Reduced expression of neural cell adhesion molecule induces metastatic dissemination of pancreatic β tumor cells

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As in the development of many human cancers, in a transgenic mouse model of β-cell carcinogenesis (Rip1Tag2), expression of neural cell adhesion molecule (NCAM) changes from the 120-kDa isoform in normal tissue to the 140/180-kDa isoforms in tumors. NCAM-deficient Rip1Tag2 mice, generated by crossing Rip1Tag2 mice with *NCAM* knockout mice, develop metastases, a tumor stage that is not seen in normal Rip1Tag2 mice. In contrast, overexpression of NCAM 120 in NCAM-deficient Rip1Tag2 mice prevents tumor metastasis. The results indicate that the loss of NCAM-mediated cell adhesion is one rate-limiting step in the actual metastatic dissemination of β tumor cells.

Neural cell adhesion molecule (NCAM) and related proteins (for example, L1, Neuroglian and insect fasciclins) form a large family of calcium-independent adhesion molecules that consist mainly of varying numbers of immunoglobulin (Ig) domains and fibronectin type III repeats[1–4]. NCAM is a cell surface sialoglycoprotein mediating homotypic and heterotypic cell–cell adhesion through a homophilic binding mechanism. Many NCAM isoforms have been reported that are all derived from a single copy gene by extensive alternative mRNA splicing. Depending on their C-terminal domains, the various NCAM isoforms are classified as glycophosphatidylinositol (GPI)-linked 120-kDa isoforms (NCAM 120) or as transmembrane 140-kDa and 180-kDa isoforms (NCAM 140 and NCAM 180, respectively). Additional heterogeneity in NCAM structure is generated by differential glycosylation, notably by alpha 2-8-linked polysialylation[5].

NCAM involvement in growth, guidance and migration of neural crest cells and neurons, axon bundling, interaction of motor axons and muscle, and several other morphogenetic events has been reported[6,7]. During embryogenesis, NCAM 140 and NCAM 180 are expressed transiently in many tissues, including epithelia[8], whereas in adult tissue, mainly NCAM 120 is expressed, mostly in the nervous system[9], in skeletal muscle cells[10] and in some neuroendocrine organs[11–13]. Despite the relatively broad expression pattern of NCAM, *NCAM* knockout mice are viable and have only minor defects in brain development[14,15]. In many human cancers, including Wilms' tumor, colon carcinoma, Ewing sarcoma, neuroblastoma, small-cell lung cancer and multiple myeloma, NCAM expression changes from NCAM 120 in normal tissue to NCAM 140 and NCAM 180 in tumors[16–20]. Moreover, in human pancreatic and colorectal cancer reduced levels of NCAM expression correlate with increased tumor malignancy[21]. However, the functional contribution of NCAM to tumor development remains unknown.

We have studied tumor progression in a transgenic mouse line that expresses SV 40 T antigen under the control of a rat insulin promoter in the β cells of pancreatic islets of Langerhans (Rip1Tag2; ref. 22). These mice reproducibly develop β-cell tumors in a multistage tumorigenesis pathway involving islet hyperplasia (~70% of all islets; refs. 23,24), the formation of new blood vessels (angiogenesis; ~20%; refs. 25,26) and, finally, solid β-cell tumors (insulinomas) that can be further categorized as well-differentiated, benign tumors (adenomas; ~2%) and dedifferentiated, invasive tumors (carcinomas; ~0.5%)[27]. Metastases are usually not found in these mice, probably because they succumb to hypoglycemia with increased tumor mass. Here we report that, as in many human cancers, in Rip1Tag2 transgenic mice the expression of NCAM also changes from NCAM 120 in normal islets of Langerhans to NCAM 140/180 in β-cell tumors. We have used loss- and gain-of-function approaches to determine the functional role of NCAM during tumor progression. The results indicate that changes in NCAM expression play an essential part in the progression to tumor metastasis and that the reduction of NCAM expression is one rate-limiting event in the metastatic dissemination of tumor cells.

