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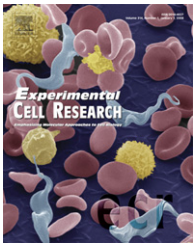

Research Article

Vascular endothelial growth factor-A stimulates Snail expression in breast tumor cells: Implications for tumor progression

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ABSTRACT

The E-cadherin transcriptional repressor Snail is a prognostic marker for metastatic breast carcinoma, as well as a critical determinant of tumor growth and recurrence. We define a non-angiogenic, autocrine function for the vascular endothelial growth factor-A (VEGF-A) in regulating Snail expression in breast tumor cells. The transfection of well-differentiated breast tumor cells with VEGF-A increases Snail mRNA and protein levels, resulting in reduced E-cadherin expression. Conversely, reducing endogenous VEGF-A expression in poorly differentiated breast tumor cells by siRNA transfection decreases Snail levels. Our studies demonstrate that VEGF and the VEGF receptor Neuropilin-1 increase Snail expression by suppressing the Glycogen Synthase Kinase-3 (GSK-3), an established inhibitor of Snail transcription and protein stability. The VEGF-A neutralizing antibody Avastin® was recently approved by the FDA for the treatment of metastatic breast cancer. We present the provocative finding that beyond its anti-angiogenic activity, Avastin® can reduce Snail expression in breast tumor cells. Collectively, this work describes a novel autocrine function for VEGF in breast tumor cells in driving the expression of Snail, a breast tumor progression factor. Based on our demonstration that Avastin® reduces Snail expression in breast tumor cells, we propose that the treatment of early stage breast cancer patients with Avastin® may impede tumor progression.

Introduction

The transition of a "normal" epithelial cell to a metastatic tumor cell requires that this cell evolve the ability to survive in foreign environments, to migrate and to invade tissue. The E-cadherin transcriptional repressor Snail is unique in its ability to impart on a cell a number of these activities associated with metastatic tumor progression. Strikingly, Snail is a prognostic marker for metastatic breast carcinoma, being expressed in node-positive, but not in low grade, node-negative specimens [1]. Early studies of Snail focused on its ability to induce an epithelial–mesenchymal transition (EMT) by acting as an E-cadherin transcriptional repressor [2,3]. However, recent studies indicate that Snail also maintains cell survival and promotes cell migration [4]. Furthermore, in mouse models of cancer, Snail is critical for tumor growth and recurrence [5,6].

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Based on its established importance for breast tumor progression, studies identifying the factors that regulate Snail expression in metastatic breast carcinoma are of utmost importance.

We and others established previously that breast tumor cells express the VEGF receptor Neuropilin-1 (NP-1) [7–9], and that an autocrine VEGF/NP-1 pathway stimulates constitutive PI3-kinase activity in breast tumor cells [7]. Similar to the expression pattern of Snail in breast tumors [1], VEGF-A expression is significantly increased in metastatic compared to non-metastatic breast tumors [10]. Furthermore, recombinant VEGF can stimulate the expression of Snail family members in pancreatic tumor cells [11]. Based on these findings, in the current work, we address the hypothesis that autocrine VEGF-A signaling in breast tumor cells stimulates Snail expression and activity. We also investigate a novel activity for a VEGF-A-directed therapeutic in suppressing tumor cell expression of Snail.

Materials and methods

Cell lines

T47D and MDA-MB-435 cells were obtained from Duke University Comprehensive Cancer Center's cell culture facility, and were cultured in RPMI/5% FBS and DMEM/5% FBS, respectively. SUM159 cells were kindly provided by Dr. Stephen Ethier, and cultured in DMEM/F12(1:1)/5% FBS. MCF-7 cells were obtained from Dr. Joseph Geradts (Duke University Medical Center), and were cultured in DMEM/5% FBS.

