p19/Arf and p53 Suppress Sentinel Lymph Node Lymphangiogenesis and Carcinoma Metastasis

Alanna Ruddell[1], Karen S. Kelly-Spratt, Momoko Furuya, Sean S. Parghi, and Christopher J. Kemp

Fred Hutchinson Cancer Research Center, Seattle WA, USA

1. Correspondence should be addressed to:

Alanna Ruddell

Fred Hutchinson Cancer Research Center

1100 Fairview Ave. N., MS-C2-023

PO Box 19024

Seattle, WA, 98109 USA

Telephone: 206-667-5496

Fax: 206-667-1623

Email: aruddell@fhcrc.org

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ABSTRACT

The ability of tumor cells to metastasize is increasingly viewed as an interaction between the primary tumor and host tissues. Deletion of the p19/Arf or p53 tumor suppressor genes accelerates malignant progression and metastatic spread of DMBA/TPA-induced squamous cell carcinomas, providing a model system to address mechanisms of metastasis. Here we show that benign pre-metastatic papillomas from wild type mice trigger lymphangiogenesis within draining lymph nodes, while there is no growth of primary tumor lymphatic vessels. Lymph node lymphangiogenesis is greatly accelerated in papilloma-bearing p19/Arf- or p53-deficient mice, which coincides with the greater propensity of these tumors to progress to carcinomas and to metastasize. The extent of accumulation of B cells within the tumor-draining lymph nodes of wild type mice predicted the level of lymph node lymphangiogenesis and metastatic potential. Arf or p53 deficiency strongly accelerated lymph node immune cell accumulation, in a manner that was associated with the extent of lymph node lymphatic sinus growth. This immune cell accumulation and lymph node lymphangiogenesis phenotype identifies host anti-tumor responses that could drive metastatic spread of cancers via the lymphatics.

INTRODUCTION

Murine cancer models have been useful to define the critical genes and multi-step pathways involved in generation of cancers. However, most mouse cancer models rarely develop metastasis to secondary organs, so that the genetics and biology of this process has been less studied. Murine or human cancers can metastasize via the vascular or lymphatic systems (Cao, 2005). Blood vessel growth (angiogenesis) in primary tumors could provide a direct route for tumor dissemination by intravasation of tumor cells into the abnormal vasculature. Lymphatic vessel growth (lymphangiogenesis) in or around tumors is similarly thought to provide a direct means to transport tumor cells to draining lymph nodes (LNs), and subsequently into the pulmonary circulation. For this reason, tumor cell detection in tumor-draining or sentinel LNs is used clinically to diagnose metastasis of human cancers including melanoma, breast, and colon cancer (Nathanson, 2003). As metastasis is the major cause of death in cancer patients, it is critical to develop mouse cancer models that reliably metastasize, to identify events required for dissemination by either pathway.

Evidence that the lymphatic system actively contributes to tumor metastasis has come from studies of lymphatic endothelial growth factors. Vascular endothelial growth factor (VEGF-A) stimulates lymphangiogenesis by activating VEGFR-2 expressed on lymphatic endothelium, however VEGF-A additionally promotes VEGFR-2-dependent blood vessel growth (Nagy *et al.*, 2002). While VEGF-A overexpression stimulates lymphangiogenesis and metastasis (Hirakawa *et al.*, 2005), and VEGFR-2 blockade inhibits metastasis

(Roberts *et al.*, 2006), these manipulations additionally alter angiogenesis and tumor size. The VEGF-C and VEGF-D growth factors selectively influence lymphatic endothelial proliferation and migration, without affecting vascular endothelium, by activating VEGFR-3 expressed on lymphatic endothelium (Baldwin *et al.*, 2004). Tumor VEGF-C or VEGF-D expression is associated with human cancers having poor prognosis (reviewed by (Stacker *et al.*, 2002)), suggesting that lymphangiogenesis promotes metastasis. Moreover, ectopic VEGF-C or VEGF-D drive tumor lymphangiogenesis and LN metastasis in murine cancers (Mandriota *et al.*, 2001; Skobe *et al.*, 2001b; Stacker *et al.*, 2001), while inhibition of VEGFR-3 signalling blocks metastasis (He *et al.*, 2002; Krishnan *et al.*, 2003; Shimizu *et al.*, 2004). However, LN metastasis can occur without tumor lymphangiogenesis (Achen and Stacker, 2006; Wong and Hynes, 2006), indicating that other alterations contribute to tumor dissemination via lymphatics.

While tumor cells can metastasize to draining LNs, an active contribution of LNs to tumor dissemination was not considered until recently. We discovered in an Eμ–*c-myc* lymphoma model that Myc-expressing B cell accumulation within LNs is associated with growth of LN lymphatic sinuses (Ruddell *et al.*, 2003). Lymphangiogenesis was also identified in LNs draining melanomas after subcutaneous implantation, but prior to metastasis to draining LNs (Harrell *et al.*, 2007). LN lymphatic sinus growth had functional consequences, as lymph flow through draining LNs increased 20- to 30-fold. These observations led us to propose that LN lymphangiogenesis and increased lymph flow actively drive

tumor cells to draining LNs (Ruddell *et al.*, 2003). In support of this idea, ectopic VEGF-A or VEGF-C overexpression in murine skin tumors promoted tumor lymphatic vessel growth, LN lymphangiogenesis, and increased metastasis (Hirakawa *et al.*, 2007; Hirakawa *et al.*, 2005). Our previous studies identified B cells as the natural mediators of lymphangiogenesis in melanoma-draining LNs, as B cell-deficient mice did not support LN lymphatic sinus growth (Harrell *et al.*, 2007). Taken together, these findings suggest that B cell–induced LN lymphangiogenesis promotes metastasis to draining LNs.

