Loss of Neural Cell Adhesion Molecule Induces Tumor Metastasis by Up-regulating Lymphangiogenesis

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ABSTRACT

Reduced expression of neural cell adhesion molecule (NCAM) has been implicated in the progression to tumor malignancy in cancer patients. Previously, we have shown that the loss of NCAM function causes the formation of lymph node metastasis in a transgenic mouse model of pancreatic β cell carcinogenesis (Rip1Tag2). Here we show that tumors of NCAM-deficient Rip1Tag2 transgenic mice exhibit up-regulated expression of the lymphangiogenic factors vascular endothelial growth factor (VEGF)-C and -D (17% in wild-type versus 60% in NCAM-deficient Rip1Tag2 mice) and, with it, increased lymphangiogenesis (0% in wild-type versus 19% in NCAM-deficient Rip1Tag2 mice). Repression of VEGF-C and -D function by adenoviral expression of a soluble form of their cognate receptor, VEGF receptor-3, results in reduced tumor lymphangiogenesis (56% versus 28% in control versus treated mice) and lymph node metastasis (36% versus 8% in control versus treated mice).

The results indicate that the loss of NCAM function causes lymph node metastasis via VEGF-C- and VEGF-D-mediated lymphangiogenesis. These results also establish Rip1Tag2/NCAM-deficient mice as a unique model for stochastic, endogenous tumor lymphangiogenesis and lymph node metastasis in immunocompetent mice.

INTRODUCTION

Neural cell adhesion molecule (NCAM, CD56) is a Ca2+-independent cell adhesion molecule, which mediates homotypic and heterotypic cell-cell and cell-matrix adhesion (1–3). Its role in neurite outgrowth, axon guidance, and long-term potentiation has been investigated in great detail (4). However, NCAM is also expressed in many other cell types, including epithelial cells of various organs, muscle, and pancreatic β cells. On the basis of alternative splicing of the COOH-terminal domains, NCAM isoforms are grouped into three main classes: a glycosylphosphatidylinositol-linked M1, 120,000 isoform (NCAM120) and transmembrane M1, 140,000 and M1, 180,000 isoforms (NCAM140 and NCAM180, respectively; refs. 1–3). NCAM140 and 180 are predominantly expressed during embryonic development, whereas NCAM120 is found in many different adult tissues (5, 6). In various cancer types, the expression of NCAM shifts from the M1, 120,000 isoform to the M1, 140,000 and M1, 180,000 isoforms (7). Moreover, an overall decrease in the NCAM level has been observed in a subset of tumors. For example, in colon carcinoma, pancreatic cancer, and astrocytoma, NCAM expression is markedly down-regulated, and the loss of NCAM correlates with poor prognosis (8–11). In contrast, in neuroblastoma and certain neuroendocrine tumors, cancer progression correlates with increased NCAM expression and extensive polysialylation (12–16).

Although NCAM has been extensively studied as a cell-cell adhesion molecule (2, 17), recent reports highlight the role of NCAM in signal transduction. For example, in primary neurons or PC12 cells NCAM is able to activate fibroblast growth factor receptor, thereby stimulating classical fibroblast growth factor receptor signal transduction pathways and neurite outgrowth (18–20). Previously, we have studied the functional role of NCAM during tumor progression in a transgenic mouse model of pancreatic β cell tumorigenesis (Rip1Tag2). In Rip1Tag2 transgenic mice, SV40 T antigen (Tag) is expressed under the control of the rat insulin promoter (Rip1), resulting in the development of β cell tumors in a multistage tumor progression pathway (21). Although Rip1Tag2 transgenic mice usually do not develop any metastases, on ablation of NCAM function, metastases to the regional lymph nodes of the pancreas are observed (22). We have previously shown that, similar to NCAM-mediated outgrowth in neuronal cells, in β tumor cells, NCAM associates with fibroblast growth factor receptor and stimulates classical fibroblast growth factor receptor signal transduction cascades, which lead to the activation of β1 integrin function and cell-substrate adhesion (23). Yet, how the loss of NCAM may cause the metastatic dissemination of β tumor cells to regional lymph nodes has remained elusive.