NCAM isoform switch during Rip1Tag2 tumor progression

Analysis of NCAM expression by indirect immunofluorescence on sections of normal mouse pancreas demonstrated that NCAM is exclusively expressed in endocrine cells of islets of Langerhans but not in acinar cells of the exocrine pancreas (Fig. 1*a*). NCAM expression was maintained throughout the different stages of β-cell tumorigenesis in Rip1Tag2 transgenic mice and did not change substantially during the transition from adenoma to carcinoma (Fig. 1*b* and *c*). However, immunoblotting analyses demonstrated that a change in NCAM isoform expression coincides with tumor progression: normal islets exclusively expressed the 120-kDa isoform of NCAM (NCAM 120; Fig. 1*d*, lane

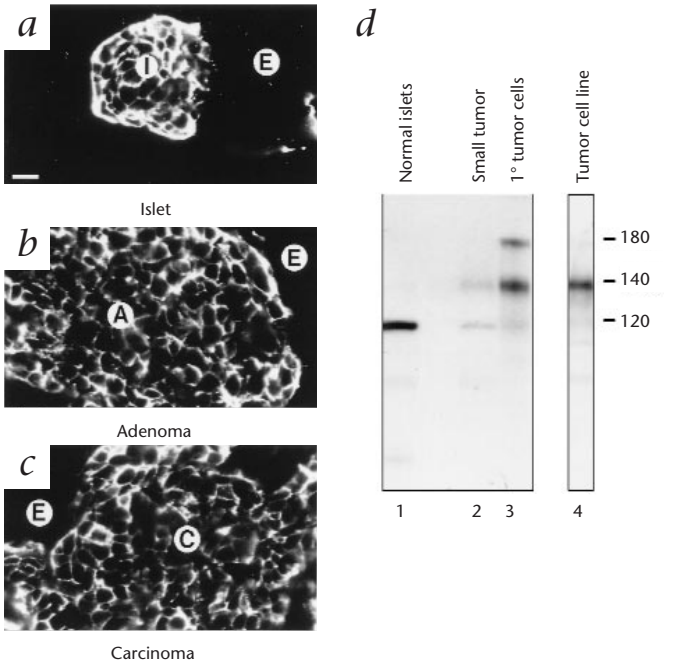

Fig. 1 NCAM expression in the stages of Rip1Tag2 tumor progression. ***a–c***, Analysis of NCAM expression by immunofluorescence. a, NCAM is expressed by the endocrine cells of normal islets of Langerhans but not by the acinar cells of exocrine pancreas. NCAM is expressed by tumor cells in adenomas (b) and carcinomas (c). A, adenoma; C, carcinoma; E, exocrine pancreas; I, islet of Langerhans. Scale bar (in *a*) represents 25 μm. ***d***, Analysis of NCAM expression by immunoblotting of cell extracts from normal islets of Langerhans (lane 1), isolated small tumors (lane 2), partially purified primary tumor cells (lane 3) and an established β tumor cell line (lane 4). NCAM isoform expression changes with tumor progression (right margin).

1), whereas small tumors began to lose expression of NCAM 120 and gain expression of the 140-kDa isoform (NCAM 140; Fig. 1*d*, lane 2). Primary tumor cells isolated from late stage tumors expressed mainly NCAM 140, with lower levels of the 180-kDa isoform (NCAM 180) and considerably reduced levels of NCAM 120 (Fig. 1*d*, lane 3). Finally, tumor cell lines established from β-cell tumors of Rip1Tag2 transgenic mice[28] expressed high levels of NCAM 140 with very low levels of NCAM 180 and NCAM 120 (Fig. 1*d*, lane 4). Immunoblotting experiments using antibodies specific for polysialic acid or polysialylated NCAM did not demonstrate any substantial polysialylation of NCAM in the islets of Langerhans or in the different stages of Rip1Tag2 tumor development (data not shown; ref. 29). The apparent lack of NCAM polysialylation is consistent with reports that NCAM is not substantially polysialylated in pancreatic β cells[30–32]. In contrast to the observed expression of NCAM 120 in adult mouse islets, rat islets express mainly NCAM 140 (refs. 11–13). However, the use of several different NCAM-specific antibodies with the treatment of cell lysates from mouse islets with neuraminidase indicated that the 120-kDa isoform is the only NCAM expressed in pancreatic islets of adult mice (Fig. 1; data not shown; ref. 29).

