Hypoxia-inducible factor 1-dependent expression of platelet-derived growth factor B promotes lymphatic metastasis of hypoxic breast cancer cells

Luana Schito\textsuperscript{a,b,c}, Sergio Rey\textsuperscript{a,b}, Marco Tafani\textsuperscript{c}, Huafeng Zhang\textsuperscript{a,b}, Carmen Chak-Lui Wong\textsuperscript{a,b}, Andrea Russo\textsuperscript{d}, Matteo A. Russo\textsuperscript{e,f,g,h,i,j,1} and Gregg L. Semenza\textsuperscript{a,b,f,h,i,j,1}

\textsuperscript{a}Vascular Program, Institute for Cell Engineering, \textsuperscript{b}McKusick-Nathans Institute of Genetic Medicine, and Departments of \textsuperscript{c}Pediatrics, \textsuperscript{d}Medicine, \textsuperscript{e}Oncology, \textsuperscript{f}Radiation Oncology, and \textsuperscript{g}Biological Chemistry, Johns Hopkins University School of Medicine, Baltimore, MD 21205; \textsuperscript{1}Department of Experimental Medicine, Sapienza University of Rome, 00161 Rome, Italy; \textsuperscript{1}Department of Surgical Pathology, Istituto di Ricovero e Cura a Carattere Scientiﬁco Regina Elena, Istituti Fisioterapici Ospitalieri, 00161 Rome, Italy; and \textsuperscript{1}Department of Cellular and Molecular Pathology, Istituto di Ricovero e Cura a Carattere Scientiﬁco San Raffaele Pisana, 00163 Rome, Italy

**AUTHOR SUMMARY**

Cancer cells can grow and invade surrounding tissue and then potentially spread to other organs and cause death. In breast cancer, this spread involves the protein hypoxia-inducible factor (HIF)-1. Breast cancer patients who have increased HIF-1α levels in their tumor biopsies have signiﬁcantly reduced survival (1, 2). Although studies have suggested correlations between HIF activation and lymphatic vessel growth (lymphangiogenesis) and lymphatic metastasis (cancer spread to the lymph nodes), whether HIFs regulate these processes directly or whether HIF inhibition might be clinically useful is not known. In this study, we investigated the role of HIFs in the lymphatic metastasis of breast cancer cells and found that HIFs play a critical role in lymphangiogenesis and lymphatic metastasis that is caused, at least in part, by increased transcription of the gene encoding platelet-derived growth factor B (PDGF-B).

In breast cancer, early spread beyond the primary tumor occurs through invasion of lymphatic vessels and passage to regional lymph nodes. From there, cancer cells can access the blood circulatory system and spread to distant organs, ultimately resulting in death (3). Because of unregulated cell growth, most breast cancers contain regions of reduced oxygen availability, or hypoxia, leading to the activation of HIFs, which are transcription factors that mediate changes in gene expression that increase O\textsubscript{2} delivery and decrease O\textsubscript{2} consumption (4). HIFs are composed of two subunits: an HIF-1β subunit that is expressed throughout the body and an O\textsubscript{2}-regulated HIF-1α or HIF-2α subunit (4).

To investigate the role of HIFs in lymphatic metastasis, we used a transplantation model in which human breast cancer cells were injected into the mammary fat pads of mice that were immunodeﬁcient and therefore unable to reject the human cells. We engineered decreased expression of HIF-1α, HIF-2α, or both HIF-1α and HIF-2α in the breast cancer cells by inserting expression vectors encoding shRNA, which are pieces of genetic material that can be used to silence (i.e., turn off) speciﬁc genes. This HIF silencing resulted in decreased lymphatic vessel density in the area of the tumor and decreased metastasis (i.e., spread) of breast cancer cells to the regional (axillary) lymph node.

We then investigated the speciﬁc signaling pathways affected by HIFs. Analysis of a panel of genes encoding proteins implicated in lymphangiogenesis (3) revealed that hypoxia induced the expression of PDGF-B mRNA and protein in an HIF-dependent manner in both breast cancer cell lines used in the experiment. Hypoxia-induced binding of HIF-1 to the human PDGFB gene was demonstrated, and a speciﬁc genetic sequence encompassing the HIF-1-binding site functioned as a hypoxia-responsive element, demonstrating that PDGFB is an HIF-1 target gene (Fig. P1). Medium from hypoxic breast cancer cells was shown to stimulate the proliferation and migration of cultured human lymphatic endothelial cells. These effects were dependent on HIF and PDGF-B.

**Fig. P1.** The HIF-1 protein activates transcription of the PDGFB gene, enhancing breast cancer lymphangiogenesis and lymphatic metastasis. (A) Under conditions of reduced oxygen availability, the HIF-1 protein is active and binds to a hypoxia-response element located within the human PDGFB gene. (B) (Left) HIF-1–PDGF-B signaling induces growth of lymphatic vessels (lymphangiogenesis) in breast tumors, allowing cancer cells (green) to invade the lymphatic vessels (red). (Right) Cancer cells travel through the lymphatic system to reach the lymph nodes (lymphatic metastasis) where they access blood vessels through which they spread throughout the body. (C) To demonstrate the clinical relevance of data obtained from cell culture and mouse models, the expression of HIF-1α and PDGF-B in breast cancer biopsy specimens was analyzed. In advanced breast cancers [grade 2 (G2) and grade 3 (G3)], a striking correlation between HIF-1α and PDGF-B expression was observed.


The authors declare no conﬂict of interest.

This is a Contributed submission.

\textsuperscript{1}To whom correspondence should be addressed. E-mail: gsemenza@jhmi.edu.

See full research article on page E2707 of www.pnas.org.

Cite this Author Summary as: PNAS 10.1073/pnas.1214019109.
expression in the breast cancer cells. Inhibition of PDGF-B expression in breast cancer cells by shRNA decreased lymphatic vessel density and lymph node metastasis. We also found that treatment of tumor-bearing mice with digoxin or imatinib, which are Food and Drug Administration-approved drugs that inhibit HIF and PDGF receptor activity, respectively, significantly decreased lymphatic vessel density and lymph node metastasis. Furthermore, analysis of human breast cancer biopsy specimens revealed a significant association between HIF-1α and PDGF-B expression and lymphatic vessel area in advanced tumors. Knowledge of HIF-1α and PDGF-B expression and lymphatic vessel area was sufficient to predict the histological grade of each breast cancer, which, together with regional lymph node status, is the major determinant of clinical outcome.

Both of the breast cancer cell lines that were analyzed in this study were derived from triple-negative breast cancers, which are characterized by the lack of expression of the estrogen receptor (ER), progesterone receptor, and HER2 receptor. Although specific therapies that target ER^+ and HER2^+ breast cancers are available, no targeted therapies have been developed for triple-negative breast cancers, which have a high recurrence rate when treated with traditional chemotherapy (5). The results of the present study and other recent findings (6, 7) indicate that inhibition of HIF activity in tumor-bearing mice by treatment with digoxin blocks primary tumor growth, metastasis via blood vessels to the lungs, and metastasis via lymphatic vessels to regional lymph nodes. Taken together, these results suggest that clinical trials of digoxin or other HIF inhibitors (4) are warranted in women with triple-negative breast cancer.