionally expressed on subsets of tumor cells. Thus, angiogenic factors may promote tumor growth not only by inducing angiogenesis but also by directly signaling through their receptors on tumor cells [13,74-76].

Our results demonstrated that melatonin treatment during hypoxia reduced the expression of VEGFR2 protein in MCF-7 and MDA-MB-231 cells. Similarly, Jardim-Perassi *et al*. [33] showed that *in vivo* treatment with melatonin reduces the expression of VEGFR2 protein in mammary tumors of athymic nude mice. Our results also showed a decrease in VEGF-C and VEGFR3 in MCF-7 cells treated with melatonin. In addition to its important role in lymphangiogenesis, the VEGF-C/VEGFR3 autocrine system in tumor cells seems to be widely involved in aspects of tumor progression including growth, invasion and metastasis, and further unknown functions may also exist [72,77,78]. Thus, the reduction of VEGF-A, VEGF-C and their receptors in tumor cells suggests that melatonin might have a dual function in inhibiting autocrine and paracrine pathways established between tumors cells and endothelium.

In addition to the VEGFRs, another important receptor protein tyrosine kinase is EGFR. The activation of this molecule promotes a wide variety of cellular responses commonly associated with tumor cell properties, including proliferation, migration, stromal invasion and resistance to cell death-inducing signals [13,79]. Our results showed that in MDA-MB-231 cells, EGFR protein expression increased significantly under hypoxic conditions and decreased after

melatonin treatment. Consistent with these results, previous studies have shown that pharmacological concentrations of melatonin are able to suppress EGFR-mediated mitogen signaling in breast cancer cells [80], which is consistent with melatonin´s antioxidant activity [81].

Moreover, melatonin treatment reduced Angiogenin protein expression in both cell lines. It is clear that Angiogenin is up-regulated in various types of human cancer and cancer cell lines [82-85], including MCF-7 and MDA-MB-231 cells [86]. Angiogenin plays a dual role in cancer progression by stimulating both tumor cells proliferation and angiogenesis, activating endothelial and smooth muscle cells and inducing a several cellular activities, as cell migration, invasion, proliferation, and angiogenesis [87,88]. Angiogenin binds to endothelial cells and undergoes nuclear translocation, an essential process for both its angiogenic activity and for angiogenesis induced by other factors including VEGF, bFGF and EGF [89]. There are no studies investigating the melatonin effects on angiogenin; however, angiogenin antagonists have been shown to inhibit the growth of angiogenesis in human tumor cells in athymic mice [90]. Thus, our results demonstrate a potential anti-angiogenic activity of melatonin by the angiogenin reduction.

Other mechanisms that promote tumor progression include the degradation of basement membrane and extracellular matrix that are required for migration of endothelial cells, a process involving MMPs [2,91]. Treatment with melatonin decreased levels of MMP-9 protein under hypoxic conditions in ERα-positive MCF-7 cells. Consistent with our results, Ordoñez *et al*. [92] demonstrated that

melatonin treatment *in vitro* modulates the motile and invasive capacity of liver cancer cells (HepG2) by decreasing MMP-9 expression. Other studies have shown that melatonin reduces MMP-9 expression in a human gastric adenocarcinoma cell line [93], HUVEC endothelial cells under inflammatory stimuli [94] and glioma cells (T98G and U251), which was associated with decreased oxidative stress [95].

Mao *et al*. [36] demonstrated that melatonin exerts an inhibitory effect on the invasion of MCF-7 breast cancer cell clones through down-regulation of the p38 pathway and inhibition of MMP-2 and MMP-9 expression and activity. In addition, Di *et al*. [96] showed that the expression of MMP-2 and MMP-9 are correlated with aromatase levels in ER-positive mammary tumors, suggesting that these MMPs are regulated by estrogen produced by aromatase. Thus, reduction of MMP-9 in ER-positive cells can be associated with proven anti-estrogenic action of melatonin.

Taken together, our results showed that among the factors evaluated, melatonin treatment reduced HIF-1α, VEGF-A, VEGF-C, MMP-9 and Angiogenin, as well as VEGFR2 and VEGFR3 receptors in MCF-7 cells. For MDA-MB-231 cells, melatonin treatment reduced HIF-1α, VEGF-A, VEGFR2, EGFR and Angiogenin.

CONCLUSION

In conclusion, our results suggest that melatonin exerts important anti-angiogenic effects by reducing angiogenic factors in breast tumor cells, especially

under hypoxic conditions. A greater number of factors were reduced in ERα-positive cells, which could be related to the anti-estrogenic activity of melatonin.

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BVJP and DAPCZ conceived the study, designed the experiments and drafted the manuscript. MRL, GMD and IHG carried out the culture experiments, the qPCR and the immunohistochemistry. GBG helped with the designed the experiments and production of the manuscript. LCF and TFB carried out the protein array experiments and assisted the manuscript production. MGM helped in qPCR analysis and the immunohistochemical statistical analysis. All authors read and approved the final manuscript. All authors read and approved the final manuscript.

LIST OF ABBREVIATIONS

ACTB = Actin beta gene

bFGF = Basic fibroblast growth factor

cDNA = Complementary deoxyribonucleic acid

$CoCl_2$ = Cobalt chloride

DAB = 3,3'-diaminobenzidine tetrahydrochloride

DEPC = Diethylpyrocarbonate

DMEM = Dulbecco's modified Eagle's medium

DMSO = Dimethyl sulfoxide

EGF = Epidermal growth factor

EGFR = Epidermal growth factor receptor

ELISA = Enzyme-linked immunosorbent assay

FBS = Fetal bovine serum

GAPDH = Glyceraldehyde-3-phosphate dehydrogenase gene

GCSF = Colony stimulating factor

HIF-1α = Hypoxia-inducible factor-1α

MMPs = Matrix metalloproteinases

MOD = Mean optical density

MTT assay = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)

PCR = Polymerase Chain Reaction

PDGF = Platelet-derived growth factor

qPCR = Quantitative Polymerase Chain Reaction

RNA = Ribonucleic acid

SEM = Mean ± standard error of the mean

VEGF-A = Vascular endothelial growth factor A

VEGF-C = Vascular endothelial growth factor C

VEGFR2 = Vascular endothelial growth factor receptor 2

CONFLICT OF INTERESTS

The author(s) confirm that this article content has no conflict of interest.

