Tumorigenesis and Neoplastic Progression

Significance of Tumor-Associated Stroma in Promotion of Intratumoral Lymphangiogenesis

Pivotal Role of a Hyaluronan-Rich Tumor Microenvironment

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Stromal cells, together with extracellular matrix components, provide a tumor microenvironment that is pivotal for cancer cell growth and progression. In our previous study using a conditional transgenic mouse model of breast cancer, the overproduction of hyaluronan, a major extracellular constituent, accelerated tumor angiogenesis through stromal cell recruitment. This finding led us to investigate the role of hyaluronan in the lymphatic vessel system. Here, we have found that microenvironmental hyaluronan promoted tumor lymphangiogenesis concurrently with the formation of stromal structures. Additionally, lymphatic vessels frequently penetrated and accumulated into stromal compartments, and up-regulation of vascular endothelial growth factor-C and -D was detected at tumor-stromal interfaces. To assess the contribution of stromal cells to lymphangiogenesis *in vivo*, we established tumor-associated fibroblasts from hyaluronan-overproducing mammary tumors and implanted them together with carcinoma cells from control tumors or MCF-7 human breast carcinoma cells in nude mice. Carcinoma cells grew rap-

idly in association with marked stromal reactions and lymphangiogenesis. Without the stromal cells, however, the tumors developed slowly with less stroma and lymphatic vessels. These findings underline the significance of tumor-associated stroma in the promotion of intratumoral lymphangiogenesis and suggest a pivotal role for the hyaluronan-rich tumor microenvironment. (Am J Pathol 2008, 172:179–193; DOI: 10.2353/ajpath.2008.070360)

Stromal contribution to the development and progression of a wide variety of tumors has been supported by extensive clinical evidence and the use of experimental mouse models of cancer pathogenesis.^{1–6} The cellular compartment of tumor stroma is composed of immune cells, inflammatory cells, pericytes, vascular endothelial cells, and fibroblasts. Each cell type can potentially communicate with others, or with tumor cells, through secretion of growth factors, chemokines, proteases, and extracellular matrix (ECM) components.^{7–9} Cumulatively, these cellular interactions influence the composition and order of the tumor microenvironment to support tumor progression by allowing angiogenesis and facilitating the invasion and metastasis of tumor cells.¹⁰ Fibroblasts contributing to tumor stroma have been termed tumor-associated fibroblasts (TAFs).^{2,11} TAFs have a potent ability to induce neovascularization by secreting angiogenic factors in solid tumor masses and may thus determine the route of metastatic spread.

Lymphangiogenesis has recently gained attention for its potential involvement in lymphatic dissemination and

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metastasis of tumor cells; clinicopathological studies have suggested a strong correlation of lymphatic microvessel density and lymph node metastasis.^{12–15} Vascular endothelial growth factors (VEGF)-C and -D, both known lymphangiogenic factors, play a critical role in the induction of lymphangiogenesis.¹² Recent studies have provided evidence that stromal cells, as well as cancer cells, are capable of secreting many potential lymphangiogenic factors that likely lead to *de novo* formation of lymphatic vessels.^{16,17} Lymphangiogenesis is therefore likely governed by interactions between tumor cells and stromal components, and stromal cells might serve as important mediators for tumor-induced lymphangiogenesis. Despite the importance of tumor-stromal interactions, there is a limited understanding of the complex cross talk between tumor cells and the surrounding stroma in lymphangiogenesis because of the insufficiency of suitable experimental *in vivo* models.

Hyaluronan (HA) is a major constituent of ECM and provides a favorable microenvironment for cell proliferation and migration by maintaining the turgidity and hydration of tissues in addition to activating intracellular signals through interaction with cell surface receptors.^{18,19} HA accumulation is closely related to the clinicopathological malignancy of human cancers.²⁰ Furthermore, the overproduction of HA in tumor cells has been implicated to be responsible for the increased deposition of intratumoral HA; our previous studies using a transgenic mouse model of breast cancer have shown that overproduction of HA in tumor cells accelerated stromal reactions accompanied by formation of intratumoral neovasculature.²¹ Using this spontaneous cancer model, we sought in this study to evaluate the role of HA in tumor lymphangiogenesis, and our results showed that microenvironmental HA indeed played a pivotal role in intratumoral lymphangiogenesis. Additionally, tumor xenograft transplantation studies further demonstrated significance of tumor-associated stroma in intratumoral lymphangiogenesis, suggesting the importance of targeting tumor stroma to prevent lymphangiogenesis and resulting cancer progression.

Materials and Methods

Generation of the Hyaluronan Synthase 2 Transgenic Mammary Tumor Model

The mammary tumor model of hyaluronan synthase 2 (Has2) transgenic mice was generated as described previously.²¹ Briefly, Has2 conditional transgenic (cTg) mice were backcrossed for nine generations to the mouse mammary tumor virus-Neu (MMTV-Neu) mammary tumor model (Charles River Laboratories International, Inc., Wilmington, MA). B6129-TgN (MMTV-cre)4Mam (MMTV-Cre) mice expressing Cre recombinase (Jackson Laboratories, Bar Harbor, ME) were backcrossed for six generations to the MMTV-Neu mice. Next, Has2:Neu bigenic mice bearing both the *Has2* and *neu* transgenes were intercrossed to Cre:Neu bigenic mice bearing both the *Cre* and *neu* transgenes. Founder lineages with a

different combination of three transgenes, MMTV-Neu (Neu), MMTV-Cre/MMTV-Neu (Cre:Neu), CAG-Neo-Has2/MMTV-Neu (Has2^{+Neo}), and CAG-Has2/MMTV-Cre/MMTV-Neu (Has2^{ΔNeo}), were thus generated and genotyped by polymerase chain reaction (PCR) analysis of genomic DNA. All animal care and experimentation were performed according to study guidelines established by the Shinshu University ethics committee for animal care, handling, and termination.