In the past few years, several major players in the regulation of lymphangiogenesis, the formation of new lymphatic vessels, have been identified. Most importantly, vascular endothelial growth factor (VEGF)-C and -D, members of the VEGF family of growth factors, have been characterized as the critical players in the induction of lymphangiogenesis in physiology and disease (24–26). VEGF-C and VEGF-D bind preferentially to VEGF receptor (VEGFR)-3 (Flt-4), which in the adult is predominantly expressed on lymphatic endothelial cells. Fully processed VEGF-C and -D bind with high affinity also to VEGFR-2 (27–29). The lymphangiogenic capability of VEGF-C and -D has been shown in many different experimental settings. For example, forced expression of VEGF-C or -D in tumor cells or in transgenic mouse models of tumorigenesis results in up-regulated lymphangiogenesis and in the formation of lymph node metastasis (24). Conversely, a soluble form of VEGFR-3 blocks lymphangiogenesis, by binding and sequestering VEGF-C and -D (30, 31). Up-regulated expression of VEGF-C, and to a lesser extent VEGF-D, also highly correlates with lymphangiogenesis and lymph node metastasis in cancer patients (24, 32). The recent discovery of a number of novel markers for lymphatic endothelium such as VEGFR-3 (Flt-4; refs. 33, 34), LYTE-1 (35, 36), podoplanin (37), and the transcription factor Prox-1 (38) has facilitated studies on the molecular mechanisms of lymphangiogenesis.

Here we report that the lymph node metastases, which are observed in NCAM-deficient Rip1Tag2 transgenic mice, are caused by up-regulated expression of VEGF-C and -D and, with it, increased tumor lymphangiogenesis. Interference with lymphangiogenesis in these mice by adenoviral expression of soluble VEGFR-3 results in a
reduction of tumor lymphangiogenesis and of lymph node metastasis, indicating that VEGF-C–driven tumor lymphangiogenesis is a metastatic route for NCAM-deficient β tumor cells. Moreover, the results establish NCAM-deficient Rip1Tag2 transgenic mice as a unique animal model for stochastic tumor lymphangiogenesis.

MATERIALS AND METHODS

Recombinant Adenoviruses. For the expression of soluble VEGFR-3, a cDNA fragment encoding the first three immunoglobulin homology domains of the extracellular portion of mouse VEGFR-3 (Flt-4; amino acids 1–332) was amplified by reverse transcription-PCR from RNA extracted from embryonic day 10.5 mice with the primer pair 5′-CCA TCG ATG AGA ATG CAG CCG GCC GCT GCC-3′-CCG CCG ATA TCA CTG ATG AAG GTC TCG TGC AC-3′ and ligated in frame to a cDNA construct encoding the mouse immunoglobulin Fc-domain (39). Recombinant E1/E3 defective adenovirus expressing soluble VEGFR-3 (AdsFlt-4) was generated with homologous recombination in E. coli as described previously (ref. 40; see supplementary information). Recombinant adenovirus expressing enhanced green fluorescent protein (AdEGFP) was used as control (41).

Cell Culture. Murine fibroblastoid cells (L cells) were cultured in DMEM supplemented with 10% FCS (Invitrogen, Carlsbad, CA), 2 mmol/L glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin sulfate. Cells were transfected with pcDNA-AdFlt-4 with LipofectAMINE (Invitrogen), and conditioned medium (48 hours conditioning) was concentrated on Centricon 1000 (Millipore, Billerica, MA).

Immunoblotting. Forty microliters of concentratedconditioned media and 10 μL of mice sera were resolved by 15% and 10% SDS-PAGE, respectively, and transferred to Immobilon-P membranes (Millipore). Membranes were probed with 1 μg/mL antibody specific for mouse immunoglobulin heavy chains (Pierce, Rockford, IL) or with 0.2 μg/mL antibody specific for mouse Flt-4 (R&D Systems, Minneapolis, MN). Unimmunostained proteins were visualized with the enhanced chemiluminescence detection system (Amersham Biosciences, Piscataway, NJ) according to the manufacturer’s recommendations.

Adenoviral Gene Delivery in Mice. Two hundred microliters of HEPES-buffered saline containing 5 × 10^9 adenoviral particles (AdsFlt-4 or control AdEGFP) were injected into the tail vein (i.v.) of Rip1Tag2;NCAM-deficient mice, 3 times (day 1, 10, and 20), starting at 9 to 10 weeks of age. Glucose (5% w/v) was added to the drinking water at 10 weeks of age to counteract hypoglycemia, which results from insulinoma development. Mice were sacrificed 4 weeks after the first injection, and tumor incidence and diameters were determined. Tumor volumes were calculated from the tumor diameter by assuming a spherical shape of the tumors, and total tumor volumes per mouse were determined.