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FIGURES

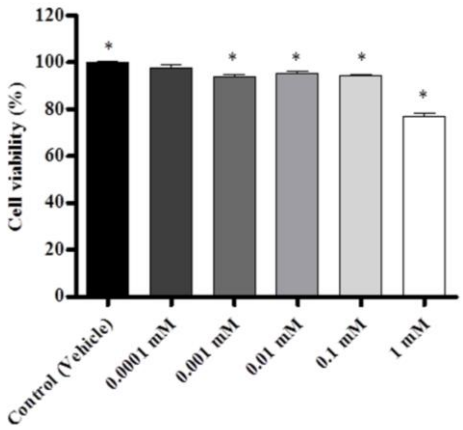

Figure 1. Analysis of cell viability by MTT assay of MCF-7 cell line treated with different concentrations of melatonin. Data are expressed as the percentage of the control group. (*) $p < 0.05$ *vs* control. Significant values determined by ANOVA followed by Bonferroni´s test (Error bars: ± standard error).

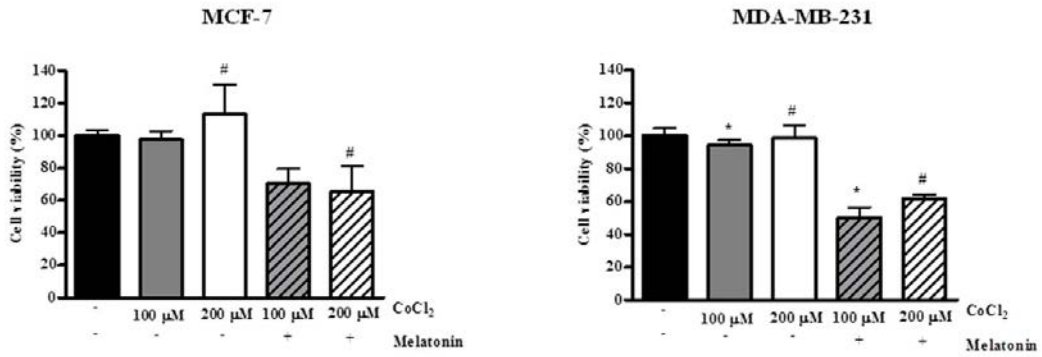

Figure 2. Analysis of cell viability by MTT assay of MCF-7 and MDA-MB-231 cells lines treated with 1 mM of melatonin under hypoxic conditions (100 uM and 200 uM of $CoCl_2$). Data are expressed as the percentage of the control group. (*) $p < 0.05$: 100 µM $CoCl_2$ *vs* 100 µM $CoCl_2$ plus 1 mM melatonin; (#) $p < 0.05$: 200 µM $CoCl_2$ *vs* 200 µM $CoCl_2$ plus 1 mM melatonin. Significant values determined by ANOVA followed by Bonferroni´s test (Error bars: ± standard error).

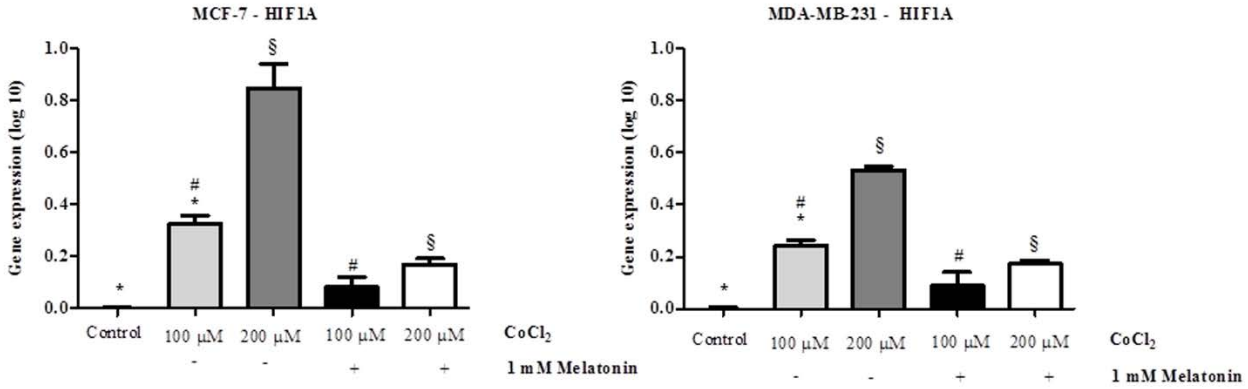

Figure 3. HIF1A gene expression in MCF-7 and MDA-MB-231 cells lines after treatment with $CoCl_2$ (100 µM and 200 µM) for induction of hypoxia and melatonin (1 mM). Value of gene expression notated as log10. (*) $p < 0.05$: Control *vs* $CoCl_2$ groups. Significant values determined by ANOVA followed by Bonferroni´s test (Error bars: ± standard error).

