Modulation of markers associated with aggressive phenotype in MDA-MB-231 breast carcinoma cells by sulforaphane.

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Metastasis as a complex process involves loss of adhesion, migration, invasion and proliferation of cancer cells. Sulforaphane (SFN) is one of naturally occurring cancer chemopreventive isothiocyanates found in cruciferous vegetables, consumption of which has been associated with reduced risk of cancer. In this study, we describe effect of SFN on various aspects determining invasive behavior of MDA-MB-231 human breast carcinoma cells.

We studied modulation of molecules associated with epithelial to mesenchymal transition (EMT), hypoxic marker CA IX and mitochondrially located peripheral benzodiazepine receptor (PBR) using flow cytometry, gene expression of matrix metalloproteinases MMP1, 3, 7, 9, 14, transcription factors POU5F1 and Twist1 mRNA by RT PCR, and cytokine production by multiplex bead assay.

SFN downregulated PBR and vimentin expression in a dose dependent manner, but significantly affected neither HIF-1α, nor CA IX protein expression, nor VEGF and GLUT1 mRNA levels. Among studied MMPs, MMP7 and MMP14 mRNA were downregulated while no apparent effect on MMP1, MMP3 and MMP9 was observed. Further, we found significant down regulation of Twist1 and POU5F1, transcription factors that mediate EMT and the self-renewal of undifferentiated embryonic stem cells. SFN reduced also the production of pro-inflammatory cytokines IL-1β, IL-6, TNF-alpha, IFN-gamma, immunomodulating cytokine IL-4 and growth factors involved in angiogenesis PDGF and VEGF. Our study shows that SFN efficacy is associated with the reversal of several biological characteristics connected with EMT or implicated in the matrix degradation and extracellular proteolysis, as well as with reduced production of pro-inflammatory cytokines and pro-angiogenic growth factors in MDA-MB-231 cells.

Keywords: sulforaphane, breast cancer, EMT, reversal of aggressiveness, cytokine production, matrix metalloproteinases

Human breast cancer progression is characterized by acquisition of migratory and invasive properties of tumor cells as they move through the breast tissue in search of blood vessels, the subsequent infiltration of blood vessels and colonization of other organs which constitutes metastasis. In many tumor types, progression and prognosis are associated with angiogenesis and some inhibitors of angiogenesis act by reducing of vascular endothelial growth factor (VEGF) production [1]. In the interaction of tumor cells with their surrounding microenvironment - stroma and extracellular matrix (ECM), molecules that are secreted by breast tumor cells and that digest other molecules located outside of cells, called matrix metalloproteinases (MMPs), play an important role [2]. Involvement of various MMPs in breast cancer progression has been studied recently [3].

The EMT is the process characterized by the loss of epithelial characteristics or the intercellular adhesion molecules and the acquisition of mesenchymal attributes in epithelial cells, important in the progression of carcinoma to metastasis providing epithelial tumor cells with the ability to migrate, invade the surrounding stroma, and disseminate in secondary organs [4]. The forced K18 expression was associated with a complete loss of the previously strong vimentin expression in the MDA-MB-231 cell line, accompanied by a dramatic reduction in the aggressiveness of the K18 transfectants in vitro and in vivo [5]. Growth factors such as EGF, FGF-2, HGF, and TGF are triggers of EMT. Besides MAPK, PI3K, ras, rho, and
src, also Wnt-, Notch-, and NF-kappaB-dependent pathways were found to contribute to EMT [6, 7].

Twist1 is a basic helix-loop-helix transcription factor that mediates EMT and has been involved in tumor progression and metastasis in several cancer types. Its overexpression has been shown in a large fraction of human cancers [8]. Twist1 mRNA was found markedly overexpressed in colorectal cancer samples compared to nontumorous colon mucosa and its mRNA levels are significantly increased in patients with nodal invasion [9]. An increase in the Twist1 promoter methylation has been shown in breast cancers [10]. Oct-3/4, also known as POU5F1, is a homeodomain transcription factor of the POU family, critically involved in the self-renewal of undifferentiated embryonic stem cells, which expression has been described also in cancer stem-like cells [11]. Its intense expression in bladder tumors was shown to be associated with further disease progression, greater metastasis, and shorter cancer-related survival compared with moderate and low expression [12].

