Genetic ablation of SOX18 function suppresses tumor lymphangiogenesis and metastasis of melanoma in mice

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ABSTRACT

The lymphatic vasculature provides a major route for tumor metastasis and inhibiting neo-lymphangiogenesis induced by tumors can reduce metastasis in animal models. Developmental biology studies have identified the transcription factor SOX18 as a critical switch for lymphangiogenesis in the mouse embryo. Here, we show that SOX18 is also critical for tumor-induced lymphangiogenesis and we demonstrate that suppressing SOX18 function is sufficient to impede tumor metastasis. Immunofluorescence analysis of murine tumor xenografts showed that SOX18 is re-expressed during tumor-induced neo-lymphangiogenesis. Tumors generated by implantation of firefly luciferase-expressing B16-F10 melanoma cells exhibited a reduced rate of metastasis to the regional draining lymph node in Sox18-deficient mice, as assessed by live bioluminescence imaging. Lower metastatic rates correlated with reduced tumoral lymphatic vessel density and diameter, and with impaired drainage of peri-tumoral injected liposomes specific for lymph vessels from the sentinel lymph nodes. Overall, our findings suggested that SOX18 induction is a key step in mediating tumor lymphangiogenesis and metastasis, and they identify SOX18 as a potential therapeutic target for metastatic blockade.
INTRODUCTION

Lymphatic vessels play crucial roles in the drainage of lymph from interstitial spaces, in regionalized immune responses and in the transport of fatty acids (1). Reduced function of the lymphatic system contributes to the development of lymphedema and various inflammatory disorders, and appears to play a role in obesity (2). Growth of lymphatic vessels from pre-existing vessels (neo-lymphangiogenesis) is regionally induced during tumorigenesis, and a number of independent studies have now shown that inhibiting tumor-induced neo-lymphangiogenesis can dramatically reduce the metastatic spread of cancer in mouse models (3, 4).

The importance of the lymphatic vasculature during cancer metastasis has emerged over the past decade, such that regional lymph node metastasis is now one of the key factors used for tumor staging and cancer prognosis (5, 6). While the fate of transitioning cancer cells in the sentinel lymph node (the first lymph node reached by metastasizing cancer cells from a primary tumor) is still debated, there is a clear gap in our knowledge of the cellular and molecular mechanisms that drive lymph node metastasis. It has been established that cancer cells can invade the pre-existing peri-tumoral lymphatic vessels to further colonize regional lymph nodes. It is also known that lymphatic vessel density correlates with the incidence of lymph node metastasis (4, 5). Lastly it has been shown that the primary tumor has the ability to induce neo-lymphangiogenesis in the lymph node itself, so as to establish a “platform” from which cancer cells can disseminate (7-9).

To date it seems that a combination of growth factors produced by the tumor and the host plays a central role in remodelling the preexisting lymphatic vasculature and controlling neo-lymphangiogenesis associated with cancer progression (6, 10). Studies in humans have
determined a direct correlation between VEGF-C / -D and lymphatic invasion, lymph node and distant organ metastasis (6, 9, 11). Neutralization of VEGF-D with specific antibody, or genetic deficiency of VEGF-D appears to suppress tumor metastasis in mice (12, 13). VEGF-C has been shown to induce inter-cellular gaps that facilitate entry of tumor cells into the lumen of the vessels (14). Anti-VEGF-R3 blocking antibody or VEGF-C/-D trap strategy (involving a soluble VEGF-R3 immunoglobulin G Fc-domain fusion protein) has been shown to reduce the rate of lymph node metastasis in mouse models by 60–70 % (5, 6, 9, 15-17). To date, modulation of the expression or activity of these growth factors or their receptors or co-receptors (e.g. Neuropilin-2 (3, 18)) has been the only therapeutic strategy considered for blocking tumor spread via inhibition of neo-lymphangiogenesis. Further molecular targets need to be identified with a view to uncovering novel approaches that could complement anti-VEGFs strategies.

Pathological lymphangiogenesis is most likely to recapitulate genetic programs involved during embryonic development. Recent progress in the field of embryogenesis has provided molecular insights into central mechanisms that underpin lymphatic vessel formation associated with tumor growth. During development, lymphatic vessels arise from pre-existing vessels (either veins or pre-existing lymphatics) by the specification, budding, migration, proliferation and differentiation of lymphatic endothelial cell (LEC) precursors (see (1) for review). In developing mouse embryos, the earliest lymphatic endothelial cells arise from a pool of precursor cells in the wall of the cardinal vein (19-21). We recently demonstrated that these precursors express the transcription factor SOX18, which drives the molecular program of lymphangiogenesis. SOX18 belongs to the SRY-related HMG domain family of developmental transcription factors; it selectively binds the heptameric consensus DNA sequence, 5'ATAATA/TCAA^T^G-3' (22, 23) and activates transcription via a trans-activation domain adjacent and C-terminal to the HMG domain (22). During embryogenesis, Sox18
gene expression begins at 9 dpc in endothelial cells in the dorso-lateral side of the cardinal vein, where it induces LEC fate by directly regulating expression of the gene Prox1 (20). By 13.5 dpc, Sox18 is expressed in LECs that form the lymphatic plexus of the embryo. At 14.5 dpc, Sox18 expression is down-regulated in lymphatic endothelial cells (20), consistent with a transient, “switching” role in LEC fate specification.