Histology and Immunohistochemistry. Pancreata were fixed overnight in 4% paraformaldehyde in PBS, dehydrated, and embedded in paraffin. Alternatively, freshly isolated tissue was snap frozen in liquid nitrogen and then embedded in OCT (Tissue Tek, Redding, CA). Five-micron sections were cut for immunohistochemical analysis. For BrdUrd labeling, mice were injected i.p. with 100 μg BrdUrd (Sigma, St. Louis, MO) per gram of body weight 50% by lymphatic vessels (50% by lymphatic vessels) and tumors that were surrounded <50% of the tumor perimeter (<50%), and tumors that were surrounded >50% by lymphatic vessels (>50%). Results are given as percentages of tumors in a given LVD class.

Abbreviations: LVD, lymphatic vessel density; n, number of tumors; N, number of mice.

* VEGF-C expression is presented as percentages of VEGF-C–positive tumors per genotype.
† Lymphatic vessels were identified by LYVE-1 immunoreactivity with light microscopy. LVD was determined by assessing the extent by which lymphatic vessels surrounded the perimeter of a tumor. Tumors from all mice of a particular genotype were then grouped into three classes: tumors that were not in significant contact with any lymphatic vessel (0%), tumors that were surrounded <50% of the tumor perimeter (<50%), and tumors that were surrounded >50% by lymphatic vessels (>50%). Results are given as percentages of tumors in a given LVD class.
‡ Mice with detectable lymph node metastasis per total number of mice.
§ P < 0.001 (x test with Yates correction for continuity).

Table 1. VEGF-C expression, tumor lymphangiogenesis, and metastasis in Rip1Tag2;NCAM-deficient mice

<table>
<thead>
<tr>
<th>Genotype</th>
<th>VEGF-C §</th>
<th>0%</th>
<th>&lt;50%</th>
<th>&gt;50%</th>
<th>Metastasis ‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rip1Tag2</td>
<td>17% §</td>
<td>73% §</td>
<td>25%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>(n = 175, N = 8)</td>
<td>(n = 154, N = 9)</td>
<td>(N = 7)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rip1Tag2:NCAM+/− and Rip1Tag2:NCAM−/−</td>
<td>60% §</td>
<td>51% §</td>
<td>30%</td>
<td>19%</td>
<td>47%</td>
</tr>
<tr>
<td>(n = 379, N = 18)</td>
<td>(n = 349, N = 8)</td>
<td>(N = 15)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rip1Tag2:Rip1VEGF-C</td>
<td>81% §</td>
<td>5%</td>
<td>12%</td>
<td>83%</td>
<td>60%</td>
</tr>
<tr>
<td>(n = 153, N = 9)</td>
<td>(n = 153, N = 9)</td>
<td>(N = 5)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
NCAM+/− and NCAM−/− Rip1Tag2 mice also do not exhibit any detectable differences in tumor progression and in the incidence of lymph node metastasis (22). Therefore, we used Rip1Tag2;NCAM+/− and Rip1Tag2;NCAM−/− mice in the same experimental groups and, for the purpose of clarity, refer to both of them as Rip1Tag2;NCAM-deficient mice.

First, we determined VEGF-C expression in β cell tumors of Rip1Tag2 and Rip1Tag2;NCAM-deficient mice by immunohistochemical staining with specific antibodies against VEGF-C. These experiments revealed a significant up-regulation of VEGF-C protein expression in NCAM-deficient tumors (Fig. 1, A–C, and F; Table 1). Although only a very small subset of wild-type β cell tumors expressed VEGF-C, it was detected in more than half of the NCAM-deficient tumors analyzed (Table 1). VEGF-C staining in β tumor cells seemed granular and was predominantly found in the perinuclear area of tumor cells (Fig. 1, A–C and F; data not shown). VEGF-C expression seemed to be independent of the tumor stage, as it was detectable in benign tumors (adenomas) as well as in malignant tumors (carcinomas; Fig. 1, B, F, and G; data not shown). Smaller adenoma expressed VEGF-C with higher incidence and more uniformly (Fig. 1B) than larger tumors, where VEGF-C expression was limited to specific areas within the tumor (Fig. 1G; and data not shown) or single scattered cells (Fig. 1C). Simultaneous immunofluorescence staining for insulin and VEGF-C revealed that VEGF-C is expressed exclusively by insulin-expressing β tumor cells, confirming that VEGF-C is produced by β tumor cells and not by other cell types.
On the other hand, not all of the insulin-expressing cells expressed VEGF-C (Fig. 1, G–I).