Histological and Immunohistochemical Analyses

Mouse mammary tumors excised from Has2^{ΔNeo} and Has2^{+Neo} mice were immediately fixed in 10% formalin or zinc fixative, dehydrated, and embedded in paraffin wax. Deparaffined sections (5 μ m thick) were rehydrated and stained with anti-CD31 (rat mAb, clone MEC13.3; BD Pharmingen, San Diego, CA), anti-podoplanin (AngioBio Co., Del Mar, CA), anti-Prox-1 (AngioBio Co.), anti-VEGF-C (Santa Cruz Biotechnologies Inc., Santa Cruz, CA), anti-VEGF-D (R&D Systems, Inc., Minneapolis, MN), anti-fibronectin (Dako Japan Co. Ltd., Kyoto, Japan), and anti-type I collagen antibodies (LSL Co. Ltd., Tokyo, Japan). The specimens were also counterstained with the biotinylated HA-binding region of aggrecan (b-HABP; Seikagaku Corp., Tokyo, Japan). Immunolocalization of the antigens were visualized using Alexa Fluor-conjugated second antibodies (Invitrogen, San Diego, CA) under a LSM 510 Meta confocal microscope (Carl Zeiss GmbH, Jena, Germany) or a Zeiss Axiovert 200 fluorescent microscope. Images were exported in TIFF format and analyzed using Metamorph software (Universal Imaging, Sunnyvale, CA). The percentage of podoplanin-positive vessels per objective field was calculated by measuring the positively stained area after immunohistochemical staining with anti-podoplanin antibody. Intratumoral lymphatic vessel refers to lymphatic vessels formed in the central part of tumors, and peritumoral lymphatic vessel indicates lymphatic vessels formed in the peripheral part of tumor tissues (within 300 μ m of the tumor-connective tissue interface). Stroma-associated vessel represents lymphatic vessels formed within stromal structures of the intra- and peritumoral parts.

Total RNA Preparation and Real-Time PCR Analysis

Total RNA was isolated using the RNeasy total RNA isolation kit (Qiagen, Inc., Valencia, CA). Reverse transcription was performed by random priming using a reverse transcription reagent (Takara Biochemicals, Shiga, Japan). Gene expression analyses of VEGF-C and -D were done using TaqMan gene expression assays (Applied Biosystems, Foster City, CA): Mm00437313-m1 for mouse VEGF-C, Hs00153458-m1 for human VEGF-C, Mm00438965-m1 for mouse VEGF-D, and Hs00189521-m1 for human VEGF-D. The expression of *Has* genes was determined using primers and probes specific for murine *Has1*, 2, and 3 genes as previously described.²² The relative amounts of GAPDH mRNA were measured using

TaqMan rodent or human GAPDH control reagents (Applied Biosystems). Relative gene expression was determined by normalizing the amount of each mRNA divided by that of the GAPDH mRNA.

Enzyme-Linked Immunosorbent Assay

Equal amount of tumor tissues (50 mg) were homogenized in lysis buffer containing 50 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 20 mmol/L phenylmethyl sulfonyl fluoride, protease inhibitor cocktail (Roche Diagnostics Corp., Basel, Switzerland). VEGF-D concentrations both in the conditioned media from cell culture and in tissue lysates were determined with a VEGF-D-Mouse DuoSet kit (R&D Systems). Each sample was tested in triplicate in separate measurements using a V_{max} kinetic microplate reader (Molecular Devices, Sunnyvale, CA). Results were calculated with SoftMAX Pro software (Molecular Devices).

Isolation of Primary Mammary Tumor Cells and TAFs

Mouse mammary tumors were excised from Has2^{ΔNeo} and Has2^{+/Neo} mice, and rinsed with phosphate-buffered saline (PBS). The tumor tissues were sliced into 1- to 4-mm³-sized pieces under sterile conditions and enzymatically digested as described previously.⁶ After digestion, large tissue debris was removed by centrifugation using a cell strainer with a 70- μ m nylon mesh (BD Falcon, Bedford, MA). The harvested cells were then cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and insulin-transferrin-selenium (Invitrogen). On reaching confluence, the TAFs were separated from the tumor cells by differential trypsinization as previously described.⁶ For the enrichment of mammary tumor cells and TAFs, differential trypsinization was repeated an additional five to eight times. The released cells were established as TAFs and the remaining adherent cells were established as mammary tumor cells by morphological verification and immunostaining using fibroblast-specific (anti-vimentin; Lab Vision, Fremont, CA), and tumor-specific (anti-pan cytokeratin; Sigma-Aldrich, St. Louis, MO) markers. Both tumor cells and TAFs were subcultured and used within nine passages.

Co-Culture of Tumor Cells and TAFs

Tumor cells were co-cultivated directly or indirectly with TAFs. Indirect co-cultivation was performed using cell culture inserts (0.4- μ m-pore size; Becton Dickinson, Franklin Lakes, NJ). Under these conditions, the diffusion of substances freely occurred without any direct contact between tumor cells and TAFs. TAFs were seeded at 3×10^5 cells/well onto bottom wells of a six-well cell culture plate. Tumor cells were seeded at 3×10^5 cells/well on cell culture inserts and set onto wells. These cells were precultured for 24 hours in Dulbecco's modified Eagle's medium containing 10% fetal calf serum before the start

of co-culture. Afterward, the tumor cells and TAFs were maintained in fresh serum-free Dulbecco's modified Eagle's medium or endothelial cell basal medium-2 (EBM-2) containing 2% fetal calf serum at 37°C in a 5% CO₂ incubator. The conditioned media and total RNA of these cells were recovered at 24 hours of incubation. In direct co-cultivation experiments, tumor cells (2×10^5 cells/dish) and TAFs (2×10^5 cells/dish) were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum for 2 days. Total RNAs were then isolated from these cells and used as templates for real-time quantitative reverse transcriptase (RT)-PCR.

Lymphatic Endothelial Cell Migration Assays

Human lymphatic endothelial cells (LECs) were isolated from patients with breast cancer and characterized by expression of lymphatic markers such as podoplanin and Prox-1 as described previously.²³ LEC migration assays were performed according to a previously published procedure.²⁴ Briefly, conditioned media of tumor cells or TAFs cultured in EBM-2 containing 2% fetal calf serum were recovered as described above. Confluent LEC cultures grown in a 24-well culture plate were scratched with a pipette tip, resulting in a cell-free zone. The remaining cells were washed with PBS and incubated with conditioned media obtained from tumor cell and TAF cultures. After 24 hours, cells were fixed with neutralized 10% formalin and distance from the starting wound edge was measured at three different areas under a Zeiss Axiovert 200 phase contrast microscope.