The CA IX belongs to family of α-carboxy anhydrases, zinc metalloenzymes that participate in many physiological and pathological processes [13]. Interestingly, in patients with Hodgkin’s disease and multiple myeloma, who developed aplastic anemia type syndrome after high dose therapy and autologous stem cell transplantation, spontaneous tumor regression was observed concomitantly with high titers of serum autoantibodies against various CAs [14]. CA IX is a cell surface isoform of CA that acts in cell adhesion and pH control. It is strongly induced by hypoxia and often associated with tumors [15]. Recently, we described its expression in the panel of human breast and ovarian carcinoma cell lines together with PBR, mitochondrial protein involved in cell proliferation and differentiation, a new marker associated with aggressiveness (invasion in breast and resistance in ovarian cancer) [16].

The consumption of cruciferous vegetables, a rich source of glucosinolates and their hydrolysis products, including indoles and isothiocyanates, has been associated with a reduced risk of cancer. Sulforaphane (SFN) is a naturally occurring cancer chemopreventive isothiocyanate found in cruciferous vegetables that is metabolized via the mercapturic acid pathway and inhibits HDAC activity in human colon, prostate, and breast cancer cells [17]. It acts through various molecular mechanisms including modulation of carcinogen metabolism by inhibition of phase I and Nrf2-mediated induction phase II detoxification enzymes, induction of anti-inflammatory responses, stimulation of anti proliferative and pro-apoptotic pathways [18]. SFN arrests proliferation and mitosis by stabilizing microtubules [19]. SFN treatment inhibited cell growth, induced a G(2)-M cell cycle block, increased expression of cyclin B1, and induced oligonucleosomal DNA fragmentation in MDA-MB-231, MDA-MB-468, MCF-7, and T47D human breast cancer cells [20] at higher concentrations (5-25µM). In this study we examine its effects on various molecules associated with tumor progression (EMT, angiogenesis, metalloproteinas, PBR, CA IX, cytokine production) using lower (2.5 and 5µM) concentrations of SFN.

### Materials and methods

**Reagents.** PBR-specific monoclonal antibody 8D7 was kindly provided by P. Casellas (Sanofi-Synthelabo Recherche, Montpellier, France) [21, 22]. Monoclonal antibody M75 specific for human carbonic anhydrase IX, directed to an epitope in the N-terminal proteoglycan (PG)-like region, was described and provided by S. Pastorekova [23]. Mouse Monoclonal to Vimentin (Human) VI-RE/1 (IgG1) PE labeled was acquired from Exbio (Bratislava, Slovakia).

Sulforaphane (4-methylsulfinylbutyl isothiocyanate, purity 97%) was purchased from ICN Biomedicals (Basingstoke, UK), dimethyloxalylglycine (DMOG) from Cayman chemical company (Tallinn, Estonia). Propidium iodide (PI) was obtained from Sigma Chemical Co. (St. Louis, MO), saponin from Riedel-de Haen (Germany). Fetal calf serum, L-Glutamine, Penicillin and Streptomycin were acquired from Biocom (Slovakia).

**Cell culture.** The human breast carcinoma cell line MDA-MB-231 was routinely cultured in RPMI supplemented with 10% fetal calf serum, 2mM L-Glutamine, 100 µg/ml penicillin and 50 µg/ml streptomycin in 5% CO₂ at 37 °C. The culture was passaged twice a week after reaching cell density of 1.0 x 10⁶ cells/ml. For SFN treatment, 1x 10⁵ cells were seeded in 6-well tissue culture plastic plate, allowed to reach the exponential growth and exposed to 2.5 µM and 5 μM SFN for 72 hours for FC analysis, 48h for cytokine detection and 24 h for RT PCR. SFN stock solution was originally dissolved in DMSO and an equal volume of DMSO (final concentration < 0.1%) was added to the control cells. For DMOG treatment, the same conditions were used with 1mM DMOG for 24h.