Thus, β-cell tumorigenesis in Rip1Tag2 transgenic mice resembles the development of many human cancers in changing NCAM expression from the 120-kDa isoform in normal tissue to the 140/180-kDa isoforms in tumor tissue. Moreover, the results indicate that the switch in NCAM isoform expression is the only important change during β-cell tumorigenesis that may affect NCAM function.

Reduced NCAM expression leads to metastasis

We studied interference with NCAM function during β-cell tumorigenesis by crossing Rip1Tag2 transgenic mice with *NCAM* knockout mice lacking all isoforms of NCAM (ref. 15). From these crosses, littermates with the following genotypes were analyzed: Rip1Tag2;*NCAM*$^{+/+}$ (RT2;+/+), Rip1Tag2;*NCAM*$^{+/-}$ (RT2;+/–) and Rip1Tag2;*NCAM*$^{-/-}$ (RT2;–/–). Compared with wild-type Rip1Tag2 littermates, mice heterozygous- or homozygous-deficient for NCAM had much faster tumor progression (Fig. 2). In approximately 50% of all mice lacking one (29 of 60 mice) or two (14 of 28 mice) functional *NCAM* alleles, there was a substantial increase in tumor burden in the pancreas. Histopathological analysis indicated that the increase in tumor volume in the pancreas is mostly due to metastases to the draining lymph nodes in the pancreas (Fig. 2). In approximately 37% of all RT2;+/– mice (22 of 60) and 39% of all RT2;–/– mice (11 of 28), metastases to distant organs were found, including lymphogenic metastases to mesenteric lymph nodes and blood-borne metastases to testis, liver, adrenals or kidney (Fig. 2). In contrast, metastases were never found in RT2;+/+ littermates and have never been observed in the hundreds of Rip1Tag2 mice in a comparable genetic background that have been analyzed[22–27].

Lymph node metastases in both RT2;+/– and RT2;–/– mice had two distinct cellular phenotypes: most had a highly malignant cellular phenotype with nuclear atypia and cellular anaplasia (Fig. 2*h*), whereas some were characterized by their relatively normal cellular appearance (Fig. 2*g*). Metastases to distant organs, including testis, liver, adrenals or kidney, were all of the malignant phenotype (Fig. 2*i*). Immunohistochemical analysis of insulin expression in the metastases demonstrated reduced and focal insulin staining, indicating that the metastases had been formed by insulin-producing β tumor cells that had lost part of their differentiation status (data not shown).

β-cell metastasis is not an epithelio–mesenchymal transition

We next characterized the progression from β-cell adenoma to carcinoma and metastasis by analyzing the expression of a number of genes that commonly serve as markers for an epithelial or a mesenchymal phenotype. Three members of the cadherin family are expressed in β cells of pancreatic islets: E-cadherin, N-cadherin and R-cadherin[32,33]. As reported for wild-type Rip1Tag2 transgenic mice[27], expression of E-cadherin was also lost during the transition from adenoma to carcinoma in RT2;+/– and RT2;–/– mice (data not shown). We found varying levels of E-cadherin expression in metastases of RT2;+/– and RT2;–/– mice. Metastases with a relatively normal cellular phenotype still expressed substantial levels of E-cadherin, albeit mostly with cytoplasmic localization (Fig. 3*a*), whereas metastases with a malignant cellular phenotype lost E-cadherin expression altogether (Figure 3*b*), indicating that E-cadherin expression and metastatic dissemination may not be linked. In contrast to E-cadherin expression, the expression of the other two cadherins expressed in β cells, N-cadherin (Fig. 3*c* and *d*) and R-Cadherin (data not shown), did not change with the progression to metastasis, indicating that these two members of the cadherin family are regulated by different mechanisms. Vimentin, a marker for mesenchymal cells, is not expressed in the carcinoma stages[27], nor was it expressed in the metastases (data not shown), of RT2;+/– and RT2;–/– mice. The expression of epithelial polarity