In the adult mouse, SOX18 expression is not detected in LEC and is not required for the maintenance of the lymphatic phenotype. By contrast, under pathological conditions such as wound healing or tumor growth, SOX18 is re-expressed in endothelial cells of the neo-formed blood vasculature (24, 25), where it is involved in endothelial cell proliferation and migration, and the establishment of vascular integrity (25, 26). However, re-expression of this gene in LECs in pathological situations has not been evaluated.

In humans, clinical work has revealed that SOX18 is a key indicator with which to stage gastric tumor progression and might be used as a prognostic marker (27). Recently, methylation-sensitive melting analysis has identified heterogeneity of methylation levels within the promoter of SOX18 in non-small cell lung cancer (28). This high-resolution genomic approach suggests that SOX18 amongst other genes might undergo epigenetic changes during the early stages of tumorigenesis.

In this study, we use in vivo imaging to uncover Sox18 function in LECs during tumor-induced neo-lymphangiogenesis. In mouse mutants with impaired Sox18 function, lymph node metastasis of induced melanoma was impaired, correlating with reduced peri-tumoral lymphatic vessel density and function. We thus identify a novel class of molecular target that could play a pivotal role in the control of lymphatic specific spread and cancer progression.
MATERIALS AND METHODS

Mouse strains

Sox18+/− mice on a pure C57Bl/6 background were generated and genotyped as previously described (29, 30). Procedures involving animals conformed to institutional guidelines (University of Queensland Animal Ethics Committee).

Cell line

The murine melanoma cell line B16-F10-luc2 was purchased from Caliper Life Sciences in August, 2008. This cell line has been authenticated and tested for contaminants by performing PCR for ectromelia, EDIM, Hantaan, K virus, LCMV, LDEV, MAD, mCMV, MHV, MNV, MPV, MTV, MVM, Mycoplasma sp., Polyoma, PVM, REO3, Sendai, TMEV GDVII. All test results were negative (Final report of Laboratory examination, MU Research Animal Diagnostic Laboratory, Case number: 15928-2008). After authentication, to avoid maintaining the cell line in culture beyond a six month period and reaching a high passage number, aliquots were frozen and stored in liquid nitrogen. Prior to each tumor injection experiment, a fresh aliquot of cells was thawed and cultured in DMEM (Invitrogen) containing 10% FBS.

Tumor model

Tumors were induced using a lymph node metastasis melanoma cell line tagged with the firefly luciferase reporter gene (B16-F10-luc2, Caliper Life Sciences). Wild type and Sox18+/− adult mice (6-8 weeks) were injected subcutaneously on the left flank, 1 cm above the tail with 2x10⁵ B16-F10-luc2 cells (100μl) in order to trigger tumor formation. Tumor growth was observed over a period of 2 weeks until it reached a volume of around 1 cm³. Tumor volume was calculated using the formula width² x length x 0.52 (mm³).
Liposomes
Liposomes were prepared by the film hydration/extrusion method (31). DOPC and PEG-DSPE (95:5 mol %, 3.9 mmol/L total lipid concentration), co-dissolved in chloroform, were dried under nitrogen. The lipid film, kept under vacuum overnight, was then hydrated with an isotonic glucose solution (5%, w/v) containing indocyanine green (ICG) (15 μmol/L, lipid/dye molar ratio of 260). The ICG-containing dispersion was freeze-thawed 6 times and extruded 10 times through double-stacked 50-nm pore size polycarbonate membranes using a Lipex thermobarrel extruder (Northern Lipids) to yield small unilamellar vesicles. Free dye was removed by size exclusion chromatography on a PD MidiTrap G-25 column (GE Healthcare) using glucose buffer as eluent (32). Filtered liposomes had a mean diameter of 60.3 ± 2.1 nm (n=3, mean ± SD) as measured by dynamic light scattering (DelsaNano Zetasizer. Beckman-Coulter, Inc.).