**Immunocytochemical staining.** Cells were harvested by trypsinization, adjusted to 10⁶ cells/ml and washed in PBS. For PBR staining, cells were fixed in 0.5% paraformaldehyde in PBS for 5 min. After permeabilization with saponin solution (0.2% saponin in PBS containing 0.1% BSA), cells were labeled for 30 min at room temperature with 1 mg/ml of the anti-PBR 8D7 antibody. Thereafter, cells were washed twice in PBS/0.1%BSA and resuspended in FITC-conjugated goat F(ab')2 antibody to mouse IgG (Beckman Coulter) diluted 100-fold in PBS/0.1%BSA. Control samples were prepared without primary monoclonal antibody 8D7.

For CA IX staining, mouse monoclonal anti-human CA IX antibody M75 (50 µl of hybridoma medium) was added to tube containing a 50 µl aliquot of cells. Isotype-matched non-reactive anti-CD45 (Bra 55) monoclonal antibody was used as a negative control. Cells were incubated at room temperature for 30 minutes. FITC-conjugated goat anti-mouse F(ab')2 antibody was added after washing with PBS/0.1% BSA solution for another 30-minute incubation. After final washing, 5ul of PI (1mg/ml) was added to determine cell viability.

Vimentin staining was performed on ethanol-fixed cells using 5ul of PE-labeled monoclonal antibody against human vimentin (Clone VI-RE/1). PE-labeled non-reactive anti-CD45 monoclonal antibody was used as a negative control.
Finally, the cells were analyzed using a Coulter Epics Altra flow cytometer and data were collected by EXPO32 Multi-COMP Software.

**Flow cytometry measurements and data analysis.** Coulter Epics Altra flow cytometer was equipped with 488 nm excitation laser and fluorescence emission was measured using band pass (525, 575 nm) filters with FL1, FL2 (respective) photomultipliers required for utilized fluorochromes: FITC (FL1), PI, PE (FL2). Forward/side light scatter characteristic and/or PI positivity were used to exclude the cell debris and dead cells from the analysis. For each analysis, at least $1 \times 10^4$ cells were collected in a list mode. Data were analyzed with WinMDI version 2.8 software (J. Trotter, Scripps Research Institute, La Jolla, CA) and FCS Express version 3.0 (De Novo Software, Ontario, Canada). The relative fluorescence intensity (RFI) was expressed as multiple of fluorescence intensity of control samples.

**RNA isolation and RT PCR.** Total RNA of cells was extracted using Nucleo Spin RNA II Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer’s instructions. Precipitated RNA was dissolved in DEPC-treated water and reverse transcribed with the Multi Scribe Reverse Transcriptase (Applied Biosystems, USA) using random hexanucleotides as primers. PCR reactions were performed using the primers specified below. Semiquantitative PCR was carried out with Go Taq® Flexi DNA Polymerase (Promega Corporation, USA). Following an initial denaturation at 94°C for 3 min, the amplification program was as follows: denaturation at 94°C for 30 s, annealing at 58°C - 63°C for 30 s, and extension at 72°C during 40 s for a total of 32 cycles, and finally 7 min at 72°C. The details of primers, annealing temperature, amplification cycles, and PCR product size for each gene are listed in Table. The PCR products were electrophoresed on 1.6 % agarose gel, stained with ethidium bromide, and visualized with an ultraviolet (UV) transilluminator. Band intensities in RT PCR were quantified using Image J analysis software.

**Western blot analysis.** Cells were sonicated by the two low amplitude cycles (10 seconds) in a buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA and 1 mM EGTA, 2 mM sodium orthovanadate, 2 mM sodium fluoride and a mixture of protease inhibitors (1 mM PMSF, 1 mM pefabloc, 1 μM leupeptin, 1 μM pepstatin and 0.3 μM aprotinin, Calbiochem). After two cycles of short-time sonication, cell lysates were equilibrated with NP-40 and SDS, to final concentrations of detergents 1 % and 0.02 %, respectively. The cell lysates were then collected by microcentrifugation at 10 000 x g, at 2 °C for 10 min and used for SDS-PAGE. Equal amounts of lysate protein (75 μg/lane) were run in 12.5% SDS/PAGE gels and transferred to nitrocellulose membrane. Blots were developed