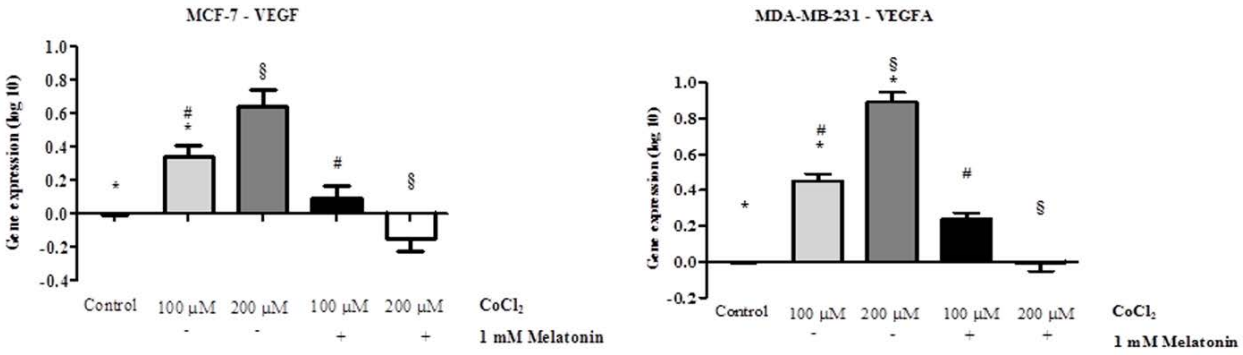

Figure 4. VEGFA gene expression in MCF-7 and MDA-MB-231 cells lines after treatment with $CoCl_2$ (100 µM and 200 µM) for induction of hypoxia and melatonin (1 mM). Value of gene expression notated as log10. (*) $p < 0.05$: Control *vs* $CoCl_2$ groups; (#) $p < 0.05$: 100 µM $CoCl_2$ *vs* 100 µM $CoCl_2$ plus 1 mM melatonin; (§) $p < 0.05$: 200 µM $CoCl_2$ *vs* 200 µM $CoCl_2$ plus 1 mM melatonin; significant values determined by ANOVA followed by Bonferroni´s test (Error bars: ± standard error).

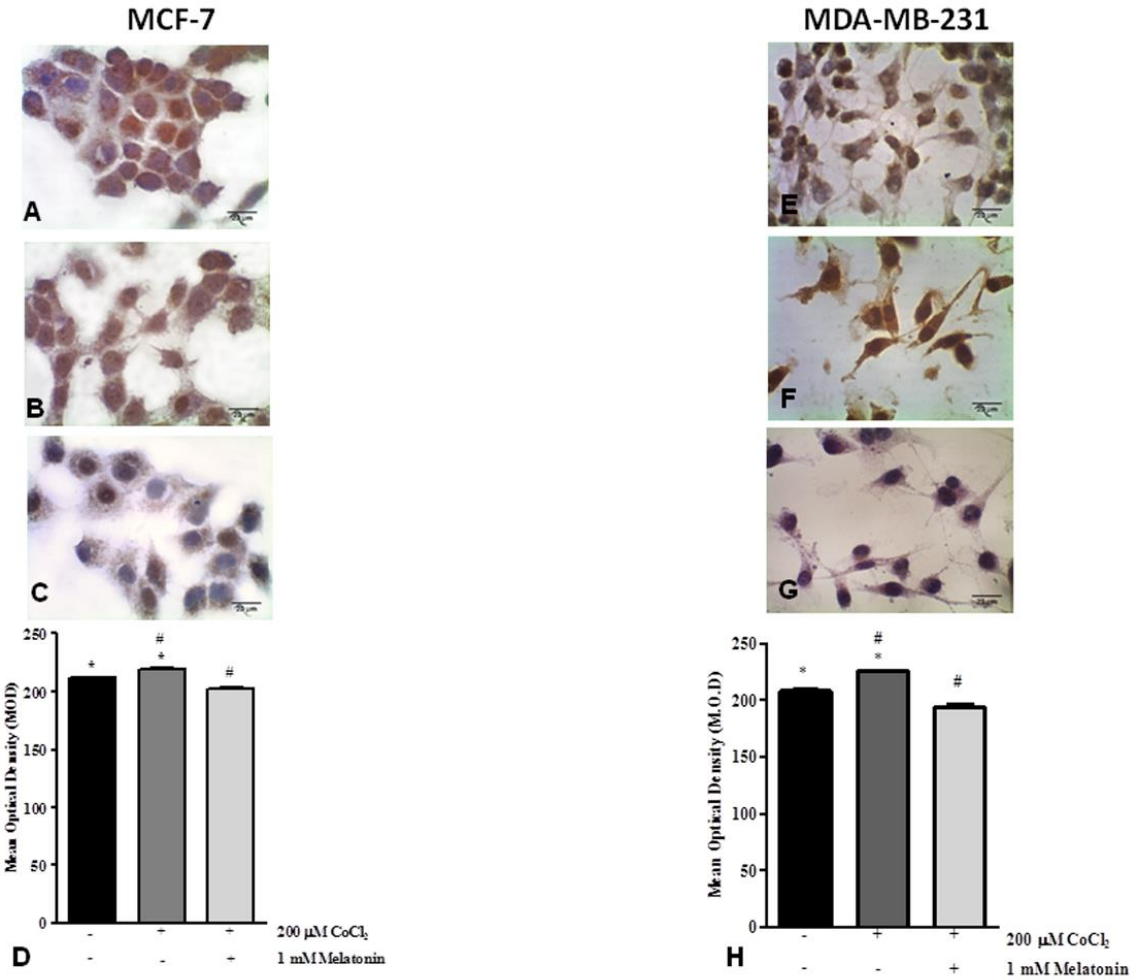

Figure 5. Immunocytochemistry of the protein HIF-1α showing MCF-7 and MDA-MB-231 cells after 24 hours of treatment with CoCl2 (200 µM) for induction of hypoxia and melatonin (1 mM). Immunocytochemistry staining (40x magnification): **A,E.** Control; **B,F.** Cells under hypoxia; **C,G.** Cells under hypoxia treated with melatonin; **D,H.** Protein quantification by mean optical density. (*) $p < 0.05$ Control *vs* Hypoxia, (#) $p < 0.05$: Hypoxia *vs* Hypoxia plus Melatonin. Significant values determined by ANOVA followed by Bonferroni´s test (Error bars: ± standard error).