In vivo near infra-red (NIR) fluorescent imaging
Mice were anesthetized with 2% isofluorane, and the fur was removed from the left flank using a shaver and topical hair remover cream. The mice were then positioned inside an IVIS Spectrum (Xenogen, Caliper Life Sciences) on their right lateral side, and precontrast injection images were taken to establish background signal intensities at the tissues of interest. The imaging parameters were as follows: λex = 745 nm, λem = 840 nm, exposure time = 6 seconds, f/stop = 2, medium binning, field of view = 6.6 x 6.6 cm². Five microliters of the liposomal ICG (15 μmol/L) were intradermally injected at the border of the primary tumor (32). Immediately after injection, serial images were acquired every 30 seconds for 25 minutes. For image analysis, Living Image software (Caliper LifeSciences) was used. Regions of interest (ROI) were placed over the inguinal lymph node. Average signal intensity values were recorded for each ROI and plotted versus time in GraphPad Prism. For assessments of flow through the inguinal lymph node, the data were analyzed via normalizing the values based on
a percentage of total enhancement (maximum average ROI signal intensity minus baseline average ROI signal intensity). Beginning at the time point of maximum enhancement in the inguinal lymph node, the data were fit to an exponential decay model as previously described (32). This analysis yielded decay rate ($K_{LN}$) and half-life values as measures of lymphatic flow through tumor draining lymph nodes.

**In vivo bioluminescent imaging**

The IVIS Spectrum was also used for *in vivo* bioluminescent imaging of B16-F10-luc2 tumors and draining lymph node metastases. Mice were injected intraperitoneally with 150 mg/kg body weight D-luciferin substrate (Caliper Life Sciences). Pilot studies revealed that the peak bioluminescent intensity of tumors was reached about 25 minutes after D-luciferin injection; therefore, this time point was chosen for imaging. Images of the tumor in the flank were taken under the following settings: exposure time = 5 seconds, f/stop = 1, medium binning, field of view = 6.6 x 6.6 cm$^2$. Afterwards, the tumor was covered with black tape and one image (exposure time = 3 minutes, f/stop = 1, medium binning, field of view = 3.9 x 3.9 cm$^2$) of the region comprising the inguinal lymph node was collected. On the final day of imaging, mice were sacrificed via cervical dislocation and images of the dissected lymph nodes were taken with exposure time of 1 minute. Living Image software was used to quantify the bioluminescent signal, reported as units of tissue radiance (photons/s/cm$^2$/sr).

**Immunofluorescence**

Tissues were fixed in 4 % paraformaldehyde (PFA) at 4 °C, and embedded in OCT. Cryosections (10 μm) were incubated first with blocking solution (100 mM maleic acid pH 7.4, 10 % horse serum in phosphate-buffered saline [PBS] containing 0.1 % Triton X-100 [PBSTx]) at room temperature for 1 hour, then with primary antibody diluted in blocking solution overnight at 4°C. Samples were washed with PBSTx at room temperature. Secondary
antibody was applied at room temperature for 1 hour. Slides were mounted in PBS/70% glycerol. Samples were analysed using a confocal microscope (Zeiss Axiovert 200 inverted, motorised XYZ) and digitally photographed.

Antibodies

Antibodies were used in the following dilutions: rabbit polyclonal anti-mouse SOX18 (Aviva systems biology), 1:1000; rabbit polyclonal anti-mouse LYVE-1 (Fitzgerald Industries), 1:1000; rat anti-mouse PECAM-1 (BD Pharmingen), 1:200; Ab-1 (clone HMB45) mouse anti-GP100 (Lab Vision, Thermo Fisher Scientific), 1:200; rabbit polyclonal anti-firefly Luciferase (abcam, Sapphire Bioscience), 1:200; goat polyclonal anti-mouse VEGF-R3 (R&D systems), 1:500. Secondary antibodies anti-rat IgG Alexa 594, anti-rabbit IgG Alexa 594, anti-chicken IgG Alexa 488, and anti-mouse IgG Alexa 488 (Molecular Probes) were used at a dilution of 1:200.

Quantitation of lymphatic vessel density and diameter

The lymphatic vessel density quantitation was performed as previously described (33). Briefly, apical photographs of frozen sections 20 x magnification were coded using LVAP plug-in (Image J software) and overlaid with an 80 x 5 grid. The number of lymphatic vessels (double stained with LYVE-1 and PECAM) and blood vessels (stained with PECAM) per square were counted in a blinded fashion, and averaged for an entire image. The lymphatic vessel diameter was measured and the average patent luminal area was quantitated by using Image J software analysis as previously described (33). The averaged field counts for each parameter were collated for all sections before graphical comparisons between phenotypes were generated, and their respective statistical significance determined by Student’s t test.
Statistical analysis

Statistical significance of differences between paired groups was determined using the Student's *t* test. We calculated 95% confidence intervals for all quantitative analysis of lymphatic vessel density, lymphatic vessel diameter and the fluorescence intensity kinetics. Unpaired *t* tests were used for statistical analysis between groups for the decay rate and half-life assessment. All statistical tests were two-sided and *P* values less than 0.05 were considered to be statistically significant.
RESULTS