markers, including Na/K-ATPase (Fig. 3*e* and *f*), the tight junction protein ZO-1 (data not shown) and F-actin (data not shown), was only slightly downregulated or disorganized in the metastases. The data indicate that in NCAM-deficient Rip1Tag2 mice, progression to tumor metastasis does not directly affect expression of markers for the epithelial or mesenchymal status of the tumor cells. Consistent with this, the overall number of primary tumors and the ratio of adenomas to carcinomas were similar among the Rip1Tag2 mice with different *NCAM* genotypes (Table 1), indicating that changes in NCAM expression mainly affect the metastatic dissemination of β tumor cells.

Table 1 Distribution of tumor types in NCAM-deficient Rip1Tag2 transgenic mice[a]

	Tumor Type[b]		
	Adenoma	Carcinoma	Metastasis
NCAM$^{+/+}$	50.0%	50.0%	0%
NCAM$^{+/-}$	42.8%	42.0%	15.2%
NCAM$^{-/-}$	46.0%	37.8%	16.2%

[a]The incidence of a particular tumor type is presented as the percentage of the total tumor number of a given NCAM genotype. Littermates were autopsied at 14–16 weeks of age. [b]Histological sections from 28 tumors of 5 Rip1Tag2/*NCAM*$^{+/+}$ mice, from 112 tumors of 27 Rip1Tag2/*NCAM*$^{+/-}$ mice and from 37 tumors of 11 Rip1Tag2/*NCAM*$^{-/-}$ mice were analyzed by hematoxylin and eosin staining to determine tumor type. Metastasis was never found in hundreds of age-matched Rip1Tag2/*NCAM*$^{+/+}$ mice that originated from other breedings in the same genetic background.

NCAM gene dosage is important for metastasis formation

There was a considerable increase in metastastic dissemination of β tumor cells in Rip1Tag2 lacking only one functional *NCAM* allele (RT2;+/–), indicating that even the reduction of NCAM-mediated cell–cell interaction in the *NCAM*$^{+/-}$ background has a substantial effect on tumor progression. This result is consistent with the observation that *NCAM*$^{+/-}$ mice have the same developmental phenotype as *NCAM*$^{-/-}$ mice[29]. Genomic imprinting of *NCAM* can be excluded as an explanation, as offspring that inherited either the maternal or the paternal functional *NCAM*allele did not show any difference in incidence of metastasis formation or in other parameters of tumor progression (data not shown).

Immunofluorescence analysis of NCAM expression in the different tumor stages of RT2;+/– mice demonstrated relatively high levels of NCAM in normal islets, whereas in adenoma, carcinoma and lymph node metastases, expression of NCAM seemed to be decreased (Fig. 4*a–d*), correlating tumor progression with additional downregulation of the expression of the remaining intact *NCAM* allele. To further investigate a possible downregulation of NCAM expression during β-cell tumorigenesis, we compared the amount of expression of the various NCAM isoforms in the different tumor stages of RT2;+/– mice and RT2;+/+ mice. Single tumors and metastases of different sizes and genotypes were isolated, protein extracts were prepared, and equal amounts of protein were analyzed by immunoblotting using antibodies that recognize all three isoforms of NCAM (Fig. 4*e*). In the tumors and metastases of RT2;+/– mice, overall expression of NCAM was reduced more than 50% compared with its expression in tumors from RT2;+/+ mice. As expected, tumors of RT2;–/– mice showed no NCAM protein expression (Fig. 4*e*). The data indicate that, in addition to the isoform switch, a general downregulation of NCAM expression may be involved in the induction of β tumor cell metastasis. Of the various NCAM isoforms, expression of NCAM 120 is reproducibly lost during progression to β-cell tumor metastasis.