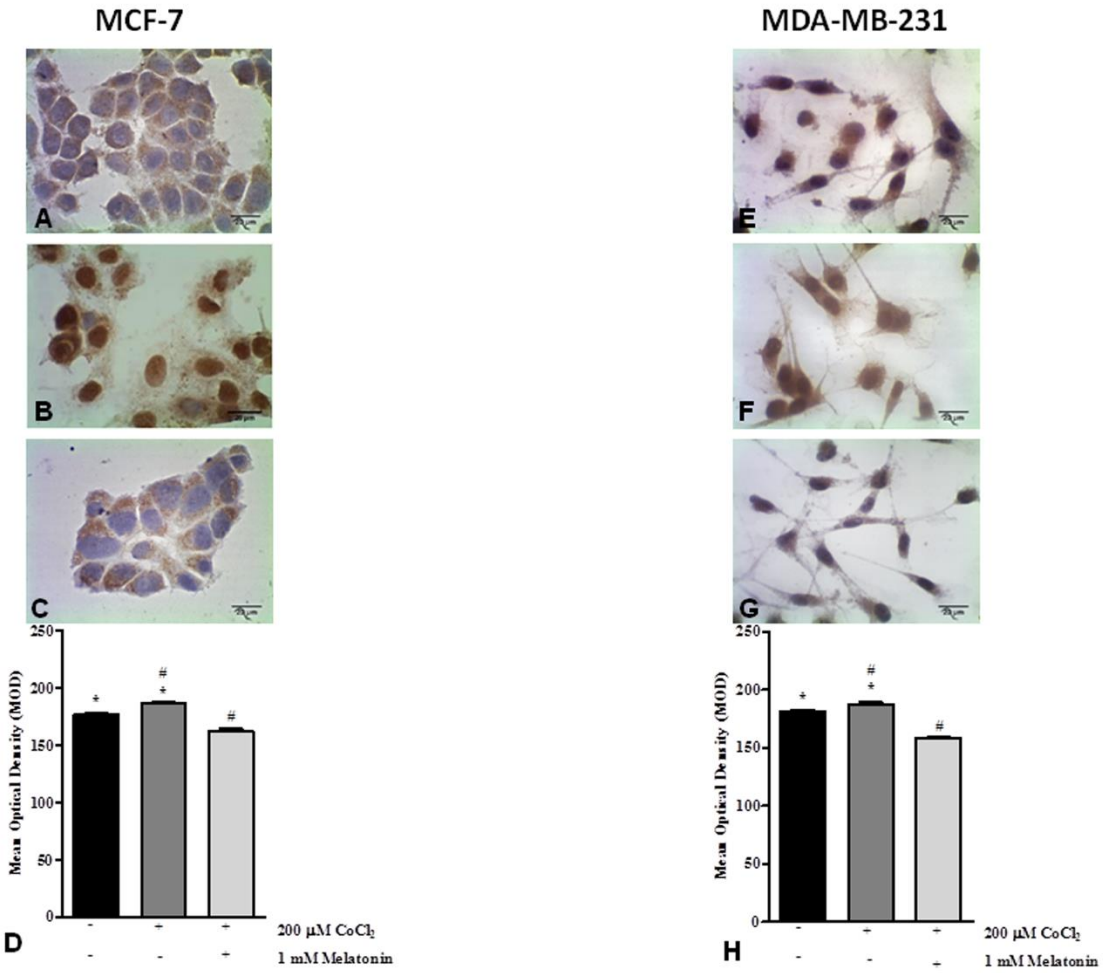

Figure 6. Immunocytochemistry of the protein VEGF-A showing MCF-7 and MDA-MB-231 cells after 24 hours of treatment with CoCl2 (200 µM) for induction of hypoxia and melatonin (1 mM). Immunocytochemistry staining (40x magnification): **A,E.** Control; **B,F.** Cells under hypoxia; **C,G.** Cells under hypoxia treated with melatonin; **D,H.** Protein quantification by mean optical density. (*) $p < 0.05$ Control *vs* Hypoxia, (#) $p < 0.05$: Hypoxia *vs* Hypoxia plus Melatonin. Significant values determined by ANOVA followed by Bonferroni´s test (Error bars: ± standard error).