SOX18 is re-expressed in LECs during tumor growth

The critical role of Sox18 in regulating lymphatic endothelial specification during embryonic development (20), and its re-expression in the blood vasculature under pathological conditions (24, 25) suggested a potential role for this transcription factor in lymphatic endothelial cells during neo-lymphangiogenesis associated with tumors. The first indication of the role of Sox18 function in tumor lymphangiogenesis was uncovered through preliminary transcriptional profiling of laser capture micro-dissected LECs from a human tumor xenotransplant (human MDA-MB-435) in nude mice. In this model, in situ hybridization for Sox18 validated that this gene was expressed in LYVE1-positive vessels (Fig. Sup.1).

We also used a second tumor model where B16 melanoma cells were injected into C57Bl/6 mice. The expression of SOX18 was assessed by immunofluorescence along with the lymphatic specific marker VEGFR-3 and the panendothelial cell marker PECAM, two weeks after cancer cell injection. SOX18 re-expression was detected throughout the tumor vasculature (Fig. 1A, asterisks) that included VEGFR-3-positive lymphatic vessels (Fig. 1A merge, arrows), VEGF-R3-negative vessels (Fig.1A merge, arrowhead), and also in a discrete population of other non-endothelial cells. Importantly, a lack of SOX18 expression was observed in the quiescent vasculature of skin tissue outside the peri-tumoral area (Fig. 1B merge, arrows and arrowheads), whereas SOX18 expression was clearly visible around hair follicles as an internal positive control (Fig.1B, hf). A schematic representation of SOX18 positive vessels is shown in Fig. 1C, and the quantification of SOX18-positive lymphatic vessels is shown as a graph on Fig. 1D. These observations confirm the expression of SOX18 in lymphatic vessels associated with tumor growth. Here, we focus on the role of SOX18
function in tumor-induced lymphatic vessels, and how its re-expression affects tumor lymphangiogenesis and metastasis.

**Defective Sox18 function is associated with impaired lymphatic drainage in a melanoma mouse model**

To understand the role of SOX18 re-expression in LECs associated with developing tumors, we assessed the impact of partial loss of Sox18 function on the lymphatic vasculature draining activity at the tumor site. Animals null for Sox18 gene die *in utero* due to the lack of lymphatic vasculature, hence experiments were performed using viable, heterozygous Sox18-mutant mice in a C57Bl/6 background. We used this genetic background so as to avoid any rescue from Sox7 and/or Sox17, which act as strain-specific modifiers of Sox18 lymphangiogenic function on other genetic backgrounds (30). To induce tumor growth, B16-F10-luc2 melanoma cells were implanted subcutaneously in the lower flank of wild type and Sox18-mutant mice. Twenty days after implantation, the function of neo-formed lymphatic vessels at the tumor periphery was assessed using liposomes loaded with the near infrared dye indocyanine green (LP-ICG) (32). Peri-tumoral injection of LP-ICG at the tumor site followed by dynamic near infrared fluorescent imaging allowed us to visualize and quantitate the bio-distribution of LP-ICG in live mice in real time.

Specific uptake of LP-ICG by lymphatic vessels rapidly led to detectable fluorescence in the tumor draining lymph node. Pictures were recorded every 30 seconds with 6 seconds exposure in order to characterise the kinetics of the dye uptake. Qualitative comparison of the signal intensity dynamics in the tumor draining lymph node of wild type and Sox18-mutant mice suggested slower dynamics of LP-ICG accumulation in the tumor draining lymph node in the Sox18-mutant mice compared to wild type mice (Fig. 2A, B).
To quantitate the data, fluorescence intensity was measured over a period of 25 minutes using the tumor draining lymph node as the region of interest (Fig. 2A, B and supplementary movies 1 and 2). We measured both the clearance rate ($K_{LN}$) of fluorescence from the primary tumor (Fig. 2C) and the half-life of the fluorescence in the tumor draining lymph node (Fig. 2D). Clearance rate was reduced in Sox18-mutants ($n=4$) to ~20% of the rate observed in wild type controls ($n=6$) (Fig. 2C), while half-life increased approximately 4-fold (Fig. 2D). These data indicate that even partial loss of Sox18 function has a dramatic effect on the tumor’s ability to support lymphatic drainage.