Tumorigenesis Assays

Tumor cells and Has2^{ΔNeo} TAFs were harvested by trypsinization and resuspended in Hanks' balanced salt solution at 5×10^6 cells/ml. The tumor cell suspensions (200 μ l) were injected with or without 5×10^5 cells of TAFs into the mammary fat pads of mice (8-week-old female BALB/c nu/nu mice; SLC, Shizuoka, Japan). Alternatively, MCF-7 human breast carcinoma cells were suspended in Hanks' balanced salt solution at 5×10^6 cells/ml, and the cell suspensions (200 μ l) were injected subcutaneously with or without 1×10^6 cells of TAFs into 8-week-old female nude mice. Aggregate of rooster comb native HA and human umbilical cord versican was prepared as described previously.²¹ Has2^{+/Neo} tumor cells were injected together with 100 μ g of rooster comb native HA or an equivalent amount of HA-versican aggregates into the mammary fat pads of mice (8-week-old female BALB/c nu/nu mice). In all mice, the appearance of visible tumors was monitored daily by palpation and tumor size was measured using a dial caliper. Tumor volume (V) was determined by the following equation: $V = (L \times W^2) \times 0.5$, where L was the length and W was the width of a tumor. Xenograft experiments were terminated at the end point where tumor size did not exceed a mean tumor diameter of 10 mm or within ~1 month. The subcutaneous tumors were dissected out and weighted at the end point of xenograft experiments.

Statistical Analysis

All results were expressed as mean $\pm$ SEM. Significance of differences was determined using the Student's *t*-test.

Results

HA Overproduction Facilitates Intratumoral Lymphangiogenesis

We previously showed that the overproduction of HA in mammary tumors accelerated angiogenesis through stromal cell recruitment. In this study, we focused our investigation on the role of the HA in lymphangiogenesis. In addressing this issue, we used a conditional transgenic mouse model of breast cancer. These mice express murine hyaluronan synthase 2 (Has2) dependently on Cre-mediated recombination. Has2 ^{Δ Neo} mice actively produce HA in Neu-initiated mammary tumors by specifically expressing Cre recombinase under the control of the MMTV promoter, whereas control Has2^{+/Neo} mice, which lack the recombinase, do not. Podoplanin is generally accepted as a specific marker for LECs,²⁵ therefore we initially immunostained mammary tumor tissues from both Has2 ^{Δ Neo} and Has2^{+/Neo} mice using this antibody and found that podoplanin-positive lymphatic microvessels were formed within Has2 ^{Δ Neo} tumors to a much greater extent than in Has2^{+/Neo} tumors (Figure 1A). These newly formed lymphatic vessels were generally negative for CD31, unlike newly formed blood vessels, which stained strongly for CD31 (Figure 1A). We then confirmed all podoplanin-positive vessels to be lymphatic by immunostaining with Prox-1, another reliable marker for LECs.²⁶ Double immunofluorescence demonstrated that Prox-1 nuclear staining was well co-localized with podoplanin-positive structures (Figure 1A). Quantitative analysis of the average density of lymphatic vessels (LMVD) demonstrated that intratumoral lymphatics were 16-fold greater in Has2 ^{Δ Neo} mice than control Has2^{+/Neo} mice (Figure 1B). On the contrary, peritumoral lymphatic vessels were only 1.5-fold greater in Has2 ^{Δ Neo} mice than control mice, suggesting that the effects of HA are mainly exerted on intratumoral lymphangiogenesis. Microscopically, intratumoral and peritumoral lymphatics were different in their structure; most intratumoral lymphatics were flat and collapsed, whereas the peritumoral lymphatic structures had open lumina (Figure 1C).

Formation of Intratumoral Lymphatics in Association with HA-Rich Stroma

Distribution of HA was examined using biotinylated HABC, a specific probe for HA, relative to the localization of lymphatic vessels (Figure 2A). HA staining was detected at the intercellular boundaries of Has2 ^{Δ Neo} tumor cells, particularly in the tumor stroma. Many HA-rich stromal structures were formed both in the central and peripheral parts of Has2 ^{Δ Neo} tumors, and podoplanin-positive lymphatic vessels were mostly found in association

with HA-rich stroma surrounding Has2 ^{Δ Neo} tumor cell islets. However, only a few HA-rich stromal structures were detected in Has2^{+/Neo} tumors, and lymphatic vessels penetrating the peritumoral part were not associated with these structures. These results underscored the significance of tumor-stromal cell interaction in the promotion of intratumoral lymphangiogenesis. To investigate the relationship between tumor stroma and lymphatic vessels, we performed double immunostaining with antibodies specific for podoplanin and stromal fibronectin or type I collagen (Figure 2B). In Has2 ^{Δ Neo} tumors, podoplanin-positive lymphatic microvessels were found exclusively within the intratumoral stroma, but not between tumor cells. Morphometric analysis indicated that the density of stroma-associated lymphatic vessels was $0.2 \pm 0.1\%$ microvessels/field in control Has2^{+/Neo} tumors, and markedly elevated in HA-overproducing Has2 ^{Δ Neo} tumors ($5.3 \pm 0.5\%$, Figure 2C). CD31-positive blood vessels were also found predominantly in intratumoral stroma, but some vessels were encountered between tumor cells without intervening stroma (data not shown).

Establishment and Characterization of Tumor Cells and TAFs

To evaluate the contribution of tumor and stromal cells to lymphangiogenesis, mammary tumors were surgically excised from individual mice of each genotype, and independent lines of tumor cells and TAFs were established by several rounds of differential trypsinization. The uniformity of the established cells was confirmed by immunofluorescent staining for cytokeratin and vimentin antibodies. Approximately 90% of cells in the tumor cell culture uniformly expressed epithelial cytokeratin but not vimentin, indicating that almost all of the cells were of epithelial origin. The mesenchymal origin of isolated TAFs was evidenced by the lack of cytokeratin expression, the presence of vimentin, and their spindle-cell morphology. The proportion of vimentin-positive cells was more than 96% in the TAF culture.

PCR analysis using genomic DNA isolated from tumor cells and TAFs demonstrated that deletion of the Neo cassette was achieved by Cre-mediated recombination in Has2 ^{Δ Neo} tumor cells, but not in Has2^{+/Neo} tumor cells and TAFs derived from both genotypes (Figure 3, A and B). The lack of the transgene recombination in Has2 ^{Δ Neo} TAFs therefore supports the above notion that they contained a negligible number of Has2 ^{Δ Neo} tumor cells in which the Neo resistance gene had been deleted. As determined by real-time quantitative RT-PCR, Has2 expression in Has2 ^{Δ Neo} tumor cells was 5.4-fold higher than that in Has2^{+/Neo} tumor cells (Figure 3C). TAFs expressed endogenous Has1 as well as Has2 (Figure 3C). Interestingly, endogenous Has2 expression in Has2 ^{Δ Neo} TAFs was equivalent to that of Has2 ^{Δ Neo} tumor cells and 6.7-fold higher than that of Has2^{+/Neo} tumor cells. Has3 expression was not detected at all in either tumor cells or TAFs. Next, *in vitro* co-culture studies were conducted to investigate the synergistic effects of these cells on the