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**Table 1: Primer sequences and PCR amplification conditions**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers (5’→3’)</th>
<th>Annealing t. (°C)</th>
<th>cycles</th>
<th>product size (bp)</th>
</tr>
</thead>
</table>
| MMP1   | S: cagagatgaatgcgggttttc  
A: caggaaacaaccttcttgga  | 58 | 32 | 460 |
| MMP3   | S: gttggttaaggagaaagacagtgg  
A: catgacagcacaagagaatataa  | 58 | 32 | 506 |
| MMP7   | S: gaggcatgctgagcatactag  
A: cgttccactgtaaatgagggta  | 58 | 32 | 307 |
| MMP9   | S: atcttcaagggcatacttact  
A: ccagaaagactaaggtaaagag  | 58 | 32 | 187 |
| MMP14  | S: gcaaatctgtctttctaaag  
A: tgggtcttgagatcctgtaac  | 58 | 32 | 228 |
| Bactin | S: ccaaacgccagaagatgacc  
A: gatctctagaggtagctagat  | 60 | 32 | 236 |
| VEGFA  | S: cttgtcgtcactctttcact  
A: agctccggggagagacaagac  | 63 | 32 | 196 |
| CA9    | S: gctgctagctctcctttcact  
A: cccagggcagagcagcctgta  | 60 | 32 | 252 |
| HIF-1a | S: ccggtttgtggtgttgtgaa  
A: gcatcctctacgtctctggtg  | 60 | 32 | 267 |
| POUSF1 | S: ggctcagggcagaacttgagag  
A: gccagagggagacactgtg  | 63 | 32 | 226 |
| Twist1 | S: cactcctacacacctgcatt  
A: actatgtggttttgcaggccagctg  | 58 | 32 | 255 |
| GLUT1  | S: cctcttctcccagagcagcaatg  
A: ccagacagagcgggtgctgtg  | 60 | 32 | 355 |

Abbreviations: S, sense primer; A, antisense primer
with HIF1α and β-actin rabbit polyclonal antibody (Santa Cruz Biotechnology), M75 mouse monoclonal antibody [23] and secondary anti-rabbit resp. anti-mouse IgG-HRP antibodies and visualized by chemiluminescence reagent (ECL). β-actin was used to control equal gel loading.

**Multiplex microbead-based cytokine immunoassay.** Cell supernatants were collected, filtered through sterile 0.22 µm pore size filter (Millipore) and aliquots stored at −80°C until analyzed. Cytokine 27-Plex panel including interleukin (IL)-1β, IL-1 receptor antagonist (IL-1ra), IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12(p70), IL-13, IL-15, IL-17, Eotaxin, fibroblast growth factor (FGF) basic, granulocyte-colony stimulating factor (G-CSF), granulocyte-macrophage colony stimulating factor (GM-CSF), interferon (IFN)γ, interferon-gamma induced protein (IP-10), monocyte chemo-tactic protein (MCP)-1, macrophage inflammatory protein (MIP)-1α, MIP-1β, platelet-derived growth factor (PDGF)-bb, regulated upon activation normal T-cell expressed and secreted (RANTES), tumor necrosis factor (TNF)-α, and vascular endothelial growth factor (VEGF) was analyzed with the Bio-Plex Suspension Array System (Bio-Rad Laboratories).

The multiplex microbead-based cytokine immunoassay was performed according to the manufacturer’s instruction manual in 96-well filter microplates. Briefly, appropriate cytokine standards and samples (50 µl) were pipetted to microplate wells and incubated with the fluorescent dyed microspheres coupled to specific monoclonal antibody (50 µl) for 30 min on a plate shaker (300 rpm) in the dark. After a wash step, the beads were further incubated with the biotinylated detection antibody cocktail (25 µl/well) on a plate shaker for 30 min. After another wash step, streptavidin-PE (50 µl/well) was added and samples incubated for 10 min. Finally, 125 µl of assay buffer was added to each well and the plate was shaken before reading on Bio-Plex system. The cytokine concentrations were calculated using software provided by the manufacturer (Bio-Plex Manager Software).