We used a murine multi-stage model of squamous cell carcinoma to assess the contributions of lymphangiogenesis and angiogenesis within the primary tumor or draining LN to metastasis. Two-stage treatment of mice with DMBA carcinogen, followed by multiple TPA treatments, produces benign squamous cell papillomas within 3 months, which feature thickened and folded epidermis (Kemp, 2005). The epithelial cells invariably feature DMBA-induced mutation and constitutive activation of the *Ha-ras* oncogene (Kemp *et al.*, 1993; Quintanilla *et al.*, 1986). DMBA-induced papillomas can sometimes convert to carcinomas invading through the underlying dermis, and occasionally metastasizing to draining LNs and lungs. Mutations or LOH of the p19/Arf or p53 tumor suppressors arise during carcinoma conversion, and Arf- or p53-deficient mice show increased carcinomas, indicating that Arf or p53 loss is rate limiting for malignant progression (Burns *et al.*, 1991; Kelly-Spratt *et al.*, 2004; Kemp *et al.*, 1993). These tumor suppressors also block metastasis, as DMBA-TPA-treated mice deficient for Arf or p53 show increased LN and lung metastases

(Kelly-Spratt *et al.*, 2004; Kemp *et al.*, 1993), providing a genetic model to evaluate mechanisms of carcinoma metastasis.

Two complementary approaches were used to identify vessel alterations associated with squamous cell carcinoma metastasis. Firstly, lymphatic and blood vessels of papillomas, carcinomas, or tumor-draining LNs were compared to normal skin or LNs, to identify alterations promoting carcinoma dissemination in wild type mice. Secondly, vessels were compared between wild type, Arf +/-, and p53 +/- mice, to identify alterations that could explain the increased susceptibility of Arf- and p53-deficient mice to metastasis. We found that LN lymphangiogenesis is the major vessel alteration associated with metastatic potential. Moreover, B cell accumulation predicted the extent of LN lymphangiogenesis, suggesting that tumor-induced B cell accumulation promotes LN lymphatic sinus growth and metastasis.

RESULTS

Angiogenesis in carcinomas of wild type and Arf +/- mice.

Squamous cell carcinomas spread to draining LNs before they are detected in lungs (Kelly-Spratt *et al.*, 2004), suggesting that they metastasize via the lymphatics. Immunohistochemical staining assessed whether lymphatic vessels increase in primary tumors, as a mechanism to drive metastasis. LYVE-1 and 10.1.1 lymphatic endothelium-specific antibodies identified lymphatic vessels of variable size and density in normal dermis (Figure 1A). Benign

papillomas showed sparse lymphatic vessels in thickened dermis (Figure 1C), as did carcinomas, which consist of undifferentiated keratinocytes (Figure 1E).

FIGURE 1

MECA-32 immunostaining of blood vessels (Leppink *et al.*, 1989) tested whether angiogenesis is involved in tumor progression. Blood vessels were confined to the dermis in normal skin (arrowhead, Figure 1B) or papillomas (Figure 1D). Carcinomas contained abundant and enlarged blood vessels, typical of angiogenic tumors (Figure 1F). These findings agree with previous measurements of 4-fold increased blood vessel density in DMBA/TPA-induced carcinomas, while intra- or peri-tumoral lymphatic vessels showed little growth (Hirakawa *et al.*, 2003). Thus angiogenesis rather than lymphangiogenesis is the major vessel alteration in carcinomas from wild type mice.

Lymphatic and blood vessels were characterized in tumors from Arf +/- mice, to determine whether vessel growth could accelerate metastasis in this genotype. Lymphatic vessels were sparse in papillomas (Figure 1G) and carcinomas (Figure 1I) from Arf +/- mice. Carcinomas from Arf-deficient mice showed angiogenesis (Figure 1J) similar to that in wild type mice (Figure 1F). Thus accelerated metastasis in Arf +/- mice is not due to enhanced tumor lymphatic or blood vessel growth, relative to wild type tumors.

Lymph node lymphangiogenesis is an early response to pre-malignant tumor growth

We previously identified LN rather than tumor lymphangiogenesis as the major vessel alteration associated with metastatic melanoma growth (Harrell *et al.*, 2007). The DMBA/TPA tumor model was used to ask whether LN lymphangiogenesis arises before carcinoma conversion, or upon metastasis to LNs. First, draining inguinal LNs of mice bearing pre-metastatic papillomas were compared to those from untreated controls. Control wild type LNs showed normal lymphatic sinuses restricted to the cortex and septum between paired LNs (Figure 2A). By contrast, LNs from papilloma-bearing mice showed enlarged lymphatic sinuses throughout the LN (Figure 2E). Enlarged lymphatic sinuses are directly attributed to papilloma formation rather than TPA treatment, as TPA treatment alone did not affect lymphatic sinuses (Figure 2C).

FIGURE 2

Comparison of lymphatic sinus area in serial LN section samples demonstrated a 2.5-fold increase in papilloma-draining LNs relative to untreated control LNs, and no significant increase in LNs from TPA-treated mice (Figure 3A). Lymphatic sinus growth involves proliferation, as 10.1.1-positive lymphatic endothelium within papilloma-draining LNs often immunostained positively for the mitotic marker phosphohistone H3 (data not shown}. Thus pre-metastatic papillomas stimulate lymphangiogenesis in draining LNs, while the papilloma itself does not undergo lymphatic vessel growth.

MECA-32 immunostaining was used to test whether blood vessels of tumor-draining LNs also respond to primary tumors. Capillaries and high endothelial venules were abundant in LNs from untreated control (Figure 2B), TPA-only (Figure 2D), or DMBA/TPA-treated papilloma-bearing mice (Figure 2F). Blood vessel area (Figure 3B) or density measurements (data not shown) confirmed that tumors do not increase LN blood vessels. Thus, papillomas act over a distance to stimulate LN lymphangiogenesis, without affecting LN blood vessels.

FIGURE 3

Arf or p53 deficiency accelerates lymph node lymphangiogenesis.