Expression of NCAM 120 prevents metastasis

To produce sustained high expression of NCAM 120 throughout tumorigenesis in Rip1Tag2 transgenic mice, we generated transgenic mouse lines with a cDNA construct encoding NCAM 120 under the control of the rat insulin promoter (Rip1NCAM 120). One mouse line chosen for further analysis specifically expressed the NCAM 120 transgene in the β cells of the islets of Langerhans at levels similar to NCAM 120 expression in normal islets, with expression beginning at approximately 4 weeks of age (data not shown). These mice did not have any overt mutant phenotype in islet development or physiology; thus, they were bred with compound Rip1Tag2/*NCAM* knockout mice to generate Rip1Tag2 transgenic mice that express the *NCAM 120* transgene in RT2;+/+, RT2;+/– and RT2;–/– backgrounds. Gross morphological and detailed histopathological analysis showed that the expression of NCAM 120 in β tumor cells prevented the formation of metastases in RT2;+/– and RT2;–/– mice. None of the 11 RT2;+/– or 12

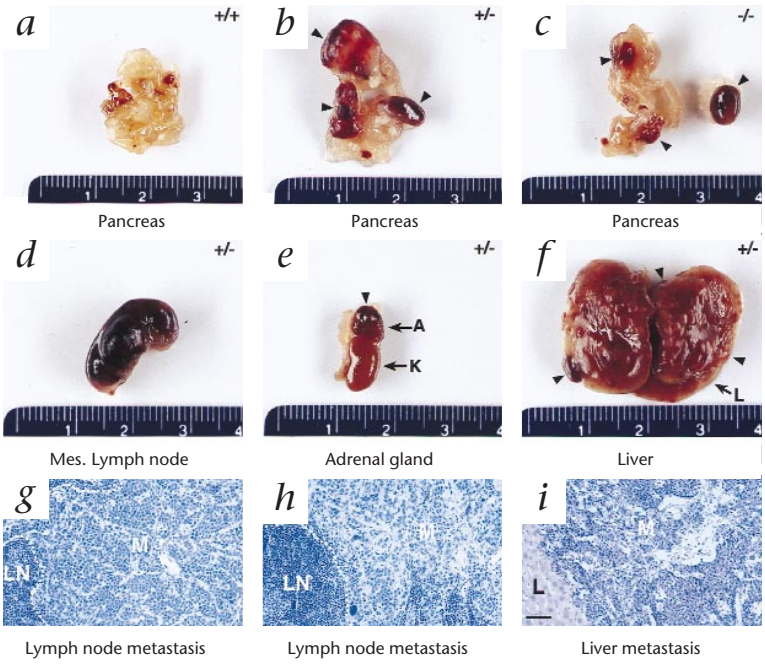

Fig. 2 Reduced *NCAM* dosage results in tumor metastasis. ***a–f***, Gross morphology of pancreata dissected from RT2;+/+ (a), RT2;+/– (b) and RT2;–/– (c) mice. There are lymph node metastases in the pancreata of RT2;+/– and RT2;–/– mice. There are distant metastases in RT2;+/– and RT2;–/– mice, as exemplified by metastases to a mesenteric lymph node (d), adrenals (e) and liver (f) in RT2;+/– mice. NCAM genotypes are indicated in each upper right corner. Arrowheads indicate metastases. A, adrenal gland; K, kidney; L, liver. Scales are in millimeters. ***g–i***, Histological analysis of β-cell metastases by hematoxylin and eosin staining. g, β-cell metastasis to the draining pancreatic lymph node of an RT2;+/– mouse with a relatively normal cellular morphology of the tumor cells. h, β-cell metastasis to the draining pancreatic lymph node of an RT2;+/– mouse with a malignant cellular morphology of the tumor cells. i, Distant β-cell metastasis to the liver of an RT2;+/– mouse. M, metastasis; L, liver parenchyma; LN, lymph node. Borders between metastases and lymph node tissue are indicated by dotted lines. Scale bar (in *i*) represents 50 μm.