Figure 1. HA overproduction leads to intratumoral lymphangiogenesis in Neu-initiated mammary tumors. **A:** Tissue sections from Has2^{ΔNeo} and Has2^{+Neo} tumors were immunostained with antibodies against CD31 (green), podoplanin (red), and Prox-1 (green). Podoplanin-positive lymphatics were more numerous in the Has2^{ΔNeo} mice compared with Has2^{+Neo} tumors. These newly formed podoplanin-positive lymphatics were generally positive for Prox-1 and negative for CD31, unlike newly formed blood vessels that stained strongly for CD31. **B:** Quantitative analysis of the average density of lymphatic vessels. After immunostaining with anti-podoplanin antibody, the percentage of podoplanin-positive vessels per objective field was calculated, as described in Materials and Methods. Intratumoral lymphatics were 16-fold greater in Has2^{ΔNeo} mice compared with tumors of control Has2^{+Neo} mice. Peritumoral lymphatic vessels were only 1.5-fold greater in Has2^{ΔNeo} mice than control mice. Data represent the mean $\pm$ SE from five random fields of three tumors per genotype. * $P < 0.05$, ** $P < 0.01$. **C:** Immunohistochemical staining for podoplanin in Has2^{ΔNeo} tumors. The intratumoral and peritumoral lymphatics (brown) are morphologically different.

Figure 2. Lymphatic vessels predominantly penetrated and accumulated in HA-rich stroma. **A:** Tissue sections from Has2^{ΔNeo} and control Has2^{+Neo} tumors were immunostained with anti-podoplanin antibody (red) and counterstained with b-HABP (green). Lymphatic vessels (**arrowheads**) were found in association with HA-rich stroma surrounding Has2^{ΔNeo} tumor cell islets, although they were only detected within the connective tissues surrounding Has2^{+Neo} tumors (**arrows**). **B:** Tissue sections from Has2^{+Neo} and Has2^{ΔNeo} tumors were double-stained with antibodies specific for podoplanin (red), type I collagen (Col I, green), and/or fibronectin (FN, green). Cell nuclei were stained with DAPI (blue). In Has2^{ΔNeo} tumors, lymphatic vessels predominantly penetrated and accumulated in stroma positively stained with type I collagen and fibronectin. **C:** Morphometric analysis of the stromal association of lymphatic vessels. The density of stroma-associated lymphatic vessels in Has2^{ΔNeo} tumors was markedly elevated when compared with control Has2^{+Neo} tumors. Data represent the mean $\pm$ SE from five random fields of three tumors per genotype. **P < 0.01.

Has expression. Co-culture of tumor cells and Has2^{ΔNeo} TAFs did not affect Has expression. Consistent with Has2 expression, the amount of HA produced by Has2^{ΔNeo} tumor cells was higher than that of Has2^{+Neo} tumor cells (Figure 3D). Both Has2^{ΔNeo} and Has2^{+Neo} TAFs synthesized higher amounts of HA than Has2^{ΔNeo} tumor cells. Co-cultivation of tumor cells and Has2^{ΔNeo} TAFs increased HA levels in an additive manner. In addition, both Has2^{ΔNeo} and Has2^{+Neo} TAFs were virtually the same in terms of the Has expression and HA production (Figure 3, C and D).

Increased Expression of VEGF-C and -D in Has2-Overexpressing Tumors

VEGF-C and -D are well-characterized lymphangiogenic factors, so real-time quantitative RT-PCR was performed to determine their expression levels in the mammary tumors (Figure 4A). Consistent with elevated lymphangiogenesis, increases in VEGF-C and -D transcripts were detected in the mammary tumors of Has2^{ΔNeo} mice. As assessed by enzyme-linked immunosorbent assay (ELISA), protein concentrations of VEGF-D were also elevated in the mammary tumors of Has2^{ΔNeo} mice (Figure

4B). We next examined the tissue localization of VEGF-C and -D by immunostaining (Figure 4C). In the Has2^{ΔNeo} tumors, VEGF-D immunoreactivity was detected adjacently to podoplanin-positive vessels formed within the stromal compartments. Similar, but broader, distributions were observed in the case of VEGF-C. Together with the fact that lymphatic vessels were predominantly accumulated within or near the stromal structures of tumors, the VEGF-C and -D immunohistochemistries suggest that tumor-stromal cell interaction promotes intratumoral lymphangiogenesis via expression of these lymphangiogenic factors.

To determine the cellular compartments responsible for the production of lymphangiogenic factors, the transcriptional levels of VEGF-C and -D were determined using total RNAs isolated from the established tumor cells and TAFs by real-time quantitative RT-PCR. As shown in Table 1, VEGF-C was highly expressed both in tumor cells and TAFs, whereas significant levels of VEGF-D were detected in TAFs. There was no significant difference in VEGF-C or -D expression levels between Has2^{ΔNeo} and Has2^{+Neo} TAFs. Protein concentrations of VEGF-D in conditioned media were also measured by ELISA and were consistent with the transcriptional levels (Table 1). We then conducted *in vitro*

Figure 3. Establishment and characterization of tumor cells and TAFs. **A:** Schematic of the transgenic construct. FLAG-tagged murine Has2 cDNA was positioned at downstream of the transgene unit including a CAG promoter (CAG Pro), a loxP sequence, the Neo-resistance gene (Neo), the SV40 poly(A) signal (pA), and a second loxP sequence. Upon recognition of the loxP site, Cre recombinase deletes the Neo cassette along with one of the loxP sequences and then joins the CAG promoter and Has2 cDNA, leading to expression of Has2 mRNA. **White, gray, and black triangles** represent the CAG promoter and the PGK-Neo and Has2-R7 primers, respectively. **B:** PCR analysis of Cre-mediated genomic DNA recombination. Genomic DNA samples were isolated from established tumor cells (T) and TAFs and analyzed by PCR as described previously.²¹ PCR screening (no. 1) with the CAG promoter and PGK-Neo primers gave the anticipated 360-bp DNA product (**arrow**) for the Has2^{ΔNeo} tumor cells and TAFs but not for the Has2^{ΔNeo} tumor cells. PCR screening (no. 2) with the CAG promoter and Has2-R7 primers gave the predicted 1880-bp DNA product (**white arrowhead**) for Has2^{ΔNeo} tumor cells and TAFs, and a 670-bp product (**black arrowhead**) for Has2^{ΔNeo} tumor cells. This change in amplified product size demonstrates that deletion of the Neo cassette was successfully achieved by Cre-mediated transgene recombination in Has2^{ΔNeo} tumor cells. **C:** Relative mRNA expression of Has1 and Has2 in tumor cells and TAFs. Total RNA samples were isolated from monoculture or co-culture of exponentially growing tumor cells and/or TAFs as described in Materials and Methods. The combinations of tumor cells and TAFs are described under each graph. Cells given in parentheses were used for RNA isolation in the case of co-cultivation. The mRNA levels of Has1 and Has2 in tumor cells and TAFs were analyzed by real-time quantitative RT-PCR and normalized to GAPDH mRNA. Data represent the mean ± SE from three experiments. **D:** HA concentration in the conditioned media of tumor cells or TAFs. The conditioned media from monoculture or co-culture of exponentially growing tumor cells and/or TAFs were harvested as described in Materials and Methods. HA concentrations in the conditioned media were determined by the IBA method as described previously.²¹ The combinations of tumor cells and TAFs are described under each graph.