Arf- or p53-deficient mice show increased conversion of papillomas to carcinomas, and more rapid metastasis (Kelly-Spratt *et al.*, 2004). We first examined LNs from papilloma-bearing Arf +/- mice, to determine whether accelerated metastasis involves alterations in draining LNs prior to malignant conversion. LNs from papilloma-bearing Arf +/- mice showed greatly increased lymphatic sinuses (Figure 2I) relative to LNs from age-matched papilloma-bearing wild type mice (Figure 2E). This was confirmed by quantifying lymphatic sinus area, which identified significantly enhanced lymphangiogenesis in LNs from Arf-deficient versus wild type papilloma-bearing mice (Figure 3A). By contrast, Arf deficiency did not affect blood vessels in papilloma-draining LNs (Figure 2J), confirmed by quantification (Figure 3B). Arf deficiency therefore strongly and selectively promotes lymphatic sinus growth at the pre-malignant papilloma stage, prior to metastasis.

Papillomas grow more rapidly in Arf-deficient mice (Kelly-Spratt *et al.*, 2004), which could potentially explain enhancement of LN lymphangiogenesis in Arf +/- mice. This hypothesis was tested by examining papilloma-draining LNs of p53 +/- mice, which also show rapid carcinoma conversion and metastasis, however they grow more slowly than wild type papillomas. LNs from papilloma-bearing p53 +/- mice showed extensive lymphangiogenesis (Figure 2K), similar to that in Arf +/- mice (Figure 2I). Lymphatic sinus area quantitation confirmed that p53 or Arf deficiency significantly increased LN lymphangiogenesis (Figure 3A). This is not due to intrinsic differences in LN lymphatics, as control LNs from wild type (Figure 2A), Arf- (Figure 2G), or p53-deficient mice (Supplementary Figure 1A) all showed few lymphatic sinuses. Arf or p53 tumor suppressor deficiency thus specifically accelerates tumor-induced LN lymphangiogenesis in a manner which is associated with an increased probability of carcinoma conversion and metastasis, that is independent of tumor growth rate.

Lymph node lymphangiogenesis in wild type carcinoma-bearing mice.

LN metastases occasionally arise in carcinoma-bearing wild type mice. Wild type mice bearing carcinomas were examined to determine whether LN lymphangiogenesis is associated with this stage of tumor progression. These LNs consistently showed greatly increased lymphatic sinuses (Figure 4A) relative to LNs from papilloma-bearing mice (Figure 2E). Quantification confirmed that lymphatic sinus area increases 4-fold relative to untreated controls (Figure 3A). This increase is much larger than that in papilloma-draining LNs (Figure 3A),

indicating that lymphatic sinus growth continues during tumor progression in wild type mice. By contrast, carcinoma-draining LN blood vessels (Figure 4B) are similar to those of papilloma-draining LNs (Figure 2F), and this was confirmed by measuring blood vessel area (Figure 3B). These findings demonstrate that carcinoma conversion provides a strong and specific lymphatic growth stimulus within draining LNs of wild type mice.

FIGURE 4

Lymphatic sinus remodeling in carcinoma-draining LNs from Arf- and p53-deficient mice

Thus far we identified enhanced lymphangiogenesis in papilloma-bearing Arf +/- mice relative to wild type mice. We investigated whether accelerated carcinoma conversion and metastasis in Arf +/- mice further enhances LN lymphangiogenesis. Surprisingly, the LN lymphatic sinuses of carcinoma-bearing Arf +/- mice were greatly reduced (Figure 4C) relative to LNs from Arf +/- mice bearing only papillomas (Figure 2I). Quantification confirmed that lymphatic sinus area in Arf +/- LNs was not significantly increased relative to normal LNs (Figure 3A).

Arf deletion accelerates carcinoma conversion and metastasis, so that tumor cells are detected in most LNs from carcinoma-bearing Arf +/- mice (Kelly-Spratt *et al.*, 2004). Tumors were identified by their distinct morphology, and by immunostaining for podoplanin (Schacht *et al.*, 2005), or keratin (Kelly-Spratt *et al.*, 2004). The LN shown (Figure 4C) is typical of LNs that do not yet contain tumors, indicating that lymphatic sinus loss in carcinoma-bearing Arf +/- mice

begins before metastases are detected. Similar loss of LN lymphatic sinuses was identified in carcinoma-bearing p53 +/- mice (Supplementary Figure 1C).

We next analyzed LNs from Arf +/- mice containing podoplanin-positive metastases ("T", Figure 4H). Sparse lymphatic sinuses were found adjacent to and within metastases by 10.1.1 (Figure 4E), or LYVE-1 immunostaining (Figure 4G), as were MECA-32-positive blood vessels (Figure 4F). These remaining LN lymphatic sinuses could potentially mediate further tumor dissemination via the lymphatics.

Carcinoma metastases grow aggressively in Arf +/- mice, so that tumors can entirely replace LNs (Kelly-Spratt *et al.*, 2004). These LNs contained minimal lymphatic sinuses (arrow, Figure 4I), while blood vessels greatly increased (Figure 4J), indicating that tumor growth destroys lymphatic sinuses, while it concurrently stimulates angiogenesis. This same phenotype was observed in primary tumors, where lymphatic vessels were sparse (Figure 1I), while blood vessels were abundant (Figure 1J). Taken together, these findings suggest that carcinoma cells are selectively toxic for lymphatic endothelium. LN metastasis often is detected in Arf-deficient mice at the time of carcinoma conversion (Kelly-Spratt *et al.*, 2004), suggesting that the arrival of small numbers of tumor cells in draining LNs could trigger lymphatic sinus degradation, before LN metastases grow large enough to be detected.

Arf or p53 deficiency enhances macrophage accumulation in tumor-draining LNs

Our finding that LN lymphangiogenesis arises at the papilloma stage, prior to metastasis, suggests that tumors produce a long-range signal driving LN lymphatic sinus growth. Migratory immune cells could traffic from primary tumors or from the blood stream into draining LNs in response to tumors. Alternatively, tumor-derived factors could travel via the lymph to activate draining LNs. One immune cell type that could contribute is macrophages, which can secrete VEGF-A, VEGF-C, and VEGF-D to drive lymphatic and blood vessel growth (Schoppmann *et al.*, 2002; Skobe *et al.*, 2001a).