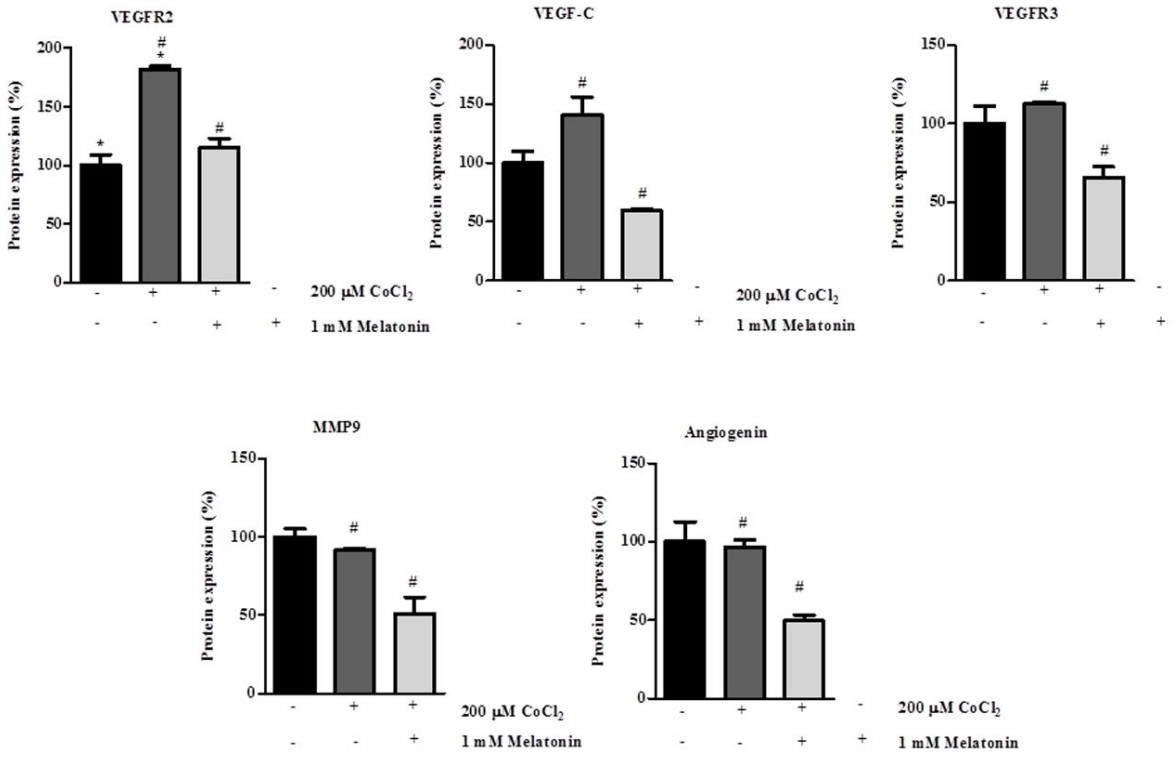

Figure 7. Significant results from quantitative analysis of growth factors using protein array kit in MCF-7 cells. Data are expressed as the percentage of the control group. (*) $p < 0.05$ Control *vs* Hypoxia, (#) $p < 0.05$: Hypoxia *vs* Hypoxia plus Melatonin. Significant values determined by ANOVA followed by Bonferroni´s test (Error bars: ± standard error).

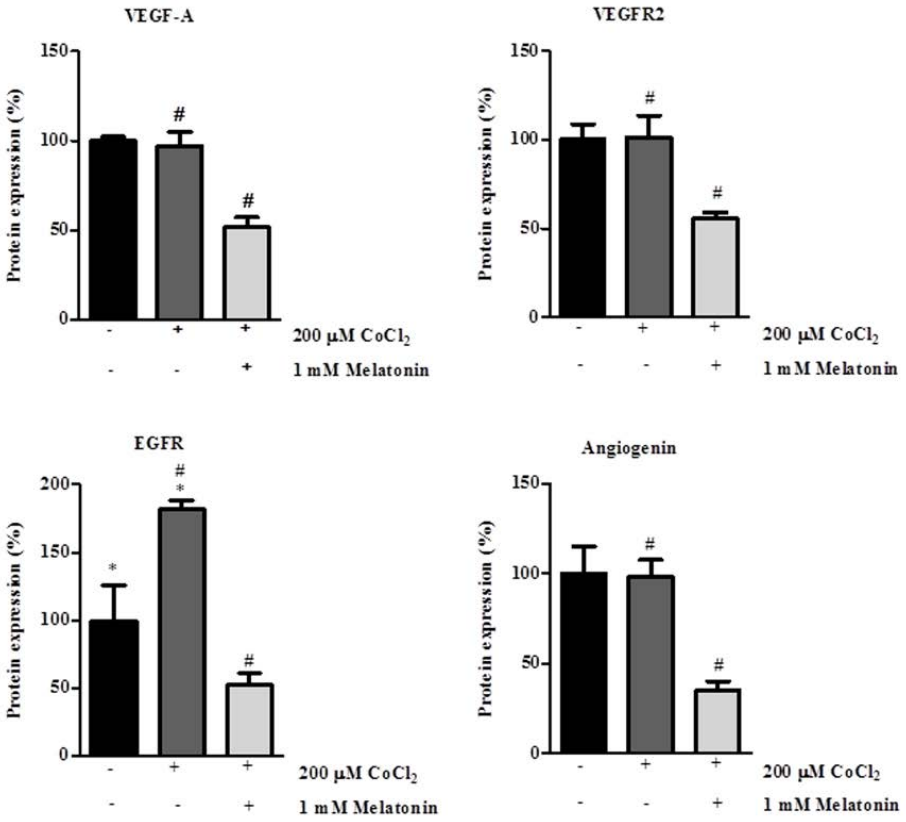

Figure 8. Significant results from quantitative analysis of growth factors using protein array kit in MDA-MB-231 cells. Data are expressed as the percentage of the control group. (#) $p < 0.05$: Hypoxia *vs* Hypoxia plus Melatonin. Significant values determined by ANOVA followed by Bonferroni´s test (Error bars: ± standard error).

TABLES

Table 1 Custom designed protein arrays kit, which consist of 20 different cytokines/factors.

	A	B	C	D	E	F	G	H
1	Positive	Positive	Negative	Negative	G-CSF	MMP9	PDGF Ra	SDF-1α
2	Positive	Positive	Negative	Negative	G-CSF	MMP9	PDGF Ra	SDF-1α
3	PDGF AA	Tie-1	RANTES	Tie-2	bFGF	VEGFA	EGF	VEGFC
4	PDGF AA	Tie-1	RANTES	Tie-2	bFGF	VEGFA	EGF	VEGFC
5	EGFR	VEGFR2	IGF-I	VEGFR3	Angiogenin	Angiostatin	Angiopoeitin-1	Angiopoeitin-2
6	EGFR	VEGFR2	IGF-I	VEGFR3	Angiogenin	Angiostatin	Angiopoeitin-1	Angiopoeitin-2
7	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Positive
8	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Positive

G-CSF = colony stimulating factor; MMP-9 = matrix metallopeptidase 9; PDGF-Ra = platelet-derived growth factor receptor, alpha polypeptide; SDF-1 α = Stromal cell-derived factor 1 alpha; PDGF-AA = platelet-derived growth factor alpha polypeptide; Tie-1 = receptor tyrosine kinase of angiopoietin-1, tyrosine kinase with immunoglobulin-like and EGF-like domains 1*;* Tie-2 = receptor tyrosine kinase of angiopoietin-1; bFGF = fibroblast growth factor (basic); VEGF-A = vascular endothelial growth factor A; EGF = epidermal growth factor; VEGF-C = vascular endothelial growth factor C; EGFR = epidermal growth factor receptor; VEGFR2 = Vascular endothelial growth factor receptor 2; IGF-I = insulin-like growth factor 1 (somatomedin C); VEGFR3 = Vascular endothelial growth factor receptor 3. All antibodies are prepared in duplicate.