**Disruption of Sox18 function impairs neo-lymphangiogenesis during tumor progression**

To investigate the cause of the impaired lymphatic drainage, we next assessed lymphatic vessel density at the tumor. Melanoma tumors were collected 20 days after implantation of B16 cells; frozen sections of tumors from wild type or Sox18-mutant animals were analysed by immunofluorescence using the lymphatic-specific marker LYVE1 and the panendothelial cell marker PECAM1. Neo-lymphangiogenesis at the tumor margin was robust in wild type animals (Fig. 3A, arrows) but poorly developed in the Sox18-heterozygous mice (Fig. 3B, arrows). Quantitation of lymphatic vessel density at tumor margin and intratumoral area confirmed a ~50% decrease in lymphatic vessel density (Fig. 3C; $n=9$). In addition to a lower lymphatic vessel density, tumor tissues of the Sox18 animals also displayed smaller vessels with diameter ~40% of wild type, as revealed by a double staining for LYVE1, PECAM (Fig. 3D) followed by quantitative analysis (Fig. 3E; $n=9$). These qualitative and quantitative analysis of tumor-induced lymphatic vessels revealed that the lack of functional lymphatic drainage in Sox18 heterozygous mice correlates with, and likely results from, impaired tumor neo-lymphangiogenesis.
Recent studies have shown that primary tumors have the capacity to actively induce sentinel lymph node neo-lymphangiogenesis prior the onset of metastasis (7, 8, 34, 35). This mechanism relies at least in part on stimulation by VEGFs that are produced by the primary tumor and transported by lymphatics to the sentinel lymph node. In order to assess lymph node neo-lymphangiogenesis as a predictive marker of metastasis, immunofluorescence using LYVE1 and PECAM antibodies was performed on tumor draining lymph nodes from wild type and Sox18-mutant mice after twenty days of B16 tumor growth. Wild type animals displayed a high density in lymphatic vessels throughout the whole tumor-draining lymph node (Fig. 4A, i, ii), whereas few lymphatic vessels were formed in the Sox18-mutant animals (Fig. 4B, i, ii). Lymph nodes from the contralateral side were used as negative control to illustrate the physiological distribution of the lymphatic vasculature in non-inflammatory conditions (Fig. 4A, B, iii). Further, histological analysis of the lymph nodes from mice of both genotypes revealed no obvious defect that would suggest impaired lymph node development in the Sox18-mutant mice (Fig. Sup. 2). Quantitation of the lymphatic vessel density in the tumor draining lymph nodes further validated the immunofluorescence analysis (Fig. 4C; \( n=5 \)). These data reveal that partial loss of Sox18 function compromised the ability of the primary tumor to induce distant neo-lymphangiogenesis in the tumor draining lymph node.

**Partial loss of Sox18 function decreases the rate of cancer cell metastasis in a melanoma mouse model**

Defective neo-lymphangiogenesis in the tumor draining lymph node of Sox18 mutant animals is an indirect indication that metastatic spread from the primary tumor might also be altered in this mutant. To investigate this possibility, B16-F10-luc2 melanoma cells expressing firefly luciferase were injected into wild type and mutant mice, and the distribution of luciferase-expressing cells was visualized *in vivo* 20 days after injection using bioluminescence imaging.
No significant difference in fluorescence intensity was observed in the primary tumors between wild type and Sox18-mutant animals, but the intensity of fluorescence was so high that the primary tumors needed to be physically masked using black tape during imaging of the metastastic cells (Fig. 5A, B). After masking, luciferase signals could be seen to be associated with the inguinal lymph node in the majority of wild type mice ($n=9$, Fig. 5A). By contrast, no signal could be observed in most Sox18-mutant mice ($n=8$, Fig. 5B). After terminal anaesthesia, tumor draining lymph nodes were dissected and ex vivo imaging was performed to confirm the origin of the luciferase signal (Fig. 5A, B, inserts). In this way, cancer cell metastasis was found to occur at a rate of 66.7% in wild type animals but only 37.5% in the Sox18-mutant animals (Fig. 5C). Thus, even heterozygous mutation of Sox18 resulted in a dramatic decrease in metastasis of induced melanoma tumors.

This difference in the frequency of metastasis rate was not due to a disparity of the primary tumor volume, which was equivalent in both wild type and mutant mice (Fig. 5D). This similar tumor growth rate between wild type and Sox18 heterozygous mice presumably reflects the fact that there is no significant difference in tumor blood vessel density between the two mice group (Fig. Sup. 3B). Gross analysis of tumor draining lymph nodes compared to contro-lateral lymph node used as internal control in wild type animals (Fig. 5E, upper panel) and Sox18 mice (Fig. 5F, upper panel) revealed no major difference at the macroscopic level, nor revealed any melanin pigmented cell clusters in either group as melanin expression is lost during migration through lymphatics (36). However, immunofluorescence analysis of lymph node frozen sections using the cancer marker GP100 (red) combined with the transgenic marker luciferase (green) confirmed that metastatic B16-F10 cancer cells more aggressively invaded wild type tissue (Fig. 5E, lower panel) than tissues from the Sox18-mutant mice (Fig. 5F, lower panel).
Taken together, these data show that the low rate of metastasis that occurs in Sox18-mutant mice correlates with poor neo-lymphangiogenesis that takes place in tumor draining lymph node and at the tumor margin. These findings establish that Sox18 function is a critical modulator of neo-lymphangiogenesis in the context of tumor biology.
DISCUSSION