co-culture studies to investigate the synergistic effects of these cells on the expression of lymphangiogenic factors. Tumor cells were co-cultivated directly or indirectly with TAFs, and their transcriptional levels of VEGF-C and -D were examined. In the Transwell indirect system, the levels of VEGF-C and -D transcripts in tumor cells were similar, even in the presence of TAFs (Table 1). Similarly, indirect co-cultivation did not affect their expression in TAFs. Direct co-cultivation also failed to enhance expression (data not shown). We then explored whether the same was true in breast carcinoma cells. The MCF-7 human breast cancer cells, which cannot express VEGF-C or -D, were co-cultured with TAFs established from Has2-overexpressing tumors. Co-cultivation did not induce *de novo* expression of

these lymphangiogenic factors in cancer cells (data not shown).

TAF Stimulation of LEC Migration

We next investigated which cell types contribute most strongly to lymphangiogenesis. Because LEC migration is a hallmark of lymphangiogenesis, wound assays were performed to determine the involvement of tumor cells and TAFs after LEC stimulation. Confluent LEC cultures in a 24-well culture plate were scraped with a pipette tip, which made a cell-free zone. These cells were incubated with conditioned media of tumor cell

Figure 4. Expression and tissue localization of lymphangiogenic factors. **A:** Relative mRNA expression of VEGF-C and -D in mammary tumor cells. Total RNA from Has2⁺Neo and Has2^ΔNeo tumors were transcribed into cDNA and the relative mRNA levels of VEGF-C and -D were analyzed by real-time quantitative RT-PCR. Increases in VEGF-C and -D transcripts were detected in the mammary tumors of the Has2^ΔNeo mice. Data are expressed as mean $\pm$ SE ($n = 8$ for Has2^ΔNeo; $n = 5$ for Has2⁺Neo); $*P < 0.05$. **B:** ELISA analysis of mouse VEGF-D protein expression in Has2⁺Neo and Has2^ΔNeo tumors. The protein concentration of VEGF-D was significantly increased in Has2^ΔNeo tumors. Data are expressed as mean $\pm$ SE ($n = 3$); $*P < 0.05$. **C:** Immunofluorescence analyses of VEGF-C and -D immunoreactivities (green) were detected adjacently to podoplanin-positive lymphatics (red) formed within stromal compartments. Cell nuclei were stained with DAPI (blue). **D:** TAF-enhanced migration of human LECs. Confluent LECs were scratched with a pipette tip and then cultured with or without tumor cell or TAF-conditioned media. The combinations of tumor cells and TAFs are described under each graph. The migrated LEC distances were measured as described in Materials and Methods. Data are expressed as mean $\pm$ SE ($n = 9$); $**P < 0.01$ versus control culture. N.S., not significant. **E:** Relative mRNA expression of VEGF-C and -D in human LECs. LECs were cultured with or without the conditioned medium of tumor cells or TAF cultures. The combinations of tumor cells and TAFs are described under each graph. Total RNA samples isolated from LECs were transcribed into cDNA and the transcriptional levels of VEGF-C and -D were analyzed by real-time quantitative RT-PCR. Data are expressed as mean $\pm$ SE ($n = 3$). $*P < 0.05$ versus control culture.

and TAF cultures. The maximal distance migrated from the starting wound edge increased in the presence of conditioned medium derived from the Has2^ΔNeo TAF culture (Figure 4D). Both Has2^ΔNeo and Has2⁺Neo TAFs had almost

the same effect on LEC migration. In contrast, there was no significant difference between tumor cell and control cultures, and co-cultivation demonstrated no synergistic effect of tumor cells on TAF-mediated LEC stimulation.

Table 1. Expression of VEGF-C and VEGF-D in Mammary Tumor Cells and TAFs

Cell type (co-cultivation with)	Relative VEGF-C mRNA*	Relative VEGF-D mRNA*	VEGF-D protein (pg/ml) [†]
Has2 ^Δ NeoT	10.1 $\pm$ 0.8 [‡]	<0.1	14.0 $\pm$ 11.5 [‡]
Has2 ^Δ NeoT (+Has2 ^Δ NeoTAF)	9.8 $\pm$ 1.2 [‡]	<0.1	
Has2 ^Δ NeoTAF (+Has2 ^Δ NeoT)	1.2 $\pm$ 0.3	0.8 $\pm$ 0.1	143.2 $\pm$ 8.1
Has2 ⁺ NeoT	8.0 $\pm$ 0.5 [‡]	<0.1	11.2 $\pm$ 3.9 [‡]
Has2 ⁺ NeoT (+Has2 ^Δ NeoTAF)	7.8 $\pm$ 0.8 [‡]	<0.1	
Has2 ^Δ NeoTAF (+Has2 ⁺ NeoT)	0.9 $\pm$ 0.1	1.0	123.6 $\pm$ 3.5
Has2 ^Δ NeoTAF	1	1	136.3 $\pm$ 4.6

Data are expressed as mean $\pm$ SE ($n = 4$).

*Relative mRNA expression was determined by quantitative real-time RT-PCR.

[†]Protein levels in conditioned media from tumor cells or TAF cultures were determined by ELISA analysis.

[‡] $P < 0.01$ versus TAF.