Discussão

IV. DISCUSSÃO

Nossos resultados mostraram que a melatonina apresentou importante ação anti-tumoral *in vitro* e *in vivo*. Inicialmente, a concentração farmacológica de melatonina (1 mM) reduziu a viabilidade de células tumorais mamárias REα-positivas (MCF-7) e triplo-receptor negativo (MDA-MB-231). Apesar de alguns estudos demonstrarem que a melatonina pode atuar em linhagens REα-negativas (LEMAN *et al.*, 2001; JUNG *et al.*, 2013), sua ação anti-proliferativa tem sido descrita principalmente em linhagens de câncer de mama RE*α*-positivo (HILL *et al.*, 1992; MAO *et al.*, 2014).

A efetividade da melatonina nesse tipo tumoral é associada principalmente à sua capacidade de interagir com a via de sinalização do estrógeno (COS; SANCHEZ-BARCELO, 2000). Além disso, sua ação anti-proliferativa é mediada também pela ativação de seu receptor MT1. Tanto linhagens REα-positivas como REα-negativas expressam esse receptor (RAM *et al.*, 2002; MAO *et al.*, 2014), no entanto, recentes pesquisas demonstraram que tumores REα-positivos, bem como a linhagem MCF-7, apresentam maior expressão de MT1 em relação a tumores triplo-receptor negativos, como a linhagem MDA-MB-231 (HILL *et al.*, 2011; JABLONSKA *et al.*, 2013).

Diversos estudos têm demonstrado que a efetividade da melatonina não é limitada aos tumores mamários REα-positivos e, ainda, que a melatonina possui outros mecanismos de ação independentes de MT1, o que dificulta a completa

compreensão de sua ação intracelular (DI BELLA *et al.*, 2013; JABLONSKA *et al.*, 2013).

De acordo com a redução da viabilidade celular *in vitro*, nossos resultados mostraram que o tratamento com melatonina *in vivo* (40 mg/kg) levou a diminuição da proliferação celular e do crescimento de tumores mamários triplo-receptor negativos, corroborando com outros estudos que demonstram que o tratamento com melatonina diminui o crescimento de diferentes tipos de câncer em animais (COS *et al.*, 1998; RAO *et al.*, 2000; MIRUNALINI S, 2010; LIU *et al.*, 2011).

Mirunalini, et al. (2010) demonstraram que o tratamento com melatonina (5 mg/mL) reduziu os efeitos do carcinógeno DMBA em ratas, levando a diminuição do crescimento do tumor mamário, do estresse oxidativo e ao aumento da capacidade antioxidante. Rao *et al.*, (2000) mostraram que o tratamento com 50 mg/kg de melatonina em camundongos transgênicos que apresentam superexpressão do oncogênese c-neu (TG.NK), foi capaz de reduzir o desenvolvimento e o tamanho dos tumores mamários em relação ao grupo controle. Outro estudo demonstrou que a melatonina nas doses de 25 mg/kg, 50 mg/kg e 100 mg/kg reduziu o crescimento do tumor gástrico de camundongos (LIU *et al.* 2011).

Além da ação anti-proliferativa, nossos resultados demonstraram que o tratamento com melatonina apresentou importante ação anti-angiogênica. *In vitro*, a melatonina reduziu a expressão de HIF-1α e, consequentemente a expressão de VEGF-A nas linhagens MCF-7 e MDA-MB-231 sob condições de hipóxia.

Esses resultados estão de acordo com outros estudos que demonstraram que a melatonina inibe a estabilização do HIF-1α em condições de hipóxia, sem alterar sua expressão gênica em células tumorais (DAI *et al.*, 2008; PARK *et al.*, 2009; PARK *et al.*, 2010; CARBAJO-PESCADOR *et al.*, 2013). Sabe-se que em normóxia, o HIF-1α é hidroxilado pela proteína contendo domínios prolil hidroxilase (PHD) e então liga-se a pVHL e é degradado. Em condições de hipóxia, a ligação HIF-1α-pVHL não ocorre e portanto a degradação do HIF-1α é bloqueada. O estudo de Park *et al.*, (2010) demonstrou que a melatonina foi capaz de restaurar essa ligação em células de carcinoma colorretal sob hipóxia, normalizando os níveis de HIF-1α. Os autores mostraram que a estabilização do HIF-1α foi resultante da ação antioxidante da melatonina, que remove espécies reativas de oxigênio, inibindo proteínas PHDs (PARK *et al.*, 2010).

Além disso, muitos estudos têm demonstrado que concentrações farmacológicas de melatonina reduzem os níveis protéicos de HIF-1α e os níveis gênicos e proteicos de VEGF-A em diferentes linhagens tumorais (DAI *et al.*, 2008; PARK *et al.*, 2009; PARK *et al.*, 2010; ALAVAREZ-GARCIA *et al.*, 2013a; CARBAJO-PESCADOR *et al.*, 2013). Kim et al. (2103) demonstraram que o tratamento com melatonina (20 mg/kg) reduziu o crescimento de adenocarcinoma renal (RENCA) em camundongos, o que foi relacionado com a diminuição protéica do HIF-1α. Além disso, o tratamento com melatonina em ratos Wistar sem neoplasia, expostos a hipóxia, levou a diminuição dos níveis protéicos de VEGF (KAUR *et al.*, 2007).

Tem-se estabelecido que as proteínas VEGF-A e VEGF-C atuam como fatores de crescimento essenciais na progressão do tumor, por meio da ligação com seus receptores VEGFRs (MATSUURA *et al.*, 2009). A interação entre VEGF-A produzido pelas células tumorais e VEGFR2 na superfície de células endoteliais é um dos sistemas mais importantes para o estabelecimento da angiogênese tumoral (WOOLARD *et al.*, 2004; MATSUURA *et al.*, 2009; COULON *et al.*, 2011). Da mesma forma, a interação parácrina entre VEGF-C e VEGFR3 é essencial para linfangiogênese no tumor (MATSUURA *et al.*, 2009; GACCHE; MESHRAM, 2013). Os VEGFRs são também expressos em células tumorais e, portanto, os fatores VEGF-A e VEGF-C promovem não só a angiogênese, mas também o crescimento de células tumorais de maneira autócrina (PRICE *et al.*, 2001; DIAS *et al.*, 2002; COSTA *et al.*, 2004; HICKLIN; ELLIS, 2005; WEIGAND *et al.*, 2005; MASOOD, 2006; KARROUM *et al.*, 2012).

