Deciphering Pro-Lymphangiogenic Programs during Mammary Involution and Postpartum Breast Cancer

Virginia F. Borges1,2, Alan M. Elder1,2 and Traci R. Lyons1,2*

1 Young Women’s Breast Cancer Translational Program, University of Colorado Cancer Center, Aurora, CO, USA,
2 Department of Medicine, Division of Medical Oncology, University of Colorado Anschutz Medical Campus, Aurora, CO, USA

Postpartum breast cancers are a highly metastatic subset of young women’s breast cancers defined as breast cancers diagnosed in the postpartum period or within 5 years of last child birth. Women diagnosed with postpartum breast cancer are nearly twice as likely to develop metastasis and to die from breast cancer when compared with nulliparous women. Additionally, epidemiological studies utilizing multiple cohorts also suggest that nearly half of all breast cancers in women aged <45 qualify as postpartum cases. Understanding the biology that underlies this increased risk for metastasis and death may lead to identification of targeted interventions that will benefit the large number of young women with breast cancer who fall into this subset. Preclinical mouse models of postpartum breast cancer have revealed that breast tumor cells become more aggressive if they are present during the normal physiologic process of postpartum mammary gland involution in mice. As involution appears to be a period of lymphatic growth and remodeling, and human postpartum breast cancers have high peritumor lymphatic vessel density (LVD) and increased incidence of lymph node metastasis (1, 2), we propose that novel insight into is to be gained through the study of the biological mechanisms driving normal postpartum mammary lymphangiogenesis as well as in the microenvironment of postpartum tumors.

Keywords: breast cancer, lymphangiogenesis, lymphatic metastasis, macrophages, postpartum

INTRODUCTION TO POSTPARTUM BREAST CANCER

Postpartum breast cancer is an under-recognized and highly metastatic subset of young women’s breast cancer, which we define as breast cancers diagnosed within 5 years of a woman’s most recent child birth (3, 4). The distinction of postpartum cases from the various interactions of breast cancer and pregnancy, or pregnancy-associated breast cancer (PABC), arose from epidemiologic studies indicating that women diagnosed with breast cancer in the postpartum years are nearly three times as likely to develop metastasis and to die from breast cancer in comparison with nulliparous women (3–6). Importantly, this research highlighted the need to clearly separate breast cancer cases as nulliparous, pregnant, or postpartum, as opposed to defining PABC as cancers diagnosed both during and in the 1–2 years after parturition as one entity, to avoid diluting the risk signal (5). Epidemiological studies utilizing multiple cohorts also identify that ~45% of all breast cancers in Caucasian women
Lymphangiogenesis and Postpartum Breast Cancer

INTRODUCTION TO THE LYMPHATIC SYSTEM AND LYMPHANGIOGENESIS

Lymphangiogenesis is the outgrowth of new lymphatic vessels, which is required for development of the immune system, fluid homeostasis, trafficking of lymphatic cells, normal wound healing, and tissue regeneration (42–47). Differential expression of lymphatic markers, which distinguish lymphatic vessels from blood vessels, has been described in detail over the past decade and has aided the field by allowing researchers to distinguish between newly formed neo- and mature lymphatics (43, 48–57). The adult lymphatic system consists of initial lymphatics, also known as lymphatic capillaries, which drain lymph fluid into pre-collecting lymphatics, followed by drainage into collecting lymphatics that then lead to the lymph node where foreign bodies can be trapped, immune reactions occur, and lymph fluid is concentrated (42, 44). While the lymphatic endothelial marker Lyve-1 is absent in the collecting lymphatics, it is highly expressed for the initial lymphatics or lymphatic capillaries (58). Furthermore, both the lymphatic capillaries and the collecting vessels exhibit high expression levels of Prox-1, VEGFR-3, and podoplanin (48–55, 59). These results suggest that Lyve-1 may be a marker that can be used to specifically measure new lymphatic formation or neo-lymphangiogenesis.

Neo-lymphangiogenesis occurs in adult tissues as an active normal response to infection, inflammation, and wound healing. Neo-lymphangiogenesis can be stimulated by the local production of the vascular endothelial growth factors VEGF-C, and -D within the damaged tissue and subsequent binding to VEGFR-2 and VEGFR-3 on nearby lymphatic endothelial cells (LEC), resulting in the expansion of lymphatics via sprouting from pre-existing lymphatic vasculature (50, 51, 59–69). Primary sources of VEGF-A, -C, and -D include fibroblasts, inflammatory cells, and macrophages (70–74). An alternative theory has emerged whereby bone marrow-derived cells, specifically macrophages, may also be recruited to contribute to lymphangiogenesis (75–77). In support of this theory, bone marrow transplanted from GFP+ mice into GFP− recipients revealed GFP+ cells localized and/or incorporated into new lymphatics during inflammation, and additional lineage tracing experiments support these findings (75, 76, 78). Furthermore, tissue-resident and bone marrow-derived macrophages express lymphatic markers, such as Lyve-1, Prox-1, and podoplanin (78–80), and the presence of macrophages at sites of neo-lymphangiogenesis during inflammation has been reported (78, 80). Thus, macrophages appear to be involved in neo-lymphangiogenesis. As macrophages are also an important part of the normal program of involution (19), we believe there is a role for macrophages in facilitating the neo-lymphangiogenesis seen during postpartum mammary involution.