RT2;–/– mice with the *NCAM 120* transgene showed signs of metastasis, whereas the RT2;+/– and RT2;–/– littermates without the *NCAM 120* transgene developed metastases with incidence and histopathology similar to the results shown in Fig. 2 (Table 2; $P = 0.00002$; two-tailed Fisher Exact Test). In contrast, expression of NCAM 120 did not cause any substantial change in tumor progression in RT2;+/+ mice (Table 2; data not shown).

These data indicate that the loss of NCAM expression is an essential event in the induction of tumor cell metastasis. Consistent with this, forced expression of NCAM 120 prevents the metastatic dissemination of β tumor cells.

Table 2 NCAM 120 prevents metastasis in NCAM-deficient Rip1Tag2 transgenic mice

	Metastasis[a]		
	NCAM$^{+/+}$	*NCAM*$^{+/-}$	*NCAM*$^{-/-}$
Rip1Tag2	0/8	7/15[b]	7/12[b]
Rip1Tag2; Rip1NCAM 120	0/7	0/11[b]	0/12[b]

[a]Metastasis to lymph nodes and distant organs was determined by gross morphological and histopathological analysis of littermate mice 12–14 weeks of age. The incidence of metastasis is given as metastasis-positive mice per total number of mice analyzed for each particular genotype. [b]$P = 0.00002$ (two-tailed Fisher Exact Test)

Discussion

Our findings provide evidence for a causal role of NCAM in the formation of metastases. Modulation of NCAM expression does not affect the number of primary tumors (tumor incidence) or the progression from benign primary tumors (adenomas) to malignant primary tumors (carcinomas). Similarly, the kinetics of primary tumor formation as well as the rate of tumor cell proliferation and apoptosis are not affected by changes in NCAM expression (data not shown). The data indicate that NCAM is functionally involved in the actual metastatic dissemination of tumor cells. The data also indicate that the role of NCAM in tumor progression is distinct from the ability of E-cadherin to suppress the transition from adenoma to carcinoma[27].

Rip1Tag2 transgenic mice with one intact *NCAM* allele have the same tumor phenotype as Rip1Tag2 mice lacking all functional *NCAM* alleles. This result may be explained by the fact that *NCAM*$^{+/-}$ mice have the same developmental phenotype as *NCAM*$^{-/-}$ mice[29]. Breeding experiments have excluded genomic imprinting to be the cause for the heterozygous phenotype (data not shown). However, quantitation of protein levels in β cell tumors of the different genotypes demonstrated an additional downregulation of NCAM expression during β-cell tumorigenesis, in particular during progression to metastases in RT2;+/– mice. The molecular nature of the downregulation of NCAM expression is unknown, yet similar NCAM dosage effects have been reported. For example, neuronal cells seem to require a threshold level of NCAM expression to respond with neurite outgrowth[34]. Moreover, downregulation of NCAM expression precedes the migration of neural crest cells[35] and correlates with increased malignancy in human pancreatic and colorectal cancer[21].

Other than the importance of the general downregulation of NCAM expression, what is the role of the NCAM isoform switch during tumor development? Is the loss of NCAM 120 or the gain of NCAM 140/180 essential for the metastatic dissemination of β tumor cells? Information to answer this question comes from our observation that tumor progression, and in particular the metastatic phenotype, are indinguishable between RT2;+/– and RT2;–/– mice. Because RT2;–/– mice do not express any NCAM and therefore are unable to gain expression of NCAM 140/180, the gain of these isoforms is unlikely to play a part in the formation of metastases. In contrast, loss of NCAM expression seems to correlate with progression to tumor metastasis. Of the different NCAM isoforms, NCAM 120 seems to be consistently lost during the development of metastases and, conversely, maintenance of NCAM 120 expression during