Table 2. TAF-Mediated Growth Acceleration of Xenografted Tumors

Xenografted tumor	Tumor volume (mm ³)	Tumor wet weight (mg)	Tumor dry weight (mg)
Has2 ^{ΔNeo} T	180.6 ± 23.2	167.9 ± 19.2	31.2 ± 3.4
Has2 ^{ΔNeo} T + Has2 ^{ΔNeo} TAF no. 5	263.8 ± 28.7*	331.5 ± 31.1*	53.1 ± 4.3*
Has2 ^{ΔNeo} T + Has2 ^{ΔNeo} TAF no. 41	281.8 ± 24.5†	374.3 ± 41.4*	60.3 ± 5.5*
Has2 ^{+/Neo} T	51.4 ± 13.8	53.4 ± 8.3	10.3 ± 2.6
Has2 ^{+/Neo} T + Has2 ^{ΔNeo} TAF no. 5	152.4 ± 39.3*	137.3 ± 12.6†	26.9 ± 5.3*
Has2 ^{+/Neo} T + Has2 ^{ΔNeo} TAF no. 41	150.8 ± 24.3*	147.7 ± 7.5†	29.2 ± 0.8†
MCF-7	15.5 ± 5.9	n.d.	n.d.
MCF-7 + Has2 ^{ΔNeo} TAF no. 5	83.9 ± 6.4†	n.d.	n.d.
MCF-7 + Has2 ^{ΔNeo} TAF no. 41	54.3 ± 8.3†	n.d.	n.d.

Independent TAF cell lines were established from two different Has2^{ΔNeo} tumors (no. 5 and no. 41). The mean tumor volume, wet weight, and dry weight were determined 14, 36, and 32 days after cell inoculation in Has2^{ΔNeo}, Has2^{+/Neo}, and MCF-7 xenografts, respectively. n.d., not determined. Data are expressed as mean ± SE (n = 4).

*P < 0.05, †P < 0.01 versus each single inoculation of tumor cells.

To explore whether tumor cells or TAFs up-regulate the VEGF-C and -D expressions of LECs, we conducted experiments in which LECs were incubated with conditioned medium from tumor cells or TAF cultures. As determined by real-time quantitative RT-PCR analysis, the expression levels of both VEGF-C and -D in LECs were slightly increased when incubated with TAF culture-conditioned medium (Figure 4E). In contrast, no such up-regulation was detected when LECs were incubated with the conditioned medium from tumor cell monoculture. Taken together, it is therefore likely that lymphangiogenesis is regulated via TAF-mediated LEC stimulation in autocrine and paracrine manners.

TAF-Mediated Acceleration of Tumor Growth, Stromal Formation, and Lymphangiogenesis

We next explored whether TAFs could promote intratumoral lymphangiogenesis. The most commonly used model for characterizing stromal effects is to combine stromal cells with tumor cells and co-implant them into animals, so we inoculated tumor cells with or without TAFs into the mammary fat pads of nude mice. Despite single inoculation, Has2^{ΔNeo} xenografts grew rapidly with intratumoral stroma as observed in spontaneous tumors (Table 2 and Figure 5A), suggesting that they developed solid tumors independently of the supplying TAFs because of their high capability of stromal induction. On the other hand, the growth of Has2^{+/Neo} xenografted tumors was primarily dependent on the co-inoculation with TAFs. As assessed by histological examinations, co-inoculation resulted in the development of tumors with stromal reactions (Figure 5A). Without the TAFs, however, the tumors developed slowly with less stroma (Table 2 and Figure 5A), and TAF alone did not generate any visible tumors during the experimental period (data not shown). Tumor volumes in the co-implanted group were approximately three times larger than those lacking TAFs (Table 2), suggesting a possible synergistic effect of co-implantation on tumor growth. Next we further examined whether there was any difference in tumor size resulting from the co-inoculation of TAFs derived from different genotypes. Similarly to the Has2^{ΔNeo} TAFs, the tumor growth of Has2^{+/Neo} xenografts was accelerated in the presence of

Has2^{+/Neo} TAFs (data not shown). To eliminate the possibility that HA overproduction caused overestimates in tumor volume because of its highly hydrated features, we compared the volume, wet weight, and dry weight of xenografted tumors. Tumor burdens were well correlated with tumor volume (Table 2).

Notably, the TAF-induced acceleration of stromal formation was well correlated with higher levels of lymphangiogenesis, as demonstrated by immunofluorescent staining for podoplanin (Figure 5). In the Has2^{+/Neo} xenografts, co-implantation of Has2^{ΔNeo} TAFs enhanced the formation of podoplanin-positive lymphatics, although the extent of lymphangiogenesis was lower than in the tumors generated from Has2^{ΔNeo} tumor cells (Figure 6). The formation of intratumoral stroma and lymphangiogenesis in co-implanted tumors were almost the same as the combination of Has2^{+/Neo} tumor cell and Has2^{+/Neo} TAFs (data not shown). The growth- and lymphangiogenesis-promoting effects of TAFs were also tested in combination with MCF-7 cells. Numerous studies have determined that *in vivo* growth of MCF-7 cells is generally dependent on β -estradiol,²⁷ so to minimize this, we subcutaneously inoculated logarithmically growing MCF-7 cells without hormones into athymic BALB/c nude mice. Under these experimental conditions, MCF-7 cells developed slowly into solid palpable tumors (Table 2). Tumor volumes in the co-implanted group were at least 3.5 times larger than those lacking TAFs, indicating that TAF synergistically affected tumor growth. Histological examination of tumor cross-sections showed formation of intratumoral stroma, particularly when TAFs were mixed in before inoculation (data not shown). Consistent with the above results, the number of podoplanin-positive lymphatic vessels was significantly increased in the presence of TAFs (Figure 6). Therefore, the *in vivo* reconstituted experiment further demonstrated the importance of tumor-associated stroma in the promotion of intratumoral lymphangiogenesis.

Lastly, we sought to confirm the role of microenvironmental HA in the promotion of stromal reactions and thereby lymphangiogenesis. To avoid the possibility that Has2 overexpression enhanced stromal cell recruitment independently from HA production, we implanted Has2^{+/Neo} tumor cells together with exogenous HA into the mammary fat pads of nude mice. In light of our