Immunohistochemical analysis of VEGF-D expression in NCAM-deficient Rip1Tag2 tumors revealed an even more variable expression pattern: whereas in some tumors a high proportion of tumor cells were expressing VEGF-D (Fig. 1D), only single, scattered VEGF-D expressing cells were found in other tumors (Fig. 1E). A comparison of VEGF-D and VEGF-C distribution on serial sections revealed that VEGF-D was frequently found to be expressed by a small subset of VEGF-C–expressing tumor cells, as exemplified in Fig. 1, panels E and F. Tumors of NCAM-expressing Rip1Tag2 mice were all negative for VEGF-D expression (data not shown).

Taken together, these results show that the loss of NCAM expression leads to the up-regulation of VEGF-C and -D expression in tumors of Rip1Tag2 mice. The lack of a clear correlation between VEGF-C and -D expression and tumor stage suggests that the expression of VEGF-C and -D may involve stochastic events that are not directly affected by tumor progression.

Reduced NCAM Expression Correlates with Tumor Lymphangiogenesis. Because Rip1Tag2;NCAM-deficient tumors exhibited increased expression of VEGF-C and -D, we verified whether this resulted in the induction of lymphangiogenesis. Immunohistochemical staining with antibodies against LYVE-1 and VEGFR-3 (Flt-4) revealed a significant increase of lymphatic vessel density in Rip1Tag2;NCAM-deficient mice (Fig. 2; Table 1). In normal control mice, lymphatic vessels were present only in the exocrine pancreas (Fig. 2A) and were not in significant contact with islets of Langerhans (43). In Rip1Tag2 mice, the majority of lymphatic vessels were also not in significant contact with β cell tumors (Fig. 2B). In contrast, almost half of the NCAM-deficient tumors were either completely or partially surrounded by lymphatic vessels (Fig. 2B, C and F; Fig. 3; Table 1). In accordance with previously published results, the highest percentage of tumors surrounded by lymphatic vessels was observed in Rip1Tag2;Rip1VEGF-C mice (Table 1), which we have used as a reference in these experiments (ref. 43; data not shown).

A small subset of Rip1Tag2;NCAM-deficient and Rip1Tag2;
Rip1VEGF-C tumors (9% and 12%, respectively) contained intratumoral structures that reacted positively with antibodies against LYVE-1 (Fig. 2D; data not shown) and podoplanin (data not shown). The majority of intratumoral lymphatic vessels was “collapsed,” although noncollapsed vessels with open lumen were sometimes observed within tumors (data not shown). Notably, the occurrence of intratumoral lymphatics did not significantly correlate with the incidence of lymph node metastasis.

In addition to LYVE-1, we used antibodies against another specific marker for lymphatic endothelial cells, podoplanin, which confirmed the increased lymphatic vessel density in NCAM-deficient tumors (Fig. 3, A, B, and D). Immunofluorescence costaining for podoplanin and LYVE-1 revealed a significant colocalization in most of the vessels stained (Fig. 3A). However, a few minor but significant differences in specificity could be observed. For example, colocalization of the two markers was best on vessels surrounding NCAM-deficient tumors, whereas some vessels in the exocrine pancreas were only weakly stained by the podoplanin antibodies (Fig. 3A). Prox-1 was also specifically expressed in lymphatic vessels of Rip1Tag2; NCAM-deficient tumors, where it colocalized with podoplanin (Fig. 2F). Antibodies against VEGFR-3 (Flt-4) decorated some intratumoral blood capillaries in addition to lymphatic vessels (data not shown), and, therefore, they were not the antibody of choice to study lymphangiogenesis in NCAM-deficient Rip1Tag2 mice and possibly in other systems.