Nesse sentido, nossos resultados mostraram que a melatonina reduziu os níveis protéicos de VEGFR2 nas linhagens MCF-7 e MDA-MB-231, bem como de VEGF-C e VEGFR3 na linhagem MCF-7. Ainda, o tratamento com melatonina *in vivo*, levou a redução de VEGFR2 e da microdensidade vascular (vWF) no tecido tumoral.

É importante ressaltar que além da avaliação de fatores angiogênicos, a angiogênese pode ser avaliada por técnicas de imagem utilizando radioisótopos acoplados com proteínas específicas. Nesse estudo, utilizou-se o Tc-99m acoplado com a proteína recombinante VEGF-c, por meio do quelante hidrazina nicotinamida (HYNIC), conforme descrito por Ali *et al.*, (2010). Agentes que

apresentam afinidade pelos receptores de VEGF, como Tc-99m-HYNIC-VEGF-c, são úteis na avaliação da angiogênese *in vivo*, uma vez que VEGFRs são altamente expressos na vasculatura do tumor em comparação com a vasculatura normal (Plate *et al.*, 1993). Quando injetado na corrente sanguínea, o VEGF-c liga-se a VEGFR2 e VEGFR3, permitindo o acúmulo do Tc-99m, que pode ser visualizado por SPECT (BACKER *et al.*, 2007; ALI *et al.*, 2010).

Assim, nossos resultados mostraram que houve menor radioatividade de Tc-99m-HYNIC-VEGF-c (SPECT) nos tumores tratados com melatonina, no entanto não houve diferença estatisticamente significante na análise semi-quantitativa das imagens. Apesar disso, a diminuição de VEGFR2 foi confirmada por imuno-histoquímica.

Além de VEGF e seus receptores, nossos resultados mostraram que o tratamento com melatonina *in vitro* levou a redução de outras proteínas pró-angiogênicas. Nas linhagens MCF-7 e MDA-MB-231 houve redução da angiogenina após o tratamento com melatonina sob condições de hipóxia. A angiogenina encontra-se superexpressa em diferentes tipos de câncer, inclusive nas linhagens MCF-7 e MDA-MB-231 (CAMPO *et al.*, 2005; TELLO-MONTOLIU *et al.*, 2006; LI; HU, 2010; RAMANI *et al.*, 2013; DUTTA *et al.*, 2014). Essa proteína atua na progressão do tumor, estimulando a proliferação de células tumorais e a angiogênese, ativando células endoteliais e células musculares lisas, e atuando na migração celular, invasão, proliferação e formação do tubo (KISHIMOTO *et al.*, 2012; ANG *et al.*, 2013). A angiogenina pode se ligar à célula endotelial e translocar-se para o núcleo, um processo essencial para

sua atividade angiogênica e indução de outros fatores pró-angiogênicos como VEGF, bFGF e EGF (NILSSON *et al.*, 2010).

Não há estudos que avaliaram os efeitos da melatonina sobre a angiogenina, no entanto, tem sido demonstrado que antagonistas de angiogenina podem inibir o crescimento tumoral em camundongos atímicos (PICCOLI *et al.*, 1998), o que sugere que a melatonina possui uma adicional ação anti-angiogênica pela redução dessa proteína.

O tratamento com melatonina *in vitro* também levou a diminuição da MMP-9 em condições de hipóxia na linhagem MCF-7. A MMP-9 atua na degradação da membrana basal e da matriz extracelular, com importante papel na invasão celular e desenvolvimento de metástases (DI *et al.*, 2005). De acordo com nossos resultados, Ordoñez et al. (ORDONEZ *et al.*, 2014) demonstraram que o tratamento com melatonina *in vitro* regula a motilidade e capacidade invasiva de células de câncer de fígado (HepG2) pela diminuição de MMP-9. Outros estudos têm demonstrado que a melatonina reduz MMP-9 em linhagens de adenocarcinoma gástrico (RUDRA *et al.*, 2013), células endoteliais (HUVEC) sob estímulo inflamatório (QIN *et al.*, 2012) e células de glioma (T98G e U251) (WANG *et al.*, 2012).

Mao *et al.* (2010) demonstraram que a melatonina exerce um efeito inibitório em clones invasivos da linhagem MCF-7, inibindo a expressão e atividade de MMP-2 e MMP-9. Além disso, Estudo de Di *et al.* (2005) demonstrou que a expressão de MMP-2 e MMP-9 relaciona-se com os níveis de aromatase em tumores mamários RE-positivos, sugerindo que essas MMPs podem

ser reguladas pelo estrógeno produzido pela aromatase. Assim, a redução da MMP-9 na linhagem REα-positiva pode ser estar relacionada à comprovada ação antiestrogênica da melatonina.

Nossos resultados mostraram que nas células MDA-MB-231, os níveis protéicos de EGFR aumentaram sob hipóxia e houve diminuição após o tratamento com melatonina. A ativação de EGFR promove cascatas intracelulares envolvidas na proliferação, migração, invasão e resistência a apoptose, com ativação da proteína quinase ativada por mitógeno (MAPK) (SCHLESSINGER, 2000; COSTA *et al.*, 2004; VAN CRUIJSEN H., 2006). A via EGFR/MAPK pode ser ativada por espécies reativas de oxigênio, assim, a ação antioxidante da melatonina pode reduzir sua ativação (BLASK *et al.*, 2002). Além disso, EGFR pode ser ativada pelo ácido 13-hidroxidienoico (13-HODE), produto da oxidação do ácido linoleico absorvido pelo tumor. A melatonina pode bloquear a absorção do ácido linoleico e conversão em 13-HODE, reduzindo assim a ativação de EGFR/MAPK e consequentemente a progressão tumoral (BLASK *et al.*, 1999; BLASK *et al.*, 2002). De acordo com nossos resultados, Margheri, *et al.,* (2012) demonstraram que o tratamento conjunto com melatonina, ácido retinóico e somastatina inibiu a via de sinalização mediada por EGFR e, consequentemente, a viabilidade de células tumorais mamárias.