PRO-LYMPHATIC PROGRAMS OBSERVED DURING POSTPARTUM MAMMARY INVOLUTION

Postpartum mammary involution has been extensively characterized using rodent mammary glands with more recent preliminary confirmation in human tissues (11, 12, 17, 19, 20, 26, 32, 35, 36, 40, 81–89). Postpartum mammary gland involution occurs in two distinct phases. During the first phase, which is reversible and lasts for 48 h (days 1–2 post weaning), apoptosis of the epithelium occurs and repopulation of the gland with adipocytes is observed. During the second phase (days 3–14), a remodeling program is initiated which results in additional cell death, increased expression of matrix remodeling proteases, degradation and remodeling of extracellular matrix components, and re-differentiation of adipocytes. Recently, we observed that neo-lymphangiogenesis occurs during postpartum mammary gland involution; however, the functional significance of these increased lymphatics has yet to be described (38, 41). Prior to our studies only a few reports had focused on mammary lymphatics, which are described below.

Expression of the VEGF family members has been characterized during the pregnancy/lactation/involution cycle, with a goal of understanding regulation of angiogenesis in the mammary gland. In these studies, VEGF-A expression was observed as increased during pregnancy and lactation where it likely drives angiogenesis and vascular permeability, which are important
for milk production. In contrast, pro-lymphangiogenic VEGFC expression levels were overall lower over the course of pregnancy and lactation, remained extremely low during the first phase of involution (days 1 and 2), and then increased nearly twofold in the second phase (days 3 and 7); this provides evidence that pro-lymphangiogenic programs may be activated during postpartum mammary involution (90). Consistent with these findings, we have observed upregulation of pro-lymphangiogenic VEGF-C and VEGF-D mRNA expression, along with their receptors, VEGFR2/3, during postpartum involution in rat mammary tissues (38) (Figure 1A). Additional studies have utilized elegant high-resolution imaging of sectioned and/or whole mounted mouse mammary glands to better understand lymphatic development during the pregnancy/lactation/involution cycle of the mammary gland. These studies revealed that VEGF-C and -D are produced locally by the mammary epithelium and myo-epithelium and that Prox-1-positive lymphatic vessels were intimately associated with the mammary epithelium and the blood vasculature.

These studies, by Betterman et al., also examined Prox-1-positive lymph vessels per area to determine density. Interestingly, they observed peak LVD during pregnancy, which decreased during lactation and involution (91). However, their analysis included only a single timepoint during involution, involution day 10, which is near the end of the involution process in mice. In contrast, while our results also reveal that the number of Lyve-1 positive vessels per area in rodent mammary glands similarly drops from pregnancy to lactation, we observe that this drop is accompanied by a subsequent rise in LVD during the early phases involution, which peaks at involution day 6 in mouse and at day 10 in rat mammary tissue (38, 41) (Figure 1B). These results suggest that neo-lymphangiogenesis occurs during the active phase of mammary remodeling in rodents. Importantly, we also analyzed podoplanin positive vessels in normal breast tissue from women who were biopsied within 10 years postpartum to determine whether the increase in lymph vessels is also evident and whether the increase persisted over time, as has been suggested by a gene signature that contains pro-angiogenic molecules angiopoietin and VEGF-A (9). The results from our study showed that women who were within 1 year of giving birth, and no longer lactating, had the highest LVD compared with never been pregnant (NBP) women. In addition, women between 3 and 10 years of giving birth also had elevated LVD compared with nulliparous suggesting that neo-lymphangiogenesis occurs during postpartum breast involution in women, and the resulting lymphatics may persist beyond the period of remodeling (38).

Consistent with a potential role for bone marrow-derived cells in neo-lymphangiogenesis, additional studies of postpartum mammary gland involution have revealed specific changes in immune cell populations, and regulation, during the involution process (12, 19, 20, 32, 36, 40, 81–83). Initial gene expression analyses during the pregnancy/lactation/involution cycle identified upregulation of genes important for acute inflammatory responses in the mammary epithelium during postpartum involution (12, 26). Specifically, Stat3 and NF-κB are primary mediators of involution in the mouse and are also known to be key mediators of acute-phase inflammatory response. Recently, and in support of these gene expression data, the postpartum mammary gland was shown to have a cascade of infiltrating immune cells, including T-cells, T regulatory cells, and dendritic cells during involution that mimic a wound-healing pattern (92). Furthermore, numerous studies have shown that macrophages are present during involution in mouse, rat, and human tissues and that macrophage ablation during the first phase of involution in mice blocks epithelial cell death and adipocyte repopulation. The details of these studies have been reviewed elsewhere (17, 20, 81). Importantly, we observe that macrophages and lymphatics may be similarly regulated during postpartum involution in mouse, rat, and human tissues (19, 38, 93) (Figure 2). We
have also shown that macrophages present during postpartum involution in rodent and human tissues express markers of an M2-polarized phenotype, such as mannose receptor, arginase-1, and CD11b (19, 36, 81). CD11b+ macrophages produce pro-lymphangiogenic factors VEGF-C and -D (78, 80, 94). Moreover, subpopulations of CD11b+ positive macrophages express lymphatic endothelial markers Lyve-1 and VEGFR-3 (95, 96). Together, these findings indicate that the CD11b+ “involution macrophages” may either stimulate lymphangiogenesis through release of pro-lymphangiogenic cytokines or through expression of lymphatic markers and incorporation into existing lymphatics; evidence for both has been published in models of inflammation and cancer (68, 78, 80, 96, 97).