To confirm the specificity of LYVE-1 and podoplanin as markers of lymphatic endothelium, we performed a costaining for LYVE-1 and for the blood vessel endothelial marker CD31 in tumors from Rip1Tag2;NCAM-deficient mice. As expected, antibodies against CD31 strongly decorated blood vessels and capillaries, whereas LYVE-1–positive lymphatic vessels showed only very weak staining for CD31 (Fig. 3C). Podoplanin/CD31 double fluorescence analysis in Rip1Tag2;NCAM-deficient tumors also revealed only a weak signal for CD31 on podoplanin-positive lymphatic vessels (Fig. 3D).

As it has been reported for Rip1Tag2;Rip1VEGF-C mice (43), tumor cell clusters were frequently found in the lumen of LYVE-1/ podoplanin-positive lymphatic vessels in Rip1Tag2;NCAM-deficient mice (arrows in Fig. 2, E and F, and Fig. 3A), suggesting that lymphatic vessels provide a metastatic route for β tumor cells in Rip1Tag2;NCAM-deficient mice.

**Lymphangiogenesis Is a Rate-Limiting Step in β Cell Lymph Node Metastasis.** In the Rip1Tag2 tumor model, both VEGF-C overexpression and NCAM deficiency resulted in the formation of lymph node metastasis (22, 43). To determine whether up-regulated lymphangiogenesis is a rate-limiting event in the formation of lymph node metastasis, we crossed Rip1Tag2;NCAM-deficient mice with Rip1Tag2;
distinct from VEGF-C/D–mediated lymphangiogenesis, may be involved as well. This conclusion is also supported by the fact that not all of the NCAM-deficient mice that exhibited increased lymphatic vessel density surrounding their tumors developed lymph node metastasis (Table 2). Conversely, there were also subsets of mice displaying lymph node metastasis in the absence of lymphangiogenesis (Table 2).

The results presented above suggest that NCAM-deficiency leads to lymph node metastasis, at least in part, through VEGF-C– and VEGF-D–mediated lymphangiogenesis. To test this hypothesis, we used a soluble form of VEGF-3 (sFlt-4) to interfere with the function of VEGF-C and -D, and potentially with tumor lymphangiogenesis and metastasis, in Rip1Tag2;NCAM-deficient mice. To systematically express high levels of sFlt-4, we generated a recombinant, replication-defective adenovirus encoding for the ligand-binding, extracellular domain of the receptor (the first three immunoglobulin domains of mouse Flt-4) with its COOH-terminus fused to a mouse immunoglobulin heavy chain (Fc) under the control of the cytomegalovirus enhancer/promoter. Intact expression of the recombinant protein was confirmed in vitro by analyzing conditioned medium of L cells transfected with an expression construct encoding sFlt-4 (Fig. 4A). Recombinant adenoviruses either expressing sFlt-4 (AdsFlt-4) or EGFP as control (AdEGFP) were then injected into the tail vein of Rip1Tag2;NCAM-deficient mice starting at 9 weeks of age, with a bolus of $5 \times 10^9$ viral particles administered at day 1, 10, and 20. From previous experiments, it was known that i.v. application of recombinant adenovirus exclusively infects hepatocytes in the liver, which in turn produce and secrete soluble proteins into the blood stream (39). The presence of sFlt-4 in the sera of mice injected i.v. with purified recombinant adenoviral particles was confirmed by immunoblotting indicating that sFlt-4 was systematically present in these mice at high levels (ranging between ~50 µg/mL and 300 µg/mL; Fig. 4, B and C).

Immunohistochemical staining for podoplanin in adenovirus-injected Rip1Tag2;NCAM-deficient mice revealed a significant reduction in the number of tumors associated with lymphatic vessels on systemic expression of sFlt-4 as compared with EGFP ($P < 0.001$, z-test; Table 3).