We performed immunohistochemical staining of tumors and draining LNs using the F4/80 antibody, to determine whether macrophages could contribute to vessel growth during tumor progression. F4/80-positive macrophages were identified within papillomas (Figure 5B), but not in normal skin (Figure 5A). Carcinomas showed extensive macrophage infiltration (Figure 5C). Papillomas and carcinomas from Arf +/- mice showed similar macrophage infiltration (data not shown), indicating that tumor macrophages do not mediate increased LN lymphangiogenesis in Arf-deficient mice.

FIGURE 5

Macrophages could potentially promote LN lymphangiogenesis by accumulating within tumor-draining LNs. F4/80-positive macrophages were restricted to normal LN capsules (arrow, Figure 5D), however they were distributed throughout papilloma-draining LNs from wild type mice (Figure 5E).

LNs from papilloma-bearing Arf +/- and p53 +/- mice (Figure 5F and G, respectively) showed increased macrophage accumulation relative to papilloma-bearing wild type mice (Figure 5E). Macrophage accumulation further increased in LNs from carcinoma-bearing Arf +/- (Figure 5I), p53 +/- (Supplementary Figure 1F), and wild type mice (Figure 5H). This macrophage accumulation was tumor-induced, as untreated LNs from wild type, p53- and Arf-deficient mice all showed few macrophages (Figure 5D, Supplementary Figure 1E, and data not shown). Macrophage accumulation could contribute to tumor-draining LN lymphangiogenesis in wild type mice, and to its enhancement in tumor-bearing Arf- and p53-deficient mice. However, maximal LN macrophage infiltration in Arf +/- or p53 +/- carcinoma-bearing mice is associated with loss of lymphatic sinuses (Figures 4C and Supplementary Figure 1C), so that macrophages do not support LN lymphangiogenesis during carcinogenesis.

Arf or p53 deficiency enhance B cell accumulation in tumor-draining lymph nodes

We previously found that lymphangiogenesis in melanoma-draining LNs is mediated by LN B cell accumulation (Harrell *et al.*, 2007). We tested whether B cell accumulation also mediates DMBA/TPA tumor-induced LN alterations. B220-positive B cells were absent from wild type (data not shown) or Arf +/- papillomas (Figure 6A). B cells were restricted to the LN cortex of untreated (Figure 6B) or TPA-treated wild type mice (data not shown). However, LNs from papilloma-bearing mice contained B cell follicles (arrows, Figure 6C), and

individual B cells throughout the cortex and medulla. Irregularly shaped follicles and individual lymphocytes were abundant throughout carcinoma-draining LNs (Figure 6D), demonstrating that B cells accumulate in tumor-draining LNs of wild type mice during tumor progression.

FIGURE 6

B lymphocyte distribution was distinct in tumor-draining LNs from Arf +/- and p53 +/- mice. Untreated Arf +/- (Figure 6G) and p53 +/- LNS (Supplementary Figure 1G) showed B cells normally restricted to the cortex. However, papilloma-drainng LNs from Arf +/- (Figure 6E) and p53 +/- mice (Figure 6F) were uniformly filled with B lymphocytes, in contrast to the discrete B cell follicles identified in LNs from papilloma-bearing wild type mice (Figure 6C). B cell localization changed again in LNs from carcinoma-bearing Arf +/- mice (Figure 6H) and p53 +/- mice (Supplementary Figure 1H), with B cells forming irregular follicles. B cells were excluded from LN regions containing metastases ("T", Figure 6I). These findings indicate that B cell behavior is highly regulated during tumor progression. Moreover, Arf and p53 can modulate host B cell response to tumor growth. Overall, the conditions yielding maximal B cell accumulation correspond to those featuring the most extensive LN lymphangiogenesis, i.e. wild type carcinomas and Arf +/- or p53 +/- papillomas (Table 1), supporting the idea that B cells promote LN lymphangiogenesis during DMBA/TPA tumor progression.

TABLE 1

Inverse correlation of VEGF-A or VEGF-C levels with LN lymphangiogenesis.

VEGF-A and VEGF-C are the major growth factors implicated in tumor-associated lymphangiogenesis (reviewed by (Cao, 2005)). We tested whether enhanced LN lymphangiogenesis in papilloma-bearing Arf- or p53-deficient mice is associated with increased production of these factors by tumors or by accumulation in draining LNs. ELISA assay revealed similar high levels of VEGF-A in wild type, Arf +/-, and p53 +/- papillomas (Figure 7A). Surprisingly, VEGF-A levels actually decreased in in papilloma-draining LNs from wild type or Arf +/- mice, relative to untreated control LNs (Figure 7B). VEGF-C expression was similar in LNs from wild type or Arf +/- papilloma-draining LNs, by immunoblotting (data not shown). VEGF-A and VEGF-C levels thus do not predict LN lymphangiogenesis, indicating that other factors regulate LN lymphatic sinus growth in wild type mice, and enhanced lymphangiogenesis in Arf- and p53-deficient mice.

FIGURE 7

DISCUSSION

The events driving successful dissemination of tumors via the lymphatics are of great interest for development of diagnostic and therapeutic strategies. We identified LN lymphangiogenesis as the major vessel alteration that precedes and predicts metastatic potential of squamous cell carcinomas. Lymphatic sinus growth reaches its maximum in carcinoma-draining LNs of wild type mice (Table 1), when tumors can begin to metastasize to draining LNs. LN

lymphangiogenesis is maximal in Arf- or p53-deficient mice bearing papillomas, which readily convert to invasive carcinomas. These findings indicate that LN lymphangiogenesis is a host response to pre-malignant tumors, which occurs prior to overt metastasis. We previously reported that LN lymphangiogenesis in pre-metastatic melanomas and lymphomas is associated with greatly increased lymph flow, which could actively drive tumor metastasis to draining LNs (Harrell *et al.*, 2007; Ruddell *et al.*, 2003). The extensive LN lymphangiogenesis in DMBA/TPA tumor-bearing mice also likely increases lymph flow to promote metastasis. Increased lymph flow may be sufficient to drive carcinoma metastasis, or additional changes could be required for successful lymphatic dissemination. Interestingly, primary tumors show minimal lymphatic vessel growth, indicating that tumor lymphangiogenesis does not contribute to metastasis in this model.