**Results**

SFN downregulated PBR expression in a dose dependent manner as seen on Fig.1A. Similarly, vimentin expression was reduced 72h after SFN treatment (Fig.1B) in the same man-
We observed slight increase of vimentin expression at both used concentration of SFN after 72 h incubation, which was not statistically significant (data not shown). Because of this observation we were interested whether corresponding changes at mRNA levels occurred. The Fig. 3 shows a faint increase of CA9 mRNA in SFN-treated cells, while no changes in HIF-1α mRNA and its downstream regulated genes were observed.

Based on our previous experiments [16] where we described higher than marginal expression of CA IX in MDA-MB-231 sparse cultures which was only weakly inducible in dense
cultures, we hypothesized possible disturbances in the hypoxic pathway regulation in MDA-MB-231 cells. Therefore we exposed these cells to disruption of O₂ sensing induced by HIF-1α pathway modulator dimethyloxalylglycine (DMOG) and examined its effect on HIF-1α, VEGFA, GLUT1 and CA9 mRNA at 24h. We found no observable changes in HIF-1α, VEGFA and GLUT1 and only ~2-fold increase of CA9 mRNA (Fig. 3). As HIF-1α mRNA is constitutively expressed in various tissues and HIF-1α protein is hardly detected under normoxia but stabilized by hypoxia, we focused our interest on HIF-1α and CA IX protein expression (investigated by western blotting) under hypoxic conditions induced by DMOG. Western blot analysis showed no significant alterations in these protein levels at 24h (Fig.4).

Because of the multiplicity of oncogenic pathways, some tumors may behave extremely aggressively. This aggressivity is dependent on tumor microenvironment, feasibility of neangiogenesis, degradation of extracellular matrix, disruption of epithelial integrity and release of cells from intercellular interactions, and acquisition of EMT. Therefore, we measured the mRNA levels of matrix metalloproteinases and transcription factors as the markers of proteolytic activity and EMT in SFN-treated MDA-MB-231, respectively. SFN downmodulated MMP14 and induced a clear decrease of MMP7, while no apparent effect on MMP1, MMP3 and MMP9 was observed. SFN decreased expression of Twist1, transcription factor that mediate EMT and transcriptional suppressor of E-cadherin and downregulated the pluripotent stem cell marker POU5F1 (Oct3/4) (Fig. 3).

Using multiplex bead array assay, we followed the production of following cytokines/chemokines after 48h SFN treatment: IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, IL-15, IL-17, Eotaxin, FGF basic, G-CSF, GM-CSF, IFN-γ, IP-10, MCP-1, MIP-1α, MIP-1β, PDGF, RANTES, TNF-α and VEGF. Fig. 5 shows reduced production of pro-inflammatory cytokines IL-1β, IL-6, TNF-α, IFN-γ, immunomodulating cytokine IL-4 and growth factors involved in angiogenesis PDGF and VEGF. Most of them were diminished by SFN in a dose dependent manner, however, some of them (IL-1b, IL-4) were reduced equally by both concentration used. Modulation of the other measured cytokines and chemokines showed no plausible concentration dependency on SFN.

**Discussion**

Better understanding of mechanisms that guide tumor growth and invasion (including EMT, epithelial-stromal cell interactions, neoangiogenesis, and degradation of ECM) and quest for agents that could inhibit these processes are important in combating dissemination in cancer patients. In our study, we explore effects of sub-toxic doses of SFN on various features of MDA-MB-231 breast cancer cells influencing their aggressiveness such as EMT, cytokine production, neoangiogenesis and degradation of ECM.

SFN downregulated PBR and vimentin expression in a dose dependent manner as measured by flow cytometry.
PBR, a mitochondrial protein involved in cell proliferation and differentiation is associated with tumor progression and aggressiveness and its high expression level was significantly correlated with a shorter disease-free survival in the lymph node-negative breast cancer patients [22]. Vimentin induction has been associated with enhanced metastasis and unfavorable clinical outcome in breast cancer and is characteristic for EMT phenotype [24]. Their downregulation induced by SFN indicates possible role of SFN in attenuation of EMT and tumor progression.