Analysis of the incidence of lymph node metastasis revealed a reduction in the number of mice with lymph node metastasis on treatment with AdsFlt-4 as compared with AdEGFP (Table 3). Notably, the one and only lymph node metastasis that has been detected in a AdsFlt-4-treated Rip1Tag2;NCAM-deficient mouse exhibited a highly malignant, anaplastic phenotype, and most likely has originated from a primary tumor of similar malignancy (supplementary Fig. S2). Because these experiments had to be performed at the very late stages of Rip1Tag2 tumor development, and for ethical reasons, the number of experimental mice had to be kept at a minimum. As a

### Table 3 Soluble Flt-4 represses tumor lymphangiogenesis and lymph node metastasis in Rip1Tag2;NCAM-deficient mice

<table>
<thead>
<tr>
<th>Podoplanin *</th>
<th>0%</th>
<th>&lt;50%</th>
<th>&gt;50%</th>
<th>Metastasis †</th>
</tr>
</thead>
<tbody>
<tr>
<td>AdEGFP</td>
<td>12.44% ‡</td>
<td>31.34% ‡</td>
<td>56.22% ‡</td>
<td>4/11 §</td>
</tr>
<tr>
<td>(n = 201, N = 12)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AdsFlt-4</td>
<td>40.52% ‡</td>
<td>31.03% ‡</td>
<td>28.45% ‡</td>
<td>1/12 §</td>
</tr>
<tr>
<td>(n = 232, N = 12)</td>
<td></td>
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</table>

Abbreviations: LVD, lymphatic vessel density; n, number of tumors; N, number of treated mice.

* LVD was determined by podoplanin immunohistochemistry with a light microscope and scored as described in Table 1. The results are given as percentages of tumors from control and AdsFlt-4-treated mice in the different LVD groups.

† Number of mice with lymph node metastasis of the total number of treated mice.

‡ Number of mice with lymph node metastasis that has been detected.

§ $P < 0.001$ (z test with Yates correction for continuity).

# Number of mice with lymph node metastasis that has been detected.

## Two-tailed Fisher exact test.

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Rip1VEGF-C mice and examined the incidence of lymph node metastasis by histopathological analysis of pancreata from these mice. In these separate breeding experiments, 20% of the Rip1Tag2;NCAM-deficient mice (n = 20), 37.5% of Rip1Tag2;Rip1VEGF-C mice (n = 8), and 80% of the composite Rip1Tag2;Rip1VEGF-C;NCAM-deficient mice (n = 10) exhibited metastasis to the regional lymph nodes of the pancreas. These results indicate that the induction of lymphangiogenesis in Rip1Tag2;NCAM-deficient mice is indeed a rate-limiting step for the metastatic dissemination of β tumor cells to the regional lymph nodes. However, because the loss of NCAM function potentiated VEGF-C–dependent lymph node metastasis in Rip1Tag2;Rip1VEGF-C;NCAM-deficient composite mice, an additional mechanism, that involves the loss of NCAM function but is
result, the number of mice in this experiment is rather low and, although a clear trend is apparent, the reduction of lymph node metastasis on AdsFlt-4 treatment is not statistically significant ($P = 0.115$, two-tailed Fisher exact test; Table 3). Besides the changes in the incidence of lymph node metastasis, no significant differences were observed for tumor incidences and volumes, tumor cell proliferation (BrdUrd-incorporation), apoptosis (TUNEL assay), and tumor angiogenesis (CD31 staining; Table 4).

Together, the results indicate that interfering with the function of VEGF-C and -D during tumor development in Rip1Tag2;NCAM-deficient mice reduces lymphangiogenesis and, to a lesser extent, lymph node metastasis but does not affect tumor cell proliferation, tumor cell apoptosis, or blood vessel angiogenesis. Thus, our data raise the possibility that, at least in the Rip1Tag2 transgenic mouse model, the loss of NCAM induces lymph node metastasis by up-regulated expression of VEGF-C and VEGF-D, which in turn induces lymphangiogenesis.

**DISCUSSION**

On the basis of changes in gene expression, alternative splicing, and differential polysialylation, the cell-cell adhesion molecule NCAM has been functionally implicated in tumor progression in a variety of cancer types (7). However, the cellular and molecular mechanisms by which changes in NCAM function may affect tumor progression have remained elusive. A first glimpse into such mechanisms has come from experiments with Rip1Tag2 transgenic mice, where the loss of NCAM contributes to the metastatic dissemination of $\beta$ cell tumors to regional lymph nodes (22). Here, we set out to identify and characterize the molecular mechanisms connecting the loss of NCAM function with lymph node metastasis. We show that, on loss of NCAM function, $\beta$ cell tumors of Rip1Tag2 transgenic mice express increased levels of the lymphangiogenic factors VEGF-C and VEGF-D. As a result, NCAM-deficient tumors exhibit up-regulated lymphangiogenesis, which in turn provides tumor cells with a route for metastatic dissemination to local lymph nodes.