LN lymphangiogenesis is associated with unusual accumulation of macrophages and B cells within tumor-draining LNs. Macrophages are normally restricted to the LN capsule, however they accumulate throughout tumor-draining LNs. Macrophages can stimulate lymphangiogenesis (Kerjaschki, 2005), so that they could promote LN lymphatic sinus growth. However, extensive macrophage accumulation within Arf +/- or p53 +/- carcinoma-draining LNs does not support lymphangiogenesis, so that macrophage abundance does not predict lymphangiogenesis (Table 1). In contrast, B cells are restricted to tumor-draining LNs, where their abundance faithfully predicts the extent of LN lymphangiogenesis observed at each stage in wild type or tumor suppressor-

deficient mice (Table 1), suggesting that B cells regulate LN lymphangiogenesis in this model. In support of this idea, LN B cell accumulation is required for melanoma-induced LN lymphangiogenesis (Harrell *et al.*, 2007), and for LN lymphangiogenesis in response to acute inflammation (Angeli *et al.*, 2006). Taken together, these studies identify a critical role of B cells to stimulate LN lymphangiogenesis.

B cell accumulation in tumor-draining LNs could represent an immune response to tumor growth, as B cell follicles appeared in LNs of papilloma-bearing wild type mice. However, B cells uniformly filled the LNs of Arf or p53-deficient mice bearing papillomas, without forming discrete follicles. Melanoma-draining LNs also uniformly filled with B cells (Harrell *et al.*, 2007) and in this case there is no humoral immune response to tumor antigens (Brown *et al.*, 2001; Skelton *et al.*, 2001). Whether or not LN B cell accumulation is associated with an immune response, the program of B cell accumulation is very different in papilloma-bearing Arf- and p53-deficient versus wild type mice. Further studies are required to understand the significance of changes in B cell localization within LNs. Tumor-induced macrophage accumulation also likely reflects some innate immune response to tumor-derived signals within primary tumors and draining LNs.

Our studies identified new shared functions of the Arf and p53 tumor suppressors in regulation of B cell and macrophage behavior, to inhibit LN lymphangiogenesis and metastasis. This could be due to tumor suppressor deficiency within the tumor cells themselves, or within other host cell types. Arf

or p53 deficiency is generated within tumor cells during carcinoma conversion (Kelly-Spratt *et al.*, 2004; Kemp, 2005), so that tumor suppressor-deficient tumor cells mediate LN immune cell accumulation and lymphangiogenesis in carcinoma-bearing wild type mice. This indicates that the accelerated LN immune cell accumulation and lymphangiogenesis in papilloma-bearing Arf +/- and p53 +/- mice is likely also due to tumor suppressor deficiency in the tumor cells themselves, rather than other cell types. Arf and p53 are commonly mutated in human cancers, where they could also promote LN lymphangiogenesis and metastasis. Our findings linking tumor suppressor deficiency, immune cell accumulation, and LN lymphangiogenesis with metastasis identify a new pathway regulating tumor dissemination via the lymphatics.

The identification of factors mediating LN lymphangiogenesis is of great interest for development of therapeutic interventions. Most studies thus far have investigated VEGF-A or VEGF-C contributions to primary tumor lymphangiogenesis and metastasis (Cao, 2005). However, we found that VEGF-A and VEGF-C abundance actually is the same or decreased in tumors or draining LNs from tumor suppressor-deficient mice relative to wild type mice. These findings do not support the simple hypothesis that tumor-derived VEGF-A or VEGF-C drains to LNs to induce lymphangiogenesis, or that immune cells accumulating within LNs produce VEGF-A or VEGF-C locally to induce lymphangiogenesis. Instead, an as yet unidentified signal could regulate LN-restricted lymphangiogenesis, which is likely to involve the accumulation of B

cells within tumor-draining LNs. However, VEGF family members could still influence LN lymphangiogenesis in a manner not reflected by tissue concentration, as their bioavailability is regulated by proteolysis (Houck *et al.*, 1992; Joukov *et al.*, 1997). B cell accumulation within LNs could provide these proteases, or instead B cells could stimulate lymphatic sinus growth by producing distinct lymphatic endothelial growth factors such as FGF-2 or PDGF-BB (Cao, 2005).

Our studies identified immune cell accumulation and LN lymphangiogenesis as the major alterations associated with metastatic potential. Similar alterations could be involved in human cancer metastasis. Tumor-reactive lymphadenopathy is common in sentinel LNs from human cancer patients (Ioachim and Ratech, 2002), involving LN enlargement, lymphocyte accumulation, and/or sinus histiocytosis (lymphatic sinus macrophage expansion), which resemble the tumor-draining LN alterations we identified in mice. Increased lymphatic vessels have been detected in LNs draining human melanoma (Dadras *et al.*, 2005), and breast cancer (Van den Eynden *et al.*, 2006), supporting the idea that LN lymphangiogenesis occurs in human cancers. Further studies should provide insight to the contribution of LN immune cell infiltration and lymphangiogenesis to tumor dissemination and metastasis of human as well as animal cancers.

MATERIALS AND METHODS

Tumor Induction

The shaved back skin of wild type, p19/Arf +/- (Kamijo *et al.*, 1997)) or p53 +/- mice (Donehower *et al.*, 1992) were treated with DMBA, followed by twice weekly applications of TPA for 15 weeks, as previously described (Kelly-Spratt *et al.*, 2004). Mice were generally sacrificed 32 weeks after DMBA treatment. Animal experiments were approved by the FHCRC Animal Care and Use Committee.