Figure 5. Tumors developing in the presence of TAFs are highly lymphangiogenic. **A:** H&E staining of histological sections from tumors xenografted in the presence or absence of *Has2*^{ΔNeo} TAFs. Tumor cells were inoculated with or without TAFs into the mammary fat pads of mice. Animals were sacrificed at the end point of experiments as described in Table 2, and the subcutaneous tumors were fixed and used for histological analyses. Despite single inoculation, *Has2*^{ΔNeo} xenografts grew with intratumoral stroma (arrowheads). When the *Has2*^{+Neo} tumor cells were co-inoculated with *Has2*^{ΔNeo} TAFs, they grew with stromal reactions (arrows). Without the TAFs, however, the tumors developed with less stroma (arrowheads). *Has2*^{+Neo} tumor cells were inoculated with native rooster comb HA (HA) or HA-versican complexes (HA/V), and animals were sacrificed 31 days after inoculation. In the presence of HA-versican complexes, *Has2*^{+Neo} xenografts grew with stromal reactions (arrows). Administration of native rooster comb HA failed to enhance stromal reactions. **B:** Podoplanin-positive lymphatic vessels in xenografted tumors. In *Has2*^{ΔNeo} xenografts, intratumoral lymphatics (red) were markedly formed, despite single inoculation. Lymphatics were also formed in *Has2*^{+Neo} tumors developed in the presence of TAFs, and fewer lymphatic vessels were detected in tumors generated from tumor cells alone. Lymphangiogenesis in *Has2*^{+Neo} xenografts was markedly enhanced in the presence of HA-versican complexes. Administration of native rooster comb HA failed to enhance lymphangiogenesis.

previous findings that exogenously added HA-versican complexes significantly promoted stromal formation and angiogenesis,²¹ we tested aggregates of high-molecular weight native HA with human umbilical cord versican.

Administration of native rooster comb HA (average molecular weight of 1000 kDa) failed to enhance stromal reactions and lymphangiogenesis in the xenograft model (Figure 5). In contrast, HA-versican complexes clearly

B

Figure 5. Continued

accelerated both responses. Taken together, the above data partially support the idea that the microenvironmental HA matrix directly or indirectly participates in the recruitment of stromal cells and lymphatic vessels.

Discussion

There is growing evidence that carcinogenesis and cancer progression are influenced and controlled by cellular interactions between tumor and stromal cells.^{2,8} Carcinoma cells actively recruit several distinct stromal cells,

such as inflammatory cells, vascular cells, and fibroblasts, within the tumor, yet the mechanisms involving these are poorly understood. In this study, the genetic manipulation of Has2 expression in mammary tumors suggested that microenvironmental HA plays an important role in the recruitment of lymphatic vessels. Using a co-implantation tumor xenograft model, we were able to further demonstrate significance of tumor-associated stroma in promotion of HA-mediated lymphangiogenesis.

TAFs are considered to play a central role in the complex process of tumor-stromal interaction.^{2,11} The release

Figure 6. Morphometric analysis of lymphatic vessel density in Has2 Δ Neo, Has2⁺Neo, and MCF-7 xenografted tumors. Podoplanin-positive lymphatics were increased in Has2⁺Neo tumors and in MCF-7 tumors developed in the presence of TAFs. Data represent the mean $\pm$ SE of five random fields from four tumors. ***P* < 0.01.

of autocrine/paracrine factors and formation of the complex ECM network are modulated by direct or indirect tumor-TAF cell interactions, which may support vascular formation as well as tumor cell proliferation and migration. A recent study showed that VEGF-A derived from TAFs is functionally significant for angiogenesis and tumorigenesis.²⁸ TAFs also promote angiogenesis by recruiting endothelial progenitor cells into carcinomas through secretion of stromal cell-derived factor-1 α (SDF-1 α).⁶ In our study, when co-implanted with control Has2⁺Neo tumor cells, TAFs gave rise to highly lymphatic tumors, suggest-

ing that TAFs are highly capable of expressing lymphangiogenic factors. It was clearly shown that TAFs expressed a high level of VEGF-D. VEGF-D has a considerable ability to induce lymphangiogenesis in solid tumor masses, and might determine the route of metastatic spread to lymph nodes.²⁹ VEGF-D is also a potent lymphangiogenic factor when delivered via an adenoviral vector into skeletal muscle.³⁰ Therefore, VEGF-D and, to a lesser extent, VEGF-C, may be responsible for the lymphangiogenesis-enhancing capability of TAFs. Previously, up-regulation of VEGF expression in human colonic fibroblasts was detected when co-cultured with colorectal cancer cells.³¹ However, our current study indicated that co-cultivation with TAFs failed to enhance the expression of VEGF-C and -D in tumor cells. The *in vitro* reconstitution experiment may therefore imply that cancer cells maintain favorable biological conditions for lymphangiogenesis through HA-induced TAF recruitment rather than via the up-regulation of lymphangiogenic factors. Despite the similar VEGF-C expression, control Has2⁺Neo tumor cells formed much less intratumoral lymphatics than Has2 Δ Neo tumor cells. Thus, this supports the idea that the stromal microenvironment as well as lymphangiogenic factors is important for the induction of intratumoral lymphangiogenesis.

The induction of lymphangiogenesis by HA overproduction is consistent with a recent report demonstrating a similar function of exogenous HA in xenografts of mouse hepatocellular carcinoma cells.³² However, the contributions of tumor-derived HA and the tumor-associated stroma to lymphangiogenesis were not addressed in that xenograft study. Furthermore, exogenous native HA isolated from rooster comb failed to promote tumor lymphangiogenesis and tumor growth in Has2⁺Neo xenografts in our hands. Such discrepancies may be attributable to the difference in HA sources used; HA was derived from umbilical cord in the former experiment and from rooster comb in our case. As demonstrated in our previous findings, commercially available umbilical cord HA contains a significant amount of versican, an HA-binding chondroitin sulfate proteoglycan, which acts as a key player in HA-mediated recruitment of host stromal cells.²¹ This may indicate that HA-promoted lymphangiogenesis shown in the former experiment is partly attributed to the ability of versican contaminants. Indeed, our present study showed a significant ability of HA-versican complexes to promote lymphangiogenesis in Has2⁺Neo xenografts, suggesting that treatment with exogenous HA-versican complexes partly mimics the physiological effects of endogenously produced HA. The spontaneous cancer model using Has2-overexpressing transgenic mice therefore allowed us to improve our understanding of the pathogenesis of HA-overproducing breast cancer cells; these transgenic experiments have led to the novel finding that tumor-derived HA induces stromal reactions and subsequent penetration of lymphatics within intratumoral stromal compartments.

Has2 overexpression may cause enhancement of stromal recruitment independent from HA production. As described above, the present findings that exogenously added HA-versican complexes significantly promoted

stromal formation and concomitant tumor lymphangiogenesis in xenograft models, offers a mechanistic explanation of how Has2 overexpression enhanced these responses. Considering the insufficient activation of lymphangiogenesis by HA-versican complexes alone, however, we should concede that there may be another mechanism by which the Has2 enzyme itself enhances tumor-induced stromal cell recruitment and lymphangiogenesis via some unknown pathways.