Apesar da melatonina diminuir EGFR *in vitro*, no estudo *in vivo* houve um aumento dessa proteína nos tumores tratados com melatonina. Esses resultados podem ser justificados pela ausência da interação com o microambiente tumoral

no estudo *in vitro*, o que sugere uma maior efetividade da melatonina no bloqueio da via de sinalização autócrina de EGFR em células tumorais.

Além disso, apesar do aumento de EGFR e da proteína IGF-I *in vivo*, a melatonina apresentou importante ação anti-tumoral, reduzindo a proliferação e o crescimento do tumor, bem como a microdensidade vascular e a expressão de VEGFR2 no tecido tumoral. Assim, o aumento dessas proteínas pode representar um mecanismo compensatório devido à inibição de VEGFR2 pela melatonina, uma vez que a ativação de VEGFR2, EGFR e do receptor de IGF-I (IGF-1R) induzem cascatas de sinalizações intracelulares interligadas, e a inibição de um receptor pode promover o aumento de outro (ARBAB, 2012; ROSENZWEIG, 2012). Além disso, estudos sugerem que no microambiente tumoral, células endoteliais podem promover a expressão de fatores angiogênicos pelas células tumorais (BUCHANAN *et al.*, 2012) e, ao contrário, a liberação desses fatores pelas células tumorais pode promover o aumento de seus receptores nas células endoteliais (SASAKI *et al.*, 2013).

Conclusões

V. CONCLUSÕES

O presente trabalho permitiu estabelecer as seguintes conclusões:

- A concentração farmacológica de melatonina reduz a viabilidade celular nas linhagens de câncer de mama REα-positivo (MCF-7) e triplo-receptor negativo (MDA-MB-231) sob condições de hipóxia;
- O tratamento com melatonina reduz os níveis de HIF-1α, e consequentemente de VEGF-A nas linhagens MCF-7 e MDA-MB-231 sob hipóxia; estando relacionado à sua ação antioxidante. Além disso, na linhagem MCF-7 a melatonina atua na redução dos fatores pró-angiogênicos VEGF-C, MMP-9 e Angiogenina, e dos receptores VEGFR2 e VEGFR3, enquanto reduz Angiogenina, VEGFR2 e EGFR na linhagem MDA-MB-231;
- O tratamento com melatonina reduz o crescimento do tumor mamário triplo-receptor negativo em camundongos, a proliferação celular (Ki-67) e apresenta ação anti-angiogênica pela redução da microdensidade vascular (vWF) e da expressão de VEGFR2;
- Esses resultados demonstram que a melatonina apresenta importante ação oncostática, atuando na redução do crescimento do tumor e de fatores pró-angiogênicos e seus receptores, o que sugere seu potencial uso terapêutico no câncer de mama.

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VI. REFERÊNCIAS

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Anexos

VII. ANEXOS

ANEXO 1: Parecer da Comissão de Ética na Experimentação Animal da Faculdade de Medicina de São José do Rio Preto.

Comissão de Ética na Experimentação Animal
CEEA
FAMERP

Faculdade de Medicina de São José do Rio Preto
Comissão de Ética na Experimentação Animal - CEEA
FAMERP Autarquia Estadual, Av. Brig. Faria Lima 5416 CEP 15090.000 Tel. 3201-5700 S.J.Rio Preto/ SP

DELIBERAÇÃO CEEA Nº 004/2011

A Comissão de Ética na Experimentação Animal da Faculdade de Medicina de São José do Rio Preto – CEEA/FAMERP, em reunião desta data analisou o projeto de pesquisa intitulado **"Avaliação da angiogênese em resposta ao tratamento com melatonina no Câncer de Mama: Estudo in vitro e in vivo" (Protocolo FAMERP nº 0127/2011)**, sob responsabilidade da Prof.ª Drª Débora Aparecida Pires Campos Zuccari, e deliberou que o mesmo está de acordo com os princípios éticos estabelecidos na Lei nº 11.794/2008 e na Resolução nº 714/2002 e foi **aprovado** por essa Comissão.

Atenção: Ao final do estudo o pesquisador deverá preencher o Formulário do Relatório Final e enviar ao CEEA.

São José do Rio Preto, 10 de fevereiro de 2011.

Profa. Dra. Glória Elisa Mendes Florido
Vice-Presidente da CEEA
FAMERP

ANEXO 2: Parecer do "Institutional Animal Care and User Committee" (IACUC), do Hospital Henry Ford, Detroit – MI, USA.

Research Administration
CFP-Basement 046
2799 West Grand Boulevard
Detroit, MI 48202-2689
(313) 916-2024 Office
(313) 916-2018 Fax

June 15, 2012

To: Ali S. Arbab, M.D., Ph.D.
Radiology

Fm: Frederick Valeriote, Ph.D., Chair
Robert Knight, Ph.D., Vice Chair
Institutional Animal Care and Use Committee

Re: **"Detection of Activation of Angiogenic Factor in Mouse Model of Breast Cancer"** (IACUC No. 1203)

Period of IACUC Approval: **June 13, 2012 through June 12, 2015**

Number of Animals Approved: 640 Athymic NCr mice

Dear Dr. Arbab:

Thank you for responding to IACUC concerns. All concerns have been adequately addressed and full approval has been granted.

The expiration date for this study is June 12, 2015. In order to remain compliant with federal regulations, a new, complete IACUC Protocol must be submitted for full board review if you intend to proceed with this line of work beyond the above indicated expiration date. A Final Report must be submitted at the completion of the 3-year approval period or upon early termination of the project.

Any adverse effects on animals must be reported to the IACUC as soon as possible.

A copy of the signed and stamped protocol indicating approval of the Institutional Animal Care and Use Committee is enclosed for your files. Please feel free to contact Wendy Parrish at 916.2315 if you have any questions regarding this matter.

Enc.

cc: Bioresources