Over the past two decades, the major focus in the analysis of molecular mechanisms that drive neo-lymphangiogenesis and cancer metastasis has been signalling through VEGF-C and –D, and their membrane receptor VEGF-R3 (4, 5, 15). Molecular mechanisms unrelated to this axis have been disregarded, leaving a gap in the current knowledge of the transcriptional control of pathological lymphangiogenesis and limiting the scope of potential novel therapeutic avenues. In this study, we applied in vivo imaging techniques combined with molecular analysis to identify Sox18 as a critical regulator of neo-lymphangiogenesis, and reveal the ability of Sox18 to modulate the capacity of melanoma cells to metastasize to the regional lymph node.

SOX18 and VEGF: Independent pathways or downstream signalling?

During tumorigenesis, a combination of growth factors and cytokines secreted by the tumor and the host tissue triggers remodelling of the pre-existing lymphatic vascular network that will further expand, increasing in vessel density but also in vessel diameter (9, 37, 38). Our finding that partial loss of Sox18 function compromises tumor lymphatic vessel density and diameter suggests that SOX18 might be also involved in the modulation of the response by the lymphatic endothelium to VEGF-D/C or interleukin signals (37, 39). These observations contrast with our previous analysis of the non-pathological lymphatic vasculature that revealed higher vessel density (Fig. Sup. 3A), with a lymphatic vascular network made of thinner and smaller vessels, in the Sox18+/- adult mice compared to wild type littermates (20). It may be that decreased Sox18 function during tumorigenesis compromises the ability of the existing lymphatic vascular bed to fully respond to growth factors secreted by the tumor.
It also has been shown that VEGF-A and –C are capable of inducing distant lymphangiogenesis in the sentinel lymph node (7, 34, 35). A potential cross-talk between VEGF pathways and SOX18 is indicated by the observation of decreased lymphatic vessel density in the tumor-draining lymph node of the Sox18 mutant mice. It remains unclear at what level VEGF-C/D are connected to the SOX18 pathway. The first level of interaction could be a modulation of SOX18 activity induced by these growth factors. Alternatively, SOX18 might drive directly the transcriptional activation of Vegf-r3 as part of a feedback positive loop to increase sensitivity to VEGF signalling.

The control of lymphatic vessel remodelling by SOX18

The expression pattern of SOX18 observed by immunofluorescence and in situ hybridisation (Fig. 1; Fig. Sup1) in this study revealed that this gene is re-expressed in VEGFR3+/LYVE1+ lymphatic vessels in the tumor margin. Small vessels such as capillaries or pre-collecting vessels are more prone to undergo remodelling when compared to bigger vessels (e.g. collecting vessels) mostly due to the lack of other cell types such as smooth muscle cells that are associated with the capillary endothelium and due to the nature of their cell-to-cell junction (40). The re-expression of Sox18 in the most plastic lymphatic vessel subtypes is an indirect indication that this transcription factor may be also involved in modulating the remodelling of the lymphatic network in a tumor microenvironment.

Sox18 function may play a central role in endothelial cell assembly, for example by controlling cell-to-cell junctions (41). A dominant negative form of Sox18 has been shown to inhibit proper assembly of cells into vessel-like structures (25), supporting a role in tube formation. In addition, SOX18 has been shown to directly regulate vascular adhesion molecule-1 expression (VCAM-1) (42). VCAM-1 plays a critical role in endothelial
activation (43), and therefore can contribute to the lymphatic remodelling during tumorigenesis.

Furthermore, interactions between tumor cell surface receptors and endothelial cell adhesion molecules are thought to contribute to tumor cell arrest and extravasation during hematogenous metastasis. It was shown that melanoma cell integrin alpha4beta1 (very late antigen-4, VLA-4) interaction with VCAM-1 is critical for tumor cell arrest (44). In addition, organ-specific increases in VCAM-1 expression correspond with reported clinical patterns of melanoma metastasis (45, 46). The re-expression of SOX18 in the lymphatic endothelium associated with tumor progression may induce the expression of this adhesion molecule, which in turn can facilitate the escape of cancer cells into lymphatics. Taken together, these observations suggest a potential role for SOX18 in modulating the interaction between lymphatic endothelium and cancer cells. Further characterization of SOX18 molecular mechanisms will be required to clarify the role of its re-expression in the neo-formed lymphatic vasculature, for example to assess whether SOX18 modulates neo-lymphangiogenesis and simultaneously facilitates the trafficking of cancer cells.