Immunostaining Analysis

Frozen tissues were serially cryosectioned to sample throughout tumors or LNs, and immunostained with MECA-32 (Developmental Studies Hybridoma Bank, University of Iowa), 10.1.1 (Farr *et al.*, 1993), 8.1.1 podoplanin (Developmental Studies Hybridoma Bank, University of Iowa (Farr *et al.*, 1993; Schacht *et al.*, 2003)), LYVE-1 (Upstate, Temecula, NY), phosphohistone H3 (Ajiro *et al.*, 1996), F4/80 (eBioscience, San Diego, CA), or FITC-labelled B220 (Caltag, Carlsbad, CA) antibodies, as previously described (Harrell *et al.*, 2007). Immunohistochemical staining was detected with purple Vector VIP, followed by Methyl Green counterstaining (Vector Laboratories, Burlingame, CA). Sections were also directly immunostained with FITC-labelled-B220 antibodies, after blocking with CD16/CD32 Fc receptor antibody (Fc Block, BD Biosciences, San Jose, CA). Carcinoma cells were detected in serial sections sampling through tumor-draining LNs, by their larger cytoplasm and by pan-keratin (Novagen, Nottingham, UK) or podoplanin immunostaining (Schacht *et al.*, 2005).

Lymphatic vessel area, blood vessel area, or blood vessel density in 3 sections sampled from at least 5 LNs were quantified in 100x magnification microscope images using NIH ImageJ software (National Institutes of Health, Bethesda, MD).

VEGF-A ELISA

Frozen tumors or lymph nodes were homogenized in phosphate-buffered saline, lysed by two freeze-thaw cycles, centrifuged, and VEGF-A was measured by ELISA (R&D Systems, Minneapolis, MN).

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Supplementary information is available at the Oncogene web site.

REFERENCES

Achen MG, Stacker SA (2006). Tumor lymphangiogenesis and metastatic spread- New players begin to emerge. *Int. J. Cancer* **119:** 1755-1760.

Ajiro K, Yoda K, Utsumi K, Nishikawa Y (1996). Alteration of cell cycle-dependent histone phosphorylations by okadaic acid. *J. Biol. Chem.* **271:** 13197-13201.

Angeli V, Ginhoux F, Llodra J, Quemeneur L, Frenette PS, Skobe M *et al* (2006). B cell-driven lymphangiogenesis in inflamed lymph nodes enhances dendritic cell mobilization. *Immunity* **24:** 203-215.

Baldwin ME, Stacker SA, Achen MG (2004). Molecular Control of Lymphangiogenesis. *Bioessays* **24:** 1030-1040.

Brown DM, Fisher TL, Wei C, Frelinger JG, Lord EM (2001). Tumours can act as adjuvants for humoral immunity. *Immunol* **102:** 486-97.

Burns PA, Kemp CJ, Gannon JV, Lane DP, Bremner R, Balmain A (1991). Loss of heterozygosity and mutational alterations of the p53 gene in skin tumours of interspecific hybrid mice. *Oncogene* **6:** 2363-9.

Cao Y (2005). Emerging mechanisms of tumour lymphangiogenesis and lymphatic metastasis. *Nature Reviews Cancer* **5:** 735-743.

Dadras S, Lange-Aschenfeldt B, Velasco P, Nguyen L, Vora A, Muzikansky A *et al* (2005). Tumor lymphangiogenesis predicts melanoma metastasis to sentinel lymph nodes. *Modern Pathology* **18:** 1232-42.

Donehower LA, Harvey M, Slagle BL, McArthur MJ, C.A. Montgomery J, Butel JS *et al* (1992). Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. *Nature* **356:** 215-221.

Farr A, Nelson A, Hosier S, Kim A (1993). A novel cytokine-responsive cell surface glycoprotein defines a subset of medullary thymic epithelium in situ. *J. Immunology* **150:** 1160-1171.

Harrell MI, Iritani BM, Ruddell A (2007). Tumor-induced sentinel lymph node lymphangiogenesis and increased lymph flow precede melanoma metastasis. *American Journal of Pathology* **170:** 774-86.

He Y, Kozaki K, Karpanen T, Koshikawa K, Yla-Herttuala S, Takahashi T *et al* (2002). Suppression of tumor lymphangiogenesis and lymph node metastasis by blocking vascular endothelial growth factor receptor 3 signaling. *Journal of the National Cancer Institute.* **94:** 819-25.

Hirakawa S, Brown LF, Kodama S, Paavonen K, Alitalo K, Detmar M (2007). VEGF-C-induced lymphangiogenesis in sentinel lymph nodes promotes tumor metastasis to distant sites. *Blood* **109:** 1010-7.

Hirakawa S, Hong YK, Harvey N, Schacht V, Matsuda K, Libermann T *et al* (2003). Identification of vascular lineage-specific genes by transcriptional profiling

of isolated blood vascular and lymphatic endothelial cells. *American Journal of Pathology.* **162:** 575-86.

Hirakawa S, Kodama S, Kunstfeld R, Kajiya K, Brown LF, Detmar M (2005). VEGF-A induces tumor and sentinel lymph node lymphangiogenesis and promotes lymphatic metastasis. *J Exp Med* **201:** 1089-99.

Houck KA, Leung DW, Rowland AM, Winer J, Ferrara N (1992). Dual regulation of vascular endothelial growth factor bioavailability by genetic and proteolytic mechanisms. *Journal of Biological Chemistry* **267:** 26031-7.

Ioachim HL, Ratech H (2002). *Ioachim's Lymph Node Pathology*. Lippincott Williams and Wilkins: Philadelphia, 623pp.

Joukov V, Sorsa T, Kumar V, Jeltsch M, Claesson-Welsh L, Cao Y *et al* (1997). Proteolytic processing regulates receptor specificity and activity of VEGF-C. *The EMBO Journal* **16:** 3898-911.

Kamijo T, Zindy F, Roussel MF, Quelle DE, Downing JR, Ashmun RA *et al* (1997). Tumor suppression at the mouse INK4a locus mediated by the alternative reading frame product p19ARF. *Cell* **91:** 649-659.

Kelly-Spratt KS, Gurley KE, Yasui Y, Kemp CJ (2004). p19Arf supports growth, malignant conversion, and metastasis of Ha-ras driven squamous cell carcinomas through p53 dependent and independent pathways. *PLOS* **2:** 1138-1149.