Because of small increase of CA IX in SFN-treated MDA-MB-231 cells obtained by flow cytometry analysis we wondered whether corresponding changes at mRNA levels occurred. We found a faint increase of CA9 mRNA in SFN-treated cells, but no changes of HIF-1α and its downstream regulated genes were observed. As CA9 is a hypoxia-regulated gene and marker of sustained hypoxia, we have used DMOG for evaluation of such induction. Although PCR analysis confirmed a higher increase of CA9 mRNA than SFN-induced upregulation, no effect of chemically induced hypoxia on other hypoxia-regulated genes was observed. Moreover, HIF-1α protein stabilization after 24h DMOG treatment was not detected, although, as reported by others, HIF-1α protein expression was found in response to chemically induced hypoxia with CoCl₂ [25]. Western blot analysis did not show significant changes in CA IX protein either. Thus the results presented support our previous hypothesis [16] that hypoxic pathway regulation in our MDA-MB-231 cells might be disturbed.

Further we studied mRNA levels of matrix metalloproteinases and transcription factors involved in EMT as markers of proteolytic activity and EMT in SFN-treated MDA-MB-231 cells. We found downmodulation of MMP14 and marked decrease of MMP7, while the levels of the other studied MMPs remained unchanged.

MMPs play an important role in breast cancer by modulating tumor-stromal interactions in a manner that favors tumor establishment and progression [26]. MMP7 over-expression correlates with breast cancer invasiveness in vitro [27]. MMP14 immunostaining was detected in 50% of brain metastasis from breast cancer, suggesting that ECM remodeling may play an important role in brain metastasis [28]. SFN may suppress breast cancer invasiveness by its downmodulation of MMP14 and MMP7.

Twist1, a prometastatic transcription factor, is overexpressed in many epithelial cancers including breast, although its regulation is not fully understood in these cancers [10]. We observed its strong downregulation by SFN, supporting an idea that SFN can attenuate EMT.

The Oct3/4 gene, a POU family transcription factor, has been noted as being specifically expressed in embryonic stem cells and in tumor cells but not in cells of differentiated tissues [29] as confirmed in human breast tissue [30]. Now we described its downregulation by SFN.

Cytokines play an important role in the communication between cells of multicellular organisms and regulate their survival, growth, differentiation and effector functions. Inflammatory mediators play a fundamental role in estrogen-dependent breast cancer growth and progression [31] as well as in tumor-mediated osteolysis [32]. In the present study SFN reduced production of several pro-inflammatory cytokines IL-1β, IL-6, TNF-α, IFN-γ, immunomodulating cytokine IL-4 and growth factors involved in angiogenesis PDGF and VEGF. Among them, bone-resorbing IL-6 plays role in tumor-mediated osteolysis [32] and TNF-α or IL-1β can up-regulate prostaglandins [31]. Nuclear factor kappa B is known as a molecular target for SFN-mediated anti-inflammatory mechanisms [33], its inhibition correlated with the suppression of NF-κB-regulated genes involved in metastasis (VEGF and MMP9) in leukemia cells [34]. Although we did not detect MMP9 mRNA changes in our experiments, we observed downregulated release of VEGF and PDGF. Down-regulation of PDGF can lead to the down-regulation of Notch-1 and inactivation of NF-κB and its target genes (VEGF) as it was already described in pancreatic cancer cell lines [35].

In oral squamous cell carcinomas, SFN-induced inhibition of COX-2 but not COX-1 was described [36]. The role of COX-2 in epithelial-stromal cell interactions and progression of ductal carcinoma in situ (DCIS) of the breast has been shown when up-regulation of COX-2 in DCIS xenografts resulted in increased VEGF and MMP14 expression [37]. We suppose that beside suppression of NF-κB, also inhibition of COX-2 by SFN might explain our observations of reduced production of VEGF and MMP14.

ITCs, naturally occurring as glucosinolate precursors in Brassica vegetables, possess chemopreventive properties and SFN, an antioxidant and a potent stimulator of natural detoxifying enzymes, exhibits tumor preventive activity in lung, prostate, breast and colon cancers [38]. It is also known as HDAC inhibitor [39]. Recently, HDAC6 has been identified as a critical regulator of EMT and a potential therapeutic target against pathological EMT [40]. Our results show that HDAC inhibitor SFN can suppress breast cancer aggressivity by means of attenuation of EMT by repressing of Twist and vimentin expression, downregulation of proteases involved in invasiveness and reduced production of pro-inflammatory cytokines and growth factors involved in angiogenesis.

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