The cellular origin of lymphatic endothelial cells during tumor-induced neo-lymphangiogenesis

According to the embryonic role of Sox18, which consists of trans-activating Proxl expression in the venous endothelium to induce lymphangiogenesis (20), our observation of SOX18 expression in both tumor blood vascular endothelial cells (25) and LECs (this study) raises the question of the cellular origin of neo-formed lymphatic vessels. There is a strong body of evidence in the literature suggesting that neo-lymphatics mainly arise from pre-existing lymphatic vessels that undergo neo-lymphangiogenesis (6, 15, 47). However, it is also possible that blood vascular endothelial cells in a tumor might be reprogrammed by
SOX18 to differentiate into LECs, akin to the mechanism by which lymphatic endothelial cells first arise in the embryo (20). In support of this concept, COUP-TFII, another transcription factor that has been shown to be essential to control PROX1 expression in venous endothelial cells to trigger the lymphatic differentiation program (21), has been also shown to be required for adult lymphangiogenesis in an animal model of cancer (48). The observation that the molecular switches responsible for driving lymphatic endothelial cell differentiation in the embryo are also essential in neo-lymphangiogenesis in cancer models raises the possibility of a multiple cellular origin of the neo-formed lymphatic vasculature in a tumor setting.

Recent studies also revealed evidence of a trans-differentiation program in which bone-marrow-derived macrophages can differentiate into lymphatic endothelium in murine tumor models (49). It is therefore important to determine the cellular differentiation mechanisms that drive tumor lymphangiogenesis. Conditional depletion of the venous endothelium or the macrophage population in a tumor model will yield a definitive answer to this question.

**SOX18 as a molecular target**

Our present data suggest that the transcription factor SOX18 acts at the nexus of the molecular mechanisms responsible for outgrowth of the lymphatic vasculature in a model of mouse melanoma. Specifically, we have illuminated how a developmental program is re-activated in the adult under pathological conditions, and the consequences of its dysregulation. Remarkably, dramatic effects on metastatic rate were observed even though the mutant mice studied were heterozygous mutants, retaining one intact copy of Sox18. In anti-lymphangiogenic approaches, there is a demand to target additional lymphangiogenic molecules that would complement the inhibition of the VEGF family to as an anti-metastatic strategy. Here we have used a classical genetic approach to establish SOX18 as a novel molecular target.
molecular target that could be used to suppress cancer metastasis with few or no side-effects likely on pre-existing lymphatic vasculature, which does not retain Sox18 expression (20). Furthermore, SOX18 has been shown to play a critical role in the initial steps of tumor angiogenesis and subsequent induction of the tumor growth, with Sox18-null mice in a mixed genetic background showing greatly reduced tumor diameter compared to wild type (25), but is not continually required in mature blood vasculature in the adult (50). We expect that effects of genetic suppression of Sox18 activity would be achievable pharmaceutically with appropriate inhibitory molecules. Hence anti-SOX18 molecules may produce a dual effect that would help suppress both cancer growth and metastasis. This approach therefore will need to be validated further via drug screening and medicinal chemistry.
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Figure legends

Figure 1. SOX18 is re-expressed in lymphatic endothelial cells during tumor growth. (A, B) Immunofluorescence analysis of B16-F10-luc2 tumor sections (including normal skin, peri-tumoral skin, tumour margin and tumor tissue) using the lymphatic specific marker VEGFR-3 (green), SOX18 (red) and PECAM (blue). SOX18 is re-expressed in tumoral blood (A, red asterisks and merge arrowhead) and lymphatic vessels (A, red asterisks and merge arrows), whereas, no SOX18 expression could be detected in quiescent lymphatic vessels in skin tissue outside the peri-tumoral area (B, merge). Normal expression of SOX18 in skin hair follicle is indicated by asterisk (B, red, hf); Framed boxes (dashed lines) are shown in higher magnification to visualise SOX18 expression in nuclei of endothelial cells. Scale bars, low magnification A and B, 100 µm; high magnification A and B, 50 µm. (C) Schematic representation of the distribution of SOX18 positive vessels during tumor growth. (D) Quantification of lymphatic vessels expressing SOX18 in and around the tumor.