Immunohistochemical analysis with antibodies against VEGF-C and -D, and against a number of well-established markers for lymphatic vessels, revealed that in a significant proportion of NCAM-deficient Rip1Tag2 tumors, both the expression of VEGF-C and -D and lymphangiogenesis are significantly up-regulated (Table 1). However, VEGF-D expression by $\beta$ tumor cells of NCAM-deficient Rip1Tag2 mice was less frequent as compared with the expression of VEGF-C (Fig. 1, $D$–$F$), implicating that VEGF-C is the major lymphangiogenic player in Rip1Tag2;NCAM-deficient mice.

Interestingly, tumors for which the diameter is partially (<50%) surrounded by lymphatic vessels were equally represented in NCAM-deficient as well as NCAM-expressing Rip1Tag2 tumors (Table 1). Such a significant interaction between $\beta$ cell tumors and lymphatic vessels can be explained by the fact that the mouse pancreas contains a rich network of lymphatic vessels, which is frequently found in the vicinity of islets of Langerhans (46). Hence, it is conceivable that outgrowing $\beta$ cell tumors may have a high chance of becoming partially surrounded by pre-existing lymphatic vessels of the pancreas. In contrast, during active tumor lymphangiogenesis, for example induced by up-regulated VEGF-C and -D expression in NCAM-deficient tumors or by transgenic expression of VEGF-C in Rip1Tag2; Rip1VEGF-C tumors, most of the tumor perimeter (>50%) is surrounded by lymphatic vessels (ref. 43; Table 1). Thus, Rip1Tag2;NCAM-deficient mice represent a model for stochastic, endogenous tumor lymphangiogenesis, a useful tool for future investigations.

Combining the lymphangiogenic effect of forced expression of VEGF-C in Rip1VEGF-C transgenic mice with the lymphangiogenesis observed in NCAM-deficient mice in composite Rip1VEGF-C;NCAM-deficient;Rip1Tag2 mice resulted in an increase of lymph node metastasis that was more than additive. This result indicates that the expression of VEGF-C and -D is a rate limiting event in the formation of lymph node metastases, but that additional mechanisms, triggered by the ablation of NCAM function, are also involved in this process in Rip1Tag2 mice. Variations in the genetic background of the different composite mouse lines may also contribute: the incidence of lymph node metastasis observed in NCAM-deficient mice varies with different genetic backgrounds (50% in ref. 22, 47% and 37%, respectively, when Rip1Tag2 were excisively crossed to NCAM knockout mice, and 20% when Rip1Tag2 mice were crossed to NCAM-deficient and Rip1VEGF-C transgenic mice). A correlation analysis between lymphangiogenesis and lymph node metastasis within single mice revealed that in a subset of NCAM-deficient mice, lymph node metastasis developed in the absence of active tumor lymphangiogenesis (Table 2). On the other hand, the finding that some mice exhibited ongoing lymphangiogenesis in their tumors and did not develop lymph node metastasis may be explained by the fact that some of the metastases were missed by the histologic analysis or that the mice were sacrificed before the formation of metastasis. Likewise, not all of the Rip1VEGF-C transgenic mice that exhibited active lymphangiogenesis developed lymph node metastasis (ref. 43; Table 2).

Systemic expression of a soluble form of VEGFR-3 (sFlt-4) by adeno viral gene delivery reduced the percentage of tumors surrounded by lymphatic vessels. Such a repression of tumor lymphangiogenesis by sFlt-4 is in accordance with previous reports on experimental tumors in chicken, mouse, and rat (31, 47, 48). Here we show for the first time that sFlt-4 is also able to reduce tumor lymphangiogenesis caused by the up-regulation of VEGF-C and -D in endogenously growing tumors not involving tumor transplantation. Soluble Flt-4 in the circulation of Rip1Tag2;NCAM-deficient mice also reduced the incidence of lymph node metastasis from 36% (4 of 11) in the control group of mice to 8% (1 of 12; Table 3), raising the possibility that loss-of-NCAM-mediated induction of lymphangiogenesis contributes to the formation of lymph node metastasis.

In contrast to the significant reduction of lymphatic vessel density, blood vessels were not affected by the adeno viral expression of sFlt4 in Rip1Tag2;NCAM-deficient mice (Table 4). This result indicates that the treatment specifically repressed lymphangiogenesis and not tumor angiogenesis, a selectivity that has been previously reported in various experimental models (48, 49).