Changes in the composition of lymphangiogenic ECM affects the function of both pre-existing and newly formed lymphatics.^{33,34} HA-rich ECM may directly modulate cellular functions and contribute to lymphatic development by interacting with cell surface HA receptors. The HA receptor LYVE-1 is expressed abundantly on the surface of lymphatic vessels,¹² thereby implicating it in fundamental roles such as maintaining either lymphatic architecture or normal lymphatic function. A recent report on LYVE-1-null mice, however, suggested that LYVE-1 is not obligatory for normal or pathological lymphangiogenesis.³⁵ This would partly explain why lymphatic vessels predominantly penetrated stromal compartments despite the accumulation of HA-rich matrices in solid tumor masses.

Multiple mechanisms may be involved in TAF-stimulated tumor growth. One possibility is that TAF-induced lymphangiogenesis may cause an increase in HA synthesis in the epithelial compartment of the tumors, thereby stimulating tumor growth in an autocrine manner. We thus determined HA localization by double-immunofluorescent staining of both HA and podoplanin and found that HA accumulation was restricted in stromal compartments of tumors formed by co-implantation of Has2^{+Neo} tumor cells and TAFs (data not shown). Furthermore, TAFs produced higher amounts of HA than Has2^{ΔNeo} and Has2^{+Neo} tumor cells *in vitro*, suggesting that they contribute greatly to the synthesis and accumulation of HA in stromal compartments where lymphatic vessels prefer to penetrate. Based on these observations, it seems unlikely that increased lymphangiogenesis causes an increase in HA synthesis in the epithelial compartment of tumors but may offer another perspective on TAF-enhanced tumor growth. Cross talk between carcinoma cells and adjacent stromal cells are important for tumor development and progression. Recently, it has been shown that fibroblasts isolated from invasive human ductal carcinomas can produce SDF-1 α , a chemokine that both enhances tumor growth and promotes angiogenesis through the recruitment of endothelial cells.⁶ We have also previously demonstrated up-regulation of SDF-1 α and concomitant promotion of tumor angiogenesis in Has2-overexpressing tumors.²¹ Thus, such chemokines and growth factors secreted from stromal cells may accelerate tumor growth directly or through enhanced angiogenesis.

Efforts to purify the potential stromal fibroblast chemotactic factors produced by tumor cells have been done, yet only a few factors such as platelet-derived growth factor have been identified.²⁸ We recently demonstrated that overproduction of HA leads to recruitment of TAFs inside tumors and subsequent formation of intratumoral

stroma.²¹ To assess the contribution of tumor-derived HA to TAF recruitment, we established several primary tumor cell lines from Has2^{ΔNeo} and control Has2^{+Neo} and implanted them into nude mice. Consistent with spontaneous tumors, xenografts of Has2^{ΔNeo} cells demonstrated remarkable formation of intratumoral stroma. On the other hand, Has2^{+Neo} tumors tended to exhibit the histological features of carcinomas with fewer stroma. Considering the fact that tumor cells themselves are sufficient for stromal reactions if they overproduce HA, we currently propose that tumor-derived HA is a novel stromal fibroblast recruitment factor. The present experiments do not address how tumor-derived HA intratumorally recruits TAFs during tumor formation, although several reliable explanations can be considered: 1) extracellular accumulation of HA in mammary carcinomas provides microenvironments amenable to easy fibroblast-penetration by increasing turgidity and hydration or by disruption of cell-to-cell junctions. 2) HA appears to promote cell motility by acting on intracellular signaling pathways through interaction with cell surface receptors. 3) In concert with HA-binding molecules, such as versican proteoglycan, HA may allow cells to prepare for proliferation and migration by enhancing cell detachment from ECM or by participating in the assembly of intracellular machinery and transmitting signals. 4) HA-rich ECM mediates the recruitment of mesenchymal stem cells, which are progenitors of TAFs. 5) Overexpression of Has2 in tumor cells induces epithelial-mesenchymal transition (EMT), which may convert tumor cells to TAFs or stimulate them to produce stromal fibroblast chemotactic factors. Because the lack of the transgene recombination in Has2^{ΔNeo} TAFs, it is unlikely that TAFs originated from tumorigenic cells that have already undergone EMT.

Clinically, intratumoral lymphatics were reported to be absent from several human tumors, such as in melanomas and colorectal and hepatocellular carcinomas, and were concentrated within the peritumoral stroma.^{36–39} On the other hand, recent pathological studies have reported the presence of intratumoral lymphatics in head and neck cancers, cutaneous melanomas, and pancreatic endocrine tumors.^{15,40,41} Recent progress in lymphatic research, especially the identification of unique molecular markers specific for LECs, have provided exciting new insight into lymphangiogenesis, yet the biological relevance of lymphatics within tumors (intratumoral lymphatics) and at the tumor periphery (peritumoral lymphatics) is still controversial. Likewise, controversial issues in the structural and functional differences of these lymphatics in promoting tumor cell dissemination remain to be determined. Unlike peritumoral lymphatics, intratumoral lymphatics are often small or collapsed, implying that these structures may not be directly involved in tumor spread.⁴² Several recent experiments in genetic and xenograft tumor models have added further controversy about the functionality of intratumoral lymphatic vessels and their importance for metastatic tumor dissemination;^{29,43–46} the expression of VEGF-D in human embryo kidney 293EBNA cells showed a strong ability to induce intratumoral lymphatic formation in solid tumors, and might determine the route of metastatic spread to lymph

nodes.²⁹ The platelet-derived growth factor-BB has also been implicated to be a survival factor as potent as VEGF-C in inducing *in vivo* intratumoral lymphangiogenesis and enhancing lymph node metastasis in mouse fibrosarcoma models.⁴³ Conversely, the loss of neural cell adhesion molecules in Rip1Tag2 transgenic mouse models resulted in the formation of intratumoral lymphatics, but their occurrence did not correlate with the incidence of lymph node metastasis.⁴⁴ Here, we revealed that intratumoral lymphatics predominantly penetrated at tumor-stroma interfaces. As noted in a previous report, no obvious sign of invasion or metastasis was found in the Has2-overexpressing mice during their short term of tumorigenesis.²¹ This, however, cannot eliminate the possibility that intratumoral lymphatics may be functional with regard to tumor dissemination. In any case, including ours, the now unsolved question is therefore the functional meaning of intratumoral lymphatics.

In conclusion, the present study shows the significance of tumor-associated stroma in the promotion of intratumoral lymphangiogenesis. Bearing in mind the pivotal role of a HA-rich microenvironment in stromal reactions, manipulating the HA biosynthesis of tumor cells may be important in preventing lymphangiogenesis and reverting malignant conversion and may lead to the development of novel anticancer therapeutic agents.

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