Antibodies

The following antibodies were used in these studies: Neuropilin-1-neutralizing antibody (R&D Systems-MAB566), mouse IgG_{2B}, (R&D Systems), Avastin® (Genentech) (kindly provided by Dr. Andrew Nixon, Duke University Medical Center), human $IgG1_{\kappa}$ (Sigma), mouse anti-E-cadherin (R&D Systems MAB1838), mouse anti-Snail [12], mouse anti-β-actin (Sigma), rabbit anti-phospho-GSK-3β (Ser9) (Cell Signaling), mouse anti-GSK-3 (Upstate), IRDye™700 Donkey anti-Mouse IgG and IRDye™800 Goat anti-Rabbit IgG (Rockland).

RNA and protein extraction

Total RNA was extracted from the indicated cells using the RNeasy kit® (Qiagen) according to the manufacturer's protocol. Proteins were extracted from cells using RIPA lysis buffer [150mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 50mM Tris (pH 8.0), 2mM phenylmethylsulfonyl fluoride, 5μg/ml aprotinin, 5μg/ml pepstatin, 50μg/ml leupeptin, and 1mM sodium orthovanadate].

Immunoblotting

Equivalent amounts of total cellular protein extracted from the cells of interest were exposed to SDS-PAGE, transferred to nitrocellulose and probed with the indicated antibodies, followed by an IRDye700 (Rockland Inc.)-conjugated secondary antibody of the appropriate species. Protein bands were visualized using Odyssey Infrared Imaging System (LI-COR Biosciences). Images were cropped and optimized for clarity using Adobe Photoshop.

pCDNA3.1-VEGF-A transfections

Cells at 70% confluence were transfected transiently with either a control vector (PCDNA3.1®, Invitrogen) or VEGF-A_{165}-expressing PCDNA3.1 vector (kindly provided by Dr. Donald Senger, Beth Israel Deaconess Medical Center) [13] using Lipofectamine2000® (Invitrogen).

Incubation of tumor cells with recombinant human $VEGF_{165}$

Cells at 70% confluence were cultured in reduced serum medium (0.5% FBS) containing 0.5ng/ml recombinant human $VEGF_{165}$ (R&D Systems).

VEGF-A siRNA transfections

Cells at 70% confluence were transfected using Lipofectamine2000® with either 100nM control siRNA (Santa Cruz) or VEGF-A siRNA (Dharmacon-{NN-N19}-NNACGCGUAACGCGGGAAUUU).

VEGF-A ELISA

VEGF-A protein levels were measured in cell lysates using a human VEGF-A ELISA kit (R&D Systems) according to the manufacturer's protocol. All samples were analyzed in triplicate.

Incubation of breast tumor cells with VEGF-neutralizing antibody

Cells were incubated for 4h with 5μg/ml Avastin® or an isotype control antibody (human $IgG1_{\kappa}$, Sigma) in reduced serum conditions (0.5% FBS). Avastin® (Genentech) was kindly provided by Dr. Andrew Nixon (Duke University Medical Center).

Quantifying mRNA

Reverse-transcription PCR (RT-PCR) for Snail, VEGF and β-Actin was performed using Superscript™ One-Step RT-PCR with Platinum®Taq (Invitrogen) with 0.6μM primers and either 0.5μg (for Snail amplification) or 0.1μg (for VEGF and β-Actin amplification) template RNA. Reverse transcription was performed at 55°C for 30min, followed by 95°C for 15min. The following PCR conditions (35 cycles) were then implemented: 1min at 94°C, 1min at 56°C, 1min at 72°C. A final extension at 72°C for 10min was performed. Primers were obtained from Invitrogen.

Snail-forward 5′-GGGCAGGTATGGAGAGGAAGA-3′.
Snail-reverse 5′-TTCTTCTGCGCTACTGCTGCG-3′.
VEGF-A-forward 5′-CGAAGTGGTGAAGTTCATGGATG-3′.
VEGF-A-reverse 5′-TTCTGTATCAGTCTTTCCTGGT-3′.
β-Actin-forward, 5′-AAATCTGGCACCACACCTTC-3′.
β-Actin-reverse, 5′-GGGGTGTTGAAAGGTCTCAAA-3′.