There has been much controversy about the functionality of intratumoral lymphatic vessels and their importance for metastatic tumor dissemination (reviewed in ref. 32). Although we clearly observed intratumoral lymphatic vessels in Rip1Tag2;NCAM-deficient mice,

| Table 4 Systemic expression of sFlt-4 does not affect general tumor growth in Rip1Tag2;NCAM-deficient mice |
|----------------------------------|-----------------|
| Tumor incidence (number per mouse) | AdEGFP | AdsFlt-4 |
| Tumor volume (mm$^3$) | $39.5 \pm 30.0$ | $30.9 \pm 45.5$ |
| Cells per field | $57.0 \pm 29.5$ | $59.6 \pm 30.9$ |
| Apoptosis | $10.9 \pm 8.1$ | $9.9 \pm 6.1$ |
| Blood vessel density | $27.4 \pm 12.5$ | $26.7 \pm 10.6$ |

**NOTE.** Values are mean ± SD. The number of treated mice was 11 and 12 for AdEGFP and AdsFlt-4, respectively.

* Cells per microscopic field (400× magnification) in S phase as determined by BrdUrd-incorporation.
† Apoptotic cells per microscopic field (400× magnification) as determined by TUNEL staining.
‡ CD31-positive vessels per microscopic field (400× magnification).
their occurrence did not correlate with the incidence of lymph node metastasis. Moreover, the lumina of these intratumoral lymphatic vessels were usually collapsed. Finally, sFlt-4 reduced only lymphatic vessel surrounding tumors and not intratumoral lymphatic vessels (data not shown). Together, these observations support the notion that intratumoral lymphatics detected in Rip1Tag2 tumors are not functional.

The correlation between the loss of NCAM function and the gain of VEGF-C and -D expression in Rip1Tag2 tumors raises a number of questions about the cellular and molecular mechanisms involved in this process. First, is the loss of NCAM directly affecting VEGF-C and -D gene expression? To address this issue, we have investigated VEGF-C expression in β tumor cell lines derived from tumors of wild-type and NCAM-deficient Rip1Tag2 mice (23). The results indicate that in cultured cells VEGF-C expression is not directly affected by changes in NCAM expression (data not shown). Hence, we speculate that up-regulated VEGF-C expression is caused by mechanisms that depend on the tissue or tumor context in Rip1Tag2 mice in vivo.

One obvious phenotypic difference between wild-type and NCAM-deficient Rip1Tag2 tumors is the dramatic tissue disaggregation of NCAM-deficient tumors. In previous work, we have shown that the loss of NCAM results in a failure to activate β1 integrin via fibroblast growth factor receptor signaling (23), a likely cause for tumor tissue disaggregation. It is conceivable that such changes in cell-matrix adhesion may be the cause for the up-regulation of VEGF-C and -D expression, for example, by changing the interstitial fluid pressure within the tumor or by causing major fluid leakages and increased tissue fluid volume. In fact, many of the β cells tumors of Rip1Tag2; NCAM-deficient mice exhibit hemorrhages filled with lymphatic fluid (23), an observation that initially motivated us to investigate lymphangiogenesis in these tumors.

Alternatively, tissue disaggregation may lead to an inflammatory response which in turn could affect lymphangiogenesis. Such mechanism is attractive, because human and mouse VEGF-C promoters contain conserved binding sites for nuclear factor κB (50), a transcription factor which plays a central role in inflammation and which has often been correlated with cancer (51). Notably, the proinflammatory cytokines interleukin-1 and tumor necrosis factor-α are able to activate both nuclear factor κB transcriptional activity and VEGF-C gene expression (52). Moreover, heregulin-β1 increases VEGF-C expression through nuclear factor κB/p38 mitogen-activated protein kinase signaling pathways in human breast cancer cells (53). Hence, it will be important to investigate whether NCAM-deficient tumors exhibit certain inflammatory responses, which type of inflammatory cells have infiltrated in these tumors, and which cytokines could possibly be responsible for the up-regulation of VEGF-C and -D in NCAM-deficient β tumor cells. Finally, future studies will also have to address whether changes in NCAM expression also correlate with up-regulated lymphangiogenesis and lymph node metastasis in cancer patients.

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