Kemp CJ (2005). Multistep skin cancer in mice as a model to study the evolution of cancer cells. *Semin Cancer Biol* **15:** 460-73.

Kemp CJ, Donehower LA, Bradley A, Balmain A (1993). Reduction of p53 gene dosage does not increase initiation or promotion but enhances malignant progression of chemically induced skin tumors. *Cell* **4:** 813-22.

Kerjaschki D (2005). The crucial role of macrophages in lymphangiogenesis. *J Clin Invest.* **115:** 2316-9.

Krishnan J, Kirkin V, Steffen A, Hegen M, Weih D, Tomarev S *et al* (2003). Differential in vivo and in vitro expression of vascular endothelial growth factor (VEGF)-C and VEGF-D in tumors and its relationship to lymphatic metastasis in immunocompetent rats. *Cancer Res* **63:** 713-22.

Leppink DM, Bishopp DK, Sedmak DD, Henry ML, Ferguson RM, Streeter PR *et al* (1989). Inducible expression of an endothelial cell antigen on murine

myocardial vasculature in association with interstitial cellular infiltration. *Transplantation* **48:** 874-877.

Mandriota SJ, Jussila L, Jeltsch M, Compagni A, Baetens D, Prevo R *et al* (2001). Vascular endothelial growth factor-C-mediated lymphangiogenesis promotes tumour metastasis. *EMBO Journal.* **20:** 672-82.

Nagy JA, Vasile E, Feng D, Sundberg C, Brown LF, Detmar MJ *et al* (2002). Vascular Permeability Factor/Vascular Endothelial Growth Factor Induces Lymphangiogenesis as well as Angiogenesis. *J. Exp. Med.* **196:** 1497-1506.

Nathanson SD (2003). Insights into the mechanisms of lymph node metastasis. *Cancer* **98:** 413-23.

Quintanilla M, Brown K, Ramsden M, Balmain A (1986). Carcinogen-specific mutation and amplification of Ha-*ras* during mouse skin carcinogenesis. *Nature* **322:** 78-80.

Roberts N, Kloos B, Cassella M, Podgrabinska S, Persaud K, Wu Y *et al* (2006). Inhibition of VEGFR-3 activation with the antagonistic antibody more potently suppresses lymph node and distant metastases than inactivation of VEGFR-2. *Cancer Research* **66:** 2650-2657.

Ruddell A, Mezquita P, Brandvold KA, Farr A, Iritani BM (2003). B lymphocyte-specific c-Myc expression stimulates early and functional expansion of the vasculature and lymphatics during lymphomagenesis. *American Journal of Pathology* **163:** 2233-2245.

Schacht V, Dadras SS, Johnson LA, Jackson DG, Hong YK, Detmar M (2005). Up-regulation of the lymphatic marker podoplanin, a mucin-type transmembrane glycoprotein, in human squamous cell carcinomas and germ cell tumors. *Am J Pathol* **166:** 913-21.

Schacht V, Ramirez MI, Hong YK, Hirakawa S, Feng D, Harvey N *et al* (2003). T1 alpha/podoplanin deficiency disrupts normal lymphatic vasculature formation and causes lymphedema. *EMBO Journal.* **22:** 3546-56.

Schoppmann SF, Birner P, Stockl J, Kalt R, Ullrich R, Caucig C *et al* (2002). Tumor-associated macrophages express lymphatic endothelial growth factors and are related to peritumoral lymphangiogenesis. *American Journal of Pathology.* **161:** 947-56.

Shimizu K, Kubo H, Yamaguchi K, Kawashima K, Ueda Y, Matsuo K *et al* (2004). Suppression of VEGFR-3 signaling inhibits lymph node metastasis in gastric cancer. *Cancer Sci.* **95:** 328-333.

Skelton D, Satake N, Kohn DB (2001). The enhanced green fluorescent protein (eGFP) is minimally immunogenic in C57BL/6 mice. *Gene Therapy* **8:** 1813-4.

Skobe M, Hamberg LM, Hawighorst T, Schirner M, Wolf GL, Alitalo K *et al* (2001a). Concurrent induction of lymphangiogenesis, angiogenesis, and macrophage recruitment by vascular endothelial growth factor-C in melanoma. *American Journal of Pathology.* **159:** 893-903.

Skobe M, Hawighorst T, Jackson DG, Prevo R, Janes L, Velasco P *et al* (2001b). Induction of tumor lymphangiogenesis by VEGF-C promotes breast cancer metastasis. *Nature Medicine.* **7:** 192-8.

Stacker SA, Baldwin MW, Achen MC (2002). The role of tumor lymphangiogenesis in metastatic spread. *FASEB Journal.* **16:** 922-934.

Stacker SA, Caesar C, Baldwin ME, Thornton GE, Williams RA, Prevo R *et al* (2001). VEGF-D promotes the metastatic spread of tumor cells via the lymphatics. *Nature Medicine.* **7:** 186-91.

Van den Eynden GG, Van der Auwera I, Van Laere SJ, Huygelen V, Colpaert CG, van Dam P *et al* (2006). Induction of lymphangiogenesis in and around axillary lymph node metastases of patients with breast cancer. *Br J Cancer* **95:** 1362-6.

Wong SY, Hynes RO (2006). Lymphatic or Hematogenous Dissemination: How Does a Metastatic Tumor Cell Decide? *Cell Cycle* **5:** 812-817.

TITLES AND LEGENDS TO FIGURES

Figure 1. Angiogenesis in carcinomas from wild type and Arf +/- mice.

Immunohistochemical staining used LYVE-1 lymphatic endothelial antibody (left panels) and MECA-32 vascular endothelial antibody (right panels) with purple staining secondary antibody detection, and methyl green counterstaining. Untreated wild type skin (A and B), wild type papilloma (C and D), wild type carcinoma (E and F), Arf +/- papilloma (G and H), and Arf +/- carcinoma (I and J) samples are shown. Lymphatic vessels (arrows) are occasionally observed in the dermis of normal skin, papillomas, and carcinomas from wild type or Arf +/- mice. Blood vessel size and density (arrowheads) increases modestly in papillomas, and extensively in carcinomas from wild type or Arf +/- mice. Scale bar 100 μm.