Figure 2. Defective Sox18 function is associated with impaired lymphatic drainage in a melanoma mouse model. (A, B) In vivo near infrared fluorescent imaging of the dynamics of flow in the tumor draining lymph node after 20 days of B16 tumor growth. Peri-tumoral injection of liposomes containing indocyanine green fluorescent dye (LP-ICG) was performed in the tumor periphery of wild type (A) and Sox18 heterozygous (B) mice. Images (pre-injection) represent the left flank of an anesthetized mouse, fur has been removed and primary tumor covered to prevent fluorescent saturation from the injection site. Dashed lines indicate the mouse body, arrow indicates fluorescent dye uptake into the tumor draining lymph node. (C, D) Quantitative assessments of the fluorescence intensity kinetics detected in the tumor draining lymph node in wild type and Sox18 mutant mice. In mutant animals the lymphatic
clearance is suppressed as represented by a decreased decay rate of signal from the primary tumor (WT, \(n=6\), Sox18 \(n=4\), \(p=0.03\)) (\(K_{LN}\), C) and increased half-life of signal (WT, \(n=6\), Sox18 \(n=4\), \(p=0.01\)) (D) remaining in the lymph node. Y-axis in (D) represents the time for clearance of half of the dye away from lymph node.

**Figure 3. Disruption of Sox18 function induces defective neo-lymphangiogenesis at the tumor periphery.** (A, B) Immunofluorescence on cryosection of B16 melanoma tumor after 20 days of growth using the lymphatic specific marker LYVE1 (green) and the panendothelial cell marker PECAM (red) reveals that lymphatic vessel outgrowth has extensively occurred in wild type animals (A, arrows) but is compromised in Sox18 mutant animals (B, arrows). Arrowheads indicate skin lymphatic vessels; Asterisks indicate tumor blood vessels. Dashed lines indicate the border between skin and tumor tissues. Scale bar, 200 \(\mu m\). (C) Quantitation of lymphatic vessels density of the tumor margin and intra-tumoral area confirmed that neo-formed lymphatics are decreased in Sox18 mutant animals when compared to wild type mice (WT, \(n=9\), Sox18 \(n=9\), \(p=0.0003\)). (D) Immunofluorescence analysis of tumor lymphatic vessels using PECAM (blue) and LYVE1 (red) antibodies revealed that in Sox18-mutant animals, these vessels have a smaller diameter. Scale bar, 100 \(\mu m\). (E) Quantitation of the diameter of tumor lymphatics further validated the decrease in vessel diameter in Sox18-mutant (WT, \(n=9\), Sox18, \(n=9\), \(p<0.0001\) a.u., arbitrary units.

**Figure 4. Impaired neo-lymphangiogenesis occurs in the tumor draining lymph node of Sox18 mutant animals.** (A, B) Immunofluorescence on cryosections of tumor draining lymph node using the lymphatic specific marker LYVE1 (green) and the panendothelial cell marker PECAM (red) reveals that neo-lymphangiogenesis is suppressed in Sox18-mutant mice (B, i) compared to wild type (A, i). Higher magnification images are shown in the centre panels (A, B, ii). Both contro-lateral lymph nodes were used as a negative control for tumor-
induced lymphangiogenesis (A, B, iii). (C) Quantitation of lymphatic vessels density validated the reduced density of vessels in Sox18-mutant lymph nodes. For each genotype analysed (n=5 mice, 3 sections/lymph node, p=0.01). Sections were performed at the same level for each lymph node to compare lymphatic vessel density. Scale bars, B, i 200 μm; B, ii 200 μm; B, iii 100 μm.

**Figure 5. Partial loss of Sox18 function causes a decrease in melanoma metastasis.** (A, B) Luciferase activity was imaged in mice 20 days post-injection of B16-F10 luciferase-expressing cancer cells to assess cancer cell metastasis. Images represent the flank of an anesthetized mouse, with the fur removed and the primary tumor masked with opaque tape. Luciferase signal could be detected originating from the inguinal lymph node in the majority of wild type mice (A, n=9, arrow). No such signal was observed in most Sox18-mutant mice (B, n=8). After terminal anaesthesia, the tumor draining lymph node was dissected and *ex vivo* live imaging performed to confirm the origin of the luciferase signal (A, B insert, lower right). The graph (C) shows the frequency of metastasis according to mouse phenotype. The graph (D) shows that tumor volumes were equivalent in both wild type and mutant mice. (E, F, upper panels) Gross analysis of tumor draining lymph node (L) compared to contralateral lymph node used an internal control (R) reveals no major difference between wild type and Sox18 mice. Immunofluorescence analysis of lymph node frozen sections using the melanoma marker GP100 (red) combined with the transgenic marker firefly luciferase (green) confirmed that metastatic B16-F10 cancer cells more aggressively invade wild type tissue (arrows) (E, lower panel) than Sox18-mutant tissues (F, lower panel). R, right; L, left; Scale bars, F upper panel, 1 mm; lower panel, 100 μm.
REFERENCES


Figure 1
Figure 2

A

B

C

D

Pre-injection

Post-injection of liposomal fluorescent dye

Wild type

Sox18−

0 0.05 0.10 0.15 0.20 (min)

0 75 50 25 0 (min)

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Genetic ablation of SOX18 function suppresses tumor lymphangiogenesis and metastasis of melanoma in mice

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