Our analysis of lymphangiogenesis during postpartum involution also revealed that administration of a selective COX-2 inhibitor, celecoxib (CXB), during postpartum involution reduced Lyve-1-positive LVD at involution day 4 (38). These results are consistent with previous observations in tumor models (98–102). While, the mechanism by which CXB blocked lymphangiogenesis was not directly revealed by these studies, our in vitro data suggested that a product of COX-2 activity, PGE$_3$, acts directly on the lymphatic endothelium via the EP2 receptor. However, PGE$_3$ can also stimulate macrophages to an M2 phenotype (103, 104); thus, it is also possible that CXB inhibits lymphangiogenesis through macrophage-dependent mechanisms. Understanding the mechanisms underlying “involution macrophage” contribution to lymphangiogenesis during normal mammary gland development could lead to insight into macrophage-mediated lymphangiogenesis during breast cancer. We postulate that postpartum involution is a developmental window that allows for studies of mechanisms driving lymphangiogenesis.

## LYMPHATIC VASCULATURE IN BREAST CANCER METASTASIS

While expansion of the lymphatic vasculature has been linked to faster healing and greater ability to fight infection, lymphangiogenesis can also be pathologic. Pathologic lymphangiogenesis has been observed in graft-versus-host disease, in chronic inflammatory diseases (e.g., Rheumatoid arthritis and inflammatory bowel disease), and in the tumor microenvironment. Lymph node metastasis, lymphatic vessel presence at the tumor margin, and invasion of tumor cells into peritumor lymphatics are all poor prognostic factors for breast cancer patients (105). Further, increased LVD in the peritumor region correlates with increased metastasis in a number of human cancers, directly implicating new lymphatic vessel formation in tumor cell dissemination (106–108). A multitude of studies have examined mechanisms driving neo-lymphangiogenesis in the breast tumor environment, and VEGF-C, VEGF-D, macrophages, and COX-2/PGE$_2$ have emerged as key players (51, 68, 72, 73, 80, 99–101, 109–118).

VEGF-C is secreted by macrophages and other lymphatic cells to stimulate lymphangiogenesis, but can also be secreted by tumor cells for the same purpose (51, 62, 72, 73, 94, 117, 119). Macrophages have also been shown to participate directly in lymphangiogenesis via inducing lymphatic vessel sprouting and incorporating into existing tumor-associated lymphatics (69, 80). COX-2 and its product PGE$_2$ also promote lymphangiogenesis in the tumor microenvironment (38, 41, 98, 99, 101, 102, 116, 120, 121). Furthermore, VEGF-D promotes lymphatic vessel dilation through a COX-2-dependent mechanism. Dilation of pre-existing, peritumor, and intratumor lymphatics allows for the intravasation of tumor cells into the lymphatic vessels and subsequent transmigration to regional lymph nodes (109, 114, 119, 122). Together these results suggest there is a connection between COX-2-mediated lymphangiogenesis and lymphogenous tumor cell spread.

## LYMPHATIC VASCULATURE IN POSTPARTUM BREAST CANCER

Postpartum breast cancers are nearly three times as likely to metastasize when compared with breast cancers in nulliparous women (5, 123, 124). Furthermore, we have shown that postpartum breast cancers have increased peritumor LVD and increased lymph node involvement (1). It is anticipated that postpartum
breast tumors will utilize mechanisms similar to those observed during postpartum involution to induce lymphangiogenesis. The first, and most obvious mechanism, is upregulation of COX-2 in the mammary epithelium (125), which results in increased PGE2 production to increase lymphangiogenesis. Indeed, in animals treated with celecoxib (CBX) during postpartum involution, the resultant postpartum tumors exhibit lower levels of LVD compared with untreated controls. In addition, COX-2 in the tumor cell appears to be required as well since tumors with stable siRNA knockdown of COX-2 exhibit decreased tumor-associated LVD. Finally, if postpartum tumors are re-implanted in nulliparous hosts they maintain their ability to drive tumor-associated lymphangiogenesis (38). Of interest, these results suggest that involution-induced pro-lymphangiogenic programs persist in tumor cells long after the process of involution is complete. These results are supported by gene-expression studies indicating that there is an involution signature observed in breast tissue of parous women that persists for 10 years postpartum (9).

In addition to a COX-2-dependent mechanism, if “involution macrophages” promote lymphangiogenesis during normal involution then postpartum tumor-associated macrophages (TAMs) may also acquire and utilize similar mechanisms to mediate lymphangiogenesis in the postpartum tumor microenvironment. CD11b+ is expressed by “involution macrophages” and by TAMs (96). TAMs also predict poor prognosis of breast cancer (126), and an association between TAMs and LVD has been reported for pancreatic cancer (127). Furthermore, TAMs express VEGF-C and may promote metastasis via lymphangiogenesis (71–73, 126, 128). Thus, the CD11b+ macrophages present during postpartum involution may promote lymphangiogenesis in a manner similar to TAMs, and we have preclinical data indicating that CD11b+ macrophages are also increased in the tumor microenvironment of involution/postpartum tumors compared with nulliparous controls (36).