Figure 2. Lymphangiogenesis in papilloma-draining LNs.

Inguinal LN sections from untreated wild type mice (A and B), TPA-treated wild type mice (C and D), papilloma-bearing wild type mice (E and F), TPA-treated Arf +/- mice (G and H), papilloma-bearing Arf +/- mice (I and J), or papilloma-bearing p53 +/- mice (K and L) were immunostained with 10.1.1 lymphatic endothelial antibody (left panels) or with MECA-32 vascular endothelial

antibody (right panels). Control LNs show lymphatic sinuses (A) in the cortex ("C") but not in the medulla ("M"), and in the septum between the paired inguinal LNs (arrow). Small blood vessels (arrowhead) and high endothelial venules (arrow) are found throughout control LNs (B). LNs from papilloma-bearing mice show increased and abnormal lymphatic sinuses compared to LNs from control or TPA only-treated wild type mice, while blood vessels do not increase. Papilloma-draining LNs from Arf +/- and p53 +/- mice show much more extensive lymphangiogenesis than LNs from wild type mice. Scale bar 100 μm.

Figure 3. Lymphatic sinus area increases in papilloma- and carcinoma-draining lymph nodes.

Quantification of lymphatic sinus area (A) shows that sinuses are significantly increased ($p < 0.0001$) in LNs draining papillomas in wild type, Arf +/-, and p53 +/- mice, and in LNs draining carcinomas in wild type mice, relative to untreated controls (WT), while TPA treatment alone had no significant effect. Lymphatic sinuses were significantly increased further in LNs from Arf +/- or p53 +/- papilloma-bearing mice relative to LNs from wild type papilloma-bearing mice ($p < 0.01$). In contrast, blood vessel area (B) is not significantly different in any condition, by two-tailed Students t test. Lymph node sections were sampled at least 3 times through 5 sectioned LNs in each condition. Standard errors are shown.

Figure 4. Dynamic changes in lymphatic sinuses of carcinoma-draining lymph nodes.

Lymph node sections were immunostained with 10.1.1, LYVE-1, or podoplanin lymphatic endothelial antibodies, or with the MECA-32 vascular endothelial antibody, as indicated. Carcinoma-draining LNs from wild type mice (A) show much more extensive lymphatic sinuses (arrow) than those of Arf +/- mice (C), while blood vessels are similar in carcinoma-draining LNs from wild type (B) and Arf +/- (D) mice. These LNs do not contain detectible metastases. Carcinoma-draining LNs from Arf +/- mice that contain metastatic tumors ("T", to left of dashed line) are shown in panels E to H. Lymphatic sinuses positive for 10.1.1 (E), LYVE-1 (G) or podoplanin (H) are observed adjacent to tumors and also within the metastasis (arrow, G). Blood vessels are found within and adjacent to LN regions containing metastases (F). Metastatic carcinoma cells sometimes replace the entire draining LN in Arf +/- mice, and these LNs show greatly reduced lymphatic sinuses (arrow, I), while they undergo extensive blood vessel growth (J). Scale bar 100 μm.

Figure 5. Macrophage accumulation in tumors and tumor-draining lymph nodes

Macrophages identified by immunohistochemical staining with F4/80 antibody are detected in papillomas (arrow, B), and are major constituents of carcinomas (C), while they are not found in untreated skin (A) from control wild type mice. Macrophages are found in the capsule (arrow) but not in the cortex

("C") or medulla ("M") of LNs from untreated mice (D). Macrophages appear throughout LNs draining papillomas of wild type mice (E), and show increased accumulation in LNs from Arf +/- (F) or p53 +/- (G) papilloma-bearing mice. Carcinoma-draining LNs from wild type (H) mice show extensive macrophage accumulation, which is further increased in carcinoma-draining LNs from Arf +/- mice (I). Scale bar 100 μm.

Figure 6. B cell accumulation in tumor-draining LNs predicts LN lymphangiogenesis.

B cells were immunostained with FITC-labelled B220 antibody in all panels, and nuclei were counterstained with DAPI in panels A and I. A). Papillomas from Arf +/- mice do not contain B220-positive B cells. B). B cells are confined to the cortex ("C"), and are rarely found in the medulla ("M") of untreated wild type mice. C). B cell follicles (arrows) and individual B cells are detected in papilloma-draining LNs from wild type mice. D). B cell follicles grow and spread in carcinoma-draining LNs from wild type mice. E). B cells completely fill papilloma-draining LNs in Arf +/- mice. F). B cells completely fill papilloma-draining LNs in p53 +/- mice. G). Untreated Arf +/- LNs show normal pattern of cortical B cells. H). Carcinoma-draining LNs of Arf +/- mice show irregular B cell follicles (arrows). I). LNs containing metastases from Arf +/- mice show exclusion of B cells from the tumor region ("T"), to the right of the dashed line. Scale bar 100 μm.

Figure 7. VEGF-A levels in papillomas or draining lymph nodes do not predict lymphangiogenesis.

A). VEGF-A abundance was measured in papillomas from wild type, Arf +/-, or p53 +/- mice by ELISA assay. B). VEGF-A abundance in control wild type LNs and in wild type or Arf +/- papilloma-draining LNs. VEGF-A levels significantly decreased in Arf +/- papilloma-draining LNs relative to wild type LNs, by two-tailed Student's t test ($p < 0.02$). Standard errors are shown.

Supplementary Figure 1

p53 deficiency has the same effect on tumor-draining lymph nodes as Arf deficiency.

Purple immunostaining lymphatic sinuses and blood vessels in p53-deficient control (A, B) and carcinoma-draining lymph nodes (C, D). F4/80 macrophages (E, F) and FITC-B220 fluorescent B cells (G, H) in control and carcinoma-draining p53 +/- lymph nodes.