**REFERENCES**

1. Lyons TR, O’Brien J, Borges VF, Conklin MW, Keely PJ, Eliceiri KW, et al. Postpartum mammary gland involution drives progression of ductal carcinoma in situ through collagen and COX-2. *Nat Med* (2011) 17:1109–15. doi:10.1038/nm.2416
2. Neill T, Zoeller J. Matrix biology highlights. *Matrix Biol* (2014) 40:1–4. doi:10.1016/j.matbio.2014.11.002
3. Borges VF, Schedin PJ. Pregnancy-associated breast cancer: an entity needing refinement of the definition. *Cancer* (2012) 118:3226–8. doi:10.1002/cncr.26643
4. Lyons TR, Schedin PJ, Borges VF. Pregnancy and breast cancer: when they collide. *J Mammary Gland Biol Neoplasia* (2009) 14:87–98. doi:10.1007/s10911-009-9119-7
5. Callihan EB, Gao D, Jindal S, Lyons TR, Manthey E, Edgerton S, et al. Postpartum diagnosis demonstrates a high risk for metastasis and merits an expanded definition of pregnancy-associated breast cancer. *Breast Cancer Res Treat* (2013) 138:549–59. doi:10.1007/s10549-013-2437-x
6. Liu Q, Wuu J, Lambe M, Hsieh SF, Ekborn A, Hsieh CC. Transient increase in breast cancer risk after giving birth: postpartum period with the highest risk (Sweden). *Cancer Causes Control* (2002) 13:299–305. doi:10.1023/A:1015287208222
7. Borges VF, Goddard E, Partridge AH, Schedin P. Abstract P6-08-08: postpartum breast cancer demonstrates increased liver and brain metastasis with a proposed role for postpartum involution. *Cancer Res* (2015). doi:10.1158/1538-7445.SABCS14-P6-08-08
8. Fornetti J, Martinson HA, Betts CR, Lyons TR, Jindal S, Guo Q, et al. Mammary gland involution as an immunotherapeutic target for postpartum breast cancer. *J Mammary Gland Biol Neoplasia* (2014) 19:213–28. doi:10.1007/s10911-014-9322-z
9. Azztalos S, Gann PH, Hayes MK, Nnon L, Beam CA, Dai Y, et al. Gene expression patterns in the human breast after pregnancy. *Cancer Prev Res (Phila)* (2010) 3:301–11. doi:10.1158/1940-6207.CAPR-09-0069
10. Abati K, Sheppard D, Werb Z. Roles of the innate immune system in mammmary gland remodeling during involution. *J Mammary Gland Biol Neoplasia* (2007) 12:37–45. doi:10.1007/s10911-007-9036-6
11. Clarkson RW, Heeley JL, Chapman R, Allet F, Hay RT, Wyllie A, et al. NF-kappaB inhibits apoptosis in murine mammary epithelia. *J Biol Chem* (2000) 275:12737–42. doi:10.1074/jbc.275.17.12737
12. Clarkson RW, Wayland MT, Lee J, Freeman T, Watson CJ. Gene expression profiling of mammary gland development reveals putative roles for death receptors and immune mediators in post-lactational regression. *Breast Cancer Res* (2004) 6:R92–109. doi:10.1186/bcr754
13. Alexander CM, Selvarajan S, Mudgett J, Werb Z. Stromelysin-1 regulates adipogenesis during mammary gland involution. *J Cell Biol* (2001) 152:693–703. doi:10.1083/jcb.152.4.693
14. Dyson V, Andres AC, Ziemiecki A. Vascular remodelling during the normal and malignant life cycle of the mammary gland. *Microsc Res Tech* (2001) 52:182–9. doi:10.1002/1097-0029(20010115)52:2<182::AID-JEMT1004>3.0.CO;2-M
15. Green KA, Lund LR. ECM degrading proteases and tissue remodelling in the mammary gland. *Bioessays* (2005) 27:894–903. doi:10.1002/bies.20281

**POTENTIAL CLINICAL IMPLICATIONS: ANTI-LYMPHANGIOGENIC THERAPY**

While it is not clear why the lymphatics are expanded during postpartum involution, it is clear that postpartum tumors hijack the lymphatic vessels in the postpartum gland to drive increased metastasis. In addition, the observed tumor-promoted neo-lymphangiogenesis offers a targetable mechanism to reduce cancer metastasis (123, 124, 129–131). Anti-lymphangiogenic therapies, such as anti-VEGFR2/3 antibodies and small molecule inhibitors that target VEGFR2/3, have been tested in clinical trials for multiple solid tumor types and have shown some successes and low toxicities (132–134). Since our studies suggest that COX-2 specific inhibitors may serve to reduce tumor-associated lymphangiogenesis, we suggest that identifying whether COX-2 inhibitors can be combined with current therapies, and/or with anti-lymphangiogenesis therapy, to reduce lymphogenous spread and metastatic recurrence should be explored for postpartum breast cancer patients.

**AUTHOR CONTRIBUTIONS**

TL was responsible for overseeing writing and editing of the manuscript, assigning contributions from VB and AE, and for preparation of the figures. VB and AE contributed to writing and editing the manuscript.

**FUNDING**

This study was supported by NCI 1 R01 CA169175-01 and Robert F. and Patricia Young Connor Endowed Chair in Young Women’s Breast Cancer Research to VB. NCI R21CA185226-01, NIH 1KL2TR001080, and UL1 TR001082 to TL. TL was also supported by funding from the Cancer League of Colorado.
35. Kreuzaler PA, Staniszewska AD, Li W, Omidvar N, Kedjouar B, Turkson J, et al. Stat3 controls lysosomal-mediated cell death in vivo. *Nat Cell Biol* (2011) 13:303–9. doi:10.1038/ncli2171

36. Martinson HA, Jindal S, Durand-Rougey C, Borges VF, Schedin P. Wound healing-like immune program facilitates postpartum mammary gland invasion and tumor progression. *Int J Cancer* (2015) 136:1803–13. doi:10.1002/ijc.29181

37. Vaught DB, Cook RS. Clearance of dying cells accelerates malignancy. *Oncotarget* (2015) 6:24590–1. doi:10.18632/oncotarget.5670

38. Lyons TR, Borges VF, Betts CB, Guo Q, Kapoor P, Martinson HA, et al. Cyclooxygenase-2-dependent lymphangiogenesis promotes nodal metastasis of postpartum breast cancer. *J Clin Invest* (2014) 124(9):3901–12. doi:10.1172/JCI73777

39. Gupta PB, Proia D, Cingoz O, Weremowicz J, Naber SP, Weinberg RA, et al. Systemic stromal effects of estrogen promote the growth of estrogen receptor-negative cancers. *Cancer Res* (2007) 67:2602–71. doi:10.1158/0008-5472.CAN-06-3895

40. Stanford JC, Young C, Hicks D, Owens P, Williams A, Vaught DB, et al. Efferocytosis produces a prometastatic landscape during postpartum mammary gland invasion. *J Clin Invest* (2014) 124:4737–52. doi:10.1172/JCI76375

41. Swartz MA. Inflammatory lymphangiogenesis in postpartum breast tissue remodeling. *J Clin Invest* (2014) 124(9):1–15. doi:10.1172/JCI77765

42. Betterman K, Harvey N. The lymphatic vasculature: development and role in shaping immunity. *Immunol Rev* (2016) 272:78–92. doi:10.1111/imr.12413

43. Tamozuka T, Altalato K. Lymphangiogenesis: molecular mechanisms and future promise. *Cell* (2010) 140:460–76. doi:10.1016/j.cell.2010.01.045

44. Butler MG, Isogai S, Weinstein BM. Lymphatic development. *Birth Defects Res C Embryo Today* (2009) 87:222–31. doi:10.1002/bdrc.20155

45. Schulte-Merker S, Sabine A, Petrova TV. Lymphatic vascular morphogenesis in development, physiology, and disease. *J Cell Biol* (2011) 193:607–18. doi:10.1083/201102094

46. Karpanen T, Mäkinen T. Regulation of lymphangiogenesis – from cell fate determination to vessel remodeling. *Exp Cell Res* (2006) 312:575–83. doi:10.1016/j.yexcr.2005.10.034

47. Karpanen T, Wirzenius M, Mäkinen T, Veikko T, Haisma HJ, Achen MG, et al. Lymphangiogenic growth factor responsiveness is modulated by postnatal vessel maturation. *Am J Pathol* (2006) 169:708–18. doi:10.2353/apjpath.2006.051200

48. Wigle JT, Harvey N, Detmar M, Lagutina I, Grosveld G, Gunn MD, et al. An essential role for Prox1 in the induction of the lymphatic endothelial cell phenotype. *EMBO J* (2002) 21:1505–13. doi:10.1093/emboj/21.7.1505

49. Pan Y, Wang WD, Yago T. Transcriptional regulation of podoplanin expression by Prox1 in lymphatic endothelial cells. *Microvasc Res* (2014) 94:96–102. doi:10.1016/j.mvr.2014.05.006

50. Norrmén C, Tammolé T, Petrova TV, Altalato K. Biological basis of therapeutic lymphangiogenesis. *Circulation* (2011) 123:1335–51. doi:10.1161/CIRCULATIONAHA.107.704998

51. Karkkainen MJ, Haiko P, Sainio M, Partanen J, Petrova TV, et al. Lymphangiogenic growth factor responsiveness is modulated by postnatal vessel maturation. *Am J Pathol* (2006) 169:708–18. doi:10.2353/apjpath.2006.051200

52. Hinkle JT, Harvey N, Detmar M, Lagutina I, Grosveld G, Gunn MD, et al. An essential role for Prox1 in the induction of the lymphatic endothelial cell phenotype. *EMBO J* (2002) 21:1505–13. doi:10.1093/emboj/21.7.1505

53. Breiteneder-Gleffe S, Soleiman A, Kowalski H, Horvat R, Amann G, Kriehuber E, et al. Angiosarcomas express mixed endothelial phenotypes of blood and lymphatic endothelial cells. *Blood* (2004) 103:2902–12. doi:10.1182/blood-2003-05-0397

54. Petrova TV, Karkkainen MJ, Taipale J, Petrova TV, et al. Lymphangiogenic growth factor responsiveness is modulated by postnatal vessel maturation. *Am J Pathol* (2006) 169:708–18. doi:10.2353/apjpath.2006.051200

55. Wigle JT, Harvey N, Detmar M, Lagutina I, Grosveld G, Gunn MD, et al. An essential role for Prox1 in the induction of the lymphatic endothelial cell phenotype. *EMBO J* (2002) 21:1505–13. doi:10.1093/emboj/21.7.1505

56. Pan Y, Wang WD, Yago T. Transcriptional regulation of podoplanin expression by Prox1 in lymphatic endothelial cells. *Microvasc Res* (2014) 94:96–102. doi:10.1016/j.mvr.2014.05.006

57. Norrmén C, Tammolé T, Petrova TV, Altalato K. Biological basis of therapeutic lymphangiogenesis. *Circulation* (2011) 123:1335–51. doi:10.1161/CIRCULATIONAHA.107.704998

58. Karkkainen MJ, Haiko P, Sainio M, Partanen J, Taipale J, Petrova TV, et al. Lymphangiogenic growth factor responsiveness is modulated by postnatal vessel maturation. *Am J Pathol* (2006) 169:708–18. doi:10.2353/apjpath.2006.051200

59. Hinkle JT, Harvey N, Detmar M, Lagutina I, Grosveld G, Gunn MD, et al. An essential role for Prox1 in the induction of the lymphatic endothelial cell phenotype. *EMBO J* (2002) 21:1505–13. doi:10.1093/emboj/21.7.1505

60. Breiteneder-Gleffe S, Soleiman A, Kowalski H, Horvat R, Amann G, Kriehuber E, et al. Angiosarcomas express mixed endothelial phenotypes of blood and lymphatic endothelial cells. *Blood* (2004) 103:2902–12. doi:10.1182/blood-2003-05-0397

61. Petrova TV, Karkkainen MJ, Taipale J, Petrova TV, et al. Lymphangiogenic growth factor responsiveness is modulated by postnatal vessel maturation. *Am J Pathol* (2006) 169:708–18. doi:10.2353/apjpath.2006.051200

62. Hinkle JT, Harvey N, Detmar M, Lagutina I, Grosveld G, Gunn MD, et al. An essential role for Prox1 in the induction of the lymphatic endothelial cell phenotype. *EMBO J* (2002) 21:1505–13. doi:10.1093/emboj/21.7.1505

63. Pan Y, Wang WD, Yago T. Transcriptional regulation of podoplanin expression by Prox1 in lymphatic endothelial cells. *Microvasc Res* (2014) 94:96–102. doi:10.1016/j.mvr.2014.05.006

64. Norrmén C, Tammolé T, Petrova TV, Altalato K. Biological basis of therapeutic lymphangiogenesis. *Circulation* (2011) 123:1335–51. doi:10.1161/CIRCULATIONAHA.107.704998

65. Karkkainen MJ, Haiko P, Sainio M, Partanen J, Taipale J, Petrova TV, et al. Vascular endothelial growth factor C is required for sprouting of the first lymphatic vessels from embryonic veins. *Nat Immunol* (2004) 5:74–80. doi:10.1038/ni0103

66. Breiteneder-Gleffe S, Soleiman A, Kowalski H, Horvat R, Amann G, Kriehuber E, et al. Angiosarcomas express mixed endothelial phenotypes of blood and lymphatic endothelial capillaries: podoplanin as a specific marker for lymphatic endothelium. *Am J Pathol* (1999) 154:385–94. doi:10.1016/S0002-9440(10)65285-6

67. Norrmén C, Ivanov KI, Cheng J, Zangger N, Delorenzi M, Jaquet M, et al. Lymphangiogenic growth factor responsiveness is modulated by postnatal vessel maturation. *Am J Pathol* (2006) 169:708–18. doi:10.2353/apjpath.2006.051200
56. Johnson NC, Dillard ME, Baluk P, McDonald DM, Harvey NL, Frase SL, et al. Lymphatic endothelial cell identity is reversible and its maintenance requires Prox1 activity. Genes Dev (2008) 22:3282–91. doi:10.1101/gad.1772708

57. Shin JW. Prox1 promotes lineage-specific expression of fibroblast growth factor (FGF) receptor-3 in lymphatic endothelium: a role for FGF signaling in lymphangiogenesis. Mol Biol Cell (2005) 17:576–84. doi:10.1091/mbc.E05-04-0368

58. Podgrabska S, Braun P, Velasco P, Klooos B, Pepper MS, Skobe M. Molecular characterization of lymphatic endothelial cells. Proc Natl Acad Sci U S A (2002) 99:16069–74. doi:10.1073/pnas.242401399

59. Klotz L, Norman S, Vieira JM, Masters M, Rohling M, Dubé KN, et al. Cardiac lymphatics are heterogeneous in origin and respond to injury. Nature (2015) 522:62–7. doi:10.1038/nature14483

60. Zheng W, Aspelund A, Alitalo K. Lymphangiogenic factors, mechanisms, and applications. J Clin Invest (2014) 124:878–87. doi:10.1172/JCI71603

61. Breier G. Lymphangiogenesis in regenerating tissue: is VEGF-C sufficient? Circ Res (2005) 96:1132–4. doi:10.1161/01.RES.0000170976.63688.ca

62. Achen MG, Jeltsch M, Kukk E, Mákinen T, Vitali A, Wilks AF, et al. Vascular endothelial growth factor D (VEGF-D) is a ligand for the tyrosine kinases VEGF receptor 2 (Flk1) and VEGF receptor 3 (Flt4). Proc Natl Acad Sci U S A (1998) 95:5438–53. doi:10.1073/pnas.95.2.548

63. Cao Y, Lindén P, Vägenbo I, Cao R, Eriksson A, Kumar V, et al. Vascular endothelial growth factor C induces angiogenesis in vivo. Proc Natl Acad Sci U S A (1998) 95:14389–94. doi:10.1073/pnas.95.24.14389

64. Yang J, Zhang L, Yu X, Yang J, Zhou X, Zhang X, et al. Lymphangiogenesis in tumor microenvironment is associated with tumor progression and poor survival. J Pathol (2015) 235:251–62. doi:10.1002/path.4585

65. Cao Z, Shang B, Zhang G, Miele L, Sarkar FH, Wang Z, et al. Tumor-derived lymphangiogenic factors and lymphatic metastasis. Cancers (Basel) (2013) 5:2273–86. doi:10.3390/cancers5122733

66. Kerjaschki D. The crucial role of macrophages in lymphangiogenesis. J Clin Invest (2005) 115:2316–9. doi:10.1172/JCI26554

67. O’Brien J, Martinson H, Durand-Rougey C, Schedin P. Macrophages are crucial for epithelial cell death and adipocyte repopulation during mammary gland involution. Development (2012) 139:269–75. doi:10.1242/dev.071696

68. Alowami S, Troup S, Al-Haddad S, Kirkpatrick I, Watson PH. Morphometric density is related to stroma and stromal proteoglycan expression. Breast Cancer Res (2003) 5:R129–35. doi:10.1186/bcr622

69. Watson CJ, Oliver CH, Khaled WT. Cytokine signalling in mammary gland involution. J Reprod Immunol (2011) 88(2):124–9. doi:10.1016/j.jri.2010.11.006

70. Walker NL, Bennett RE, Kerr JF. Cell death by apoptosis during involution of the lactating breast in mice and rats. Am J Anat (1989) 185:19–32. doi:10.1002/aja.188018502

71. Kirch MT, Schwertfeger KL, Ryder JW, Anderson SM. A atlas of mouse mammary gland development. J Mammary Gland Biol Neoplasia (2000) 5:227–41. doi:10.1023/A:10201649952305

72. Schwertfeger KL, Kirch MT, Anderson SM. Mammary gland involution is delayed by activated Akt in transgenic mice. Mol Endocrinol (2001) 15:867–81. doi:10.1210/mend.15.6.0663

73. Monks J, Rosner D, Geske FH, Lehman L, Hanson L, Neville MC, et al. Epithelial cells as phagocytes: apoptotic epithelial cells are engulfed by mammary alveolar epithelial cells and repress inflammatory mediator release. Cell Death Diff (2005) 12:107–14. doi:10.1038/sj.cdd.4401517

74. Rudolph MC, McNamman JL, Hunter L, Phang T, Neville MC. Functional development of the mammary gland: use of expression profiling and trajectory clustering to reveal changes in gene expression during pregnancy, lactation, and involution. J Mammary Gland Biol Neoplasia (2003) 8:287–307. doi:10.1023/B:JMG.0000010030.73983.57

75. Monks J, Smith-Steinhardt C, Kruk ER, Fadok VA, Henson PM. Epithelial cells remove apoptotic epithelial cells during post-lactation involution of the mouse mammary gland. Biol Reprod (2008) 78:586–94. doi:10.1095/bioreprod.107.065045

76. Pepper MS, Baetens D, Mandriota SJ, Di Sanza C, Oikemus S, Lane TF, et al. Regulation of VEGF and VEGF receptor expression in the rodent mammary gland during pregnancy, lactation, and involution. Dev Dyn (2000) 218:307–24. doi:10.1002/1097-0177(200007)218:3<307::AID-DVDY1012>3.0.CO;2-5

77. Betterman KL, Paquet-Fifield S, Asselin-Labat ML, Visvader JE, Butler LM, Stacker SA, et al. Remodeling of the lymphatic vasculature during mouse mammary gland morphogenesis is mediated via epithelial-derived lymphangiogenic stimuli. Am J Pathol (2012) 181:2225–38. doi:10.1016/j.ajpath.2012.08.035

78. Martinson H, Jindal S, Borges V, Schedin P. Immune cell influx during postpartum mammary gland involution reflects immunosuppression and tumor promotion. Cancer Res (2013):73.

79. Jindal S, Gao D, Bell P, Albrektsen G, Edgerton SM, Ambrosone CB, et al. Postpartum breast involution reveals regression of secretory lobules mediated by tissue-remodeling. Breast Cancer Res (2014) 16:R31. doi:10.1186/bcr363

80. Katare RP, Jung K, Jang C, Yang H, Schwendener RA, Baik JE, et al. Critical role of CD11b+ macrophages and VEGF in inflammatory lymphangiogenesis, antigen clearance, and inflammation resolution. Blood (2009) 113:5650–9. doi:10.1182/blood-2008-09-176776
114. Karnezis T, Chen L, Cursiefen C, Zhang Q, Joyce NC, Gabbiani G. Expression of vascular endothelial growth factor receptor-3 (VEGFR-3) on monocytes and bone marrow-derived cells in the conjunctiva. Exp Eye Res (2004) 79:553–61. doi:10.1016/j.exer.2004.06.028

115. Karpanen T, Egeblad M, Karkkainen MJ, Kubo H, Ylä-Herttuala S, Jäättelä M, et al. Vascular endothelial growth factor C promotes tumor lymphangiogenesis and intralymphatic tumor growth. Cancer Res (2001) 61:1786–90.

116. Liu H, Yang Y, Xiao J, Li Y, Liu Y, Yang H, et al. COX-2-mediated regulation of VEGF-C in association with lymphangiogenesis and lymph node metastasis in lung cancer. Anatom Rec (Hoboken) (2010) 293:1838–46. doi:10.1002/ar.21240

117. Konishi K, Hwang D, Jiang H, Barlogie B, Fuks Z, Tse W, et al. Induction of tumor lymphangiogenesis by VEGF-C promotes breast cancer metastasis. Nat Med (2001) 7:192–8. doi:10.1038/84643

118. Wang CA, Jindal S, Amin MS, Mercadante F, Deitch E, et al. SIX1 induces lymphangiogenesis and metastasis via upregulation of VEGF-C in mouse models of breast cancer. J Clin Invest (2012) 122:1895–906. doi:10.1172/JCI59585

119. Stacke SA, Caesar C, Baldwin ME, Thornton GE, Williams RA, Prevo R, et al. VEGF-D promotes the metastatic spread of tumor cells via the lymphatics. Nat Med (2001) 7:186–91. doi:10.1038/84635

120. Katoh H, Hoshino K, Ito Y, Suzuki T, Ogawa Y, Kubo H, et al. COX-2 and prostaglandin EP3/EP4 signaling regulate the tumor stromal proangiogenic microenvironment via CXCL12-CXCR4 chemokine systems. Am J Pathol (2010) 176:1469–83. doi:10.2353/apjpath.2010.090907

121. Su JL, Shih JY, Yen ML, Cheng CC, Lai CY, et al. Cyclooxygenase-2 up-regulation: a novel mechanism of lymphangiogenesis in lung adenocarcinoma. Cancer Res (2004) 64:554–64. doi:10.1158/0008-5472.CAN-03-1301

122. Mäkinen T, Veikkola T, Mustjoki S, Karpanen T, Cattin B, Nice EC, et al. Isolated lymphatic endothelial cells transduce growth, survival and migratory signals via the VEGF-C/D receptor VEGFR-3. Embo J (2001) 20:4762–73. doi:10.1093/emboj/20.17.4762

123. Hoshida T, Isaka N, Hagedoorn J, di Tomaso E, Pytowski B, et al. Imaging steps of lymphatic metastasis reveals that vascular endothelial growth factor-C increases metastasis by increasing delivery of cancer cells to lymph nodes: therapeutic implications. Cancer Res (2006) 66:8065–7. doi:10.1158/0008-5472.CAN-06-1392

124. Quagliata L, Klusmeier S, Cremers N, Pytowski B, Harvey A, Pettis RJ, et al. Inhibition of VEGF-C activation in tumor-draining lymph nodes suppresses the outgrowth of lymph node metastases in the MT-450 syngeneic rat breast cancer model. Clin Exp Metastasis (2011) 31:351–65. doi:10.1007/s10585-013-9633-2

125. Forneri J, Jindal S, Middleton KA, Borges VE, Schild P. Physiological COX-2 expression in breast epithelium associates with COX-2 levels in ducal carcinoma in situ and invasive breast cancer in young women. Am J Pathol (2014) 184:1219–29. doi:10.1016/j.ajpath.2013.12.026

126. Lewis C, Pollard J. Distinct role of macrophages in different tumor micro-environments. Cancer Res (2006) 66(2):605–12. doi:10.1158/0008-5472.CAN-05-4005

127. Kurahara H, Takao S, Maemura K, Maki T, Kuwahata T, Maeda K, et al. M2-polarized tumor-associated macrophage infiltration of regional lymph nodes is associated with nodal lymphangiogenesis and occult nodal involvement in pN0 pancreatic cancer. Pancreas (2013) 42:155–9. doi:10.1097/MPA.0b013e31825f2d1

128. Mantovani A, Romero P, Palucka AK, Marincola FM. Tumor immunity: effector response to tumour and role of the microenvironment. Lancet (2008) 371:771–83. doi:10.1016/S0140-6736(08)60241-X

129. Burton JB, Priceman SJ, Sung JL, Brakenhielm E, An DS, Pytowski B, et al. Suppression of prostate cancer nodal and systemic metastasis by blockade of the lymphangiogenic axis. Cancer Res (2008) 68:7828–37. doi:10.1158/0008-5472.CAN-08-1488

130. Pytowski B, Goldman J, Persaud K, Wu Y, Witte L, Hicklin DJ, et al. Complete neutralizing antibody. J Natl Cancer Inst (2005) 97:14–21. doi:10.1093/jnci/dji002

131. Roberts N, Klooos B, Cassella M, Podgrabinska S, Persaud K, Wu Y, et al. Inhibition of VEGF-C activation with the antagonistic antibody more potently suppresses lymph node and distant metastases than...
inactivation of VEGFR-2. Cancer Res (2006) 66:2650–7. doi:10.1158/0008-5472.CAN-05-1843
132. Saif WM, Knost JA, Chiorean EG, Kambhampati SRI, Yu D, Pytowski B, et al. Phase I study of anti-VEGF receptor-3 (VEGFR-3) monoclonal antibody (Mab) LY3022856/IMC-3C5 (JC5). J Clin Oncol (2015) 33.
133. Mross K, Frost A, Scheulen ME, Krauss J, Strumberg D, Schultheiss B, et al. Phase I study of telatinib (BAY 57-9352): analysis of safety, pharmacokinetics, tumor efficacy, and biomarkers in patients with colorectal cancer. Vasc Cell (2011) 3:16. doi:10.1186/2045-824X-3-16
134. Marx J. Cancer. Encouraging results for second-generation antiangiogenesis drugs. Science (2005) 308:1248–9.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2016 Borges, Elder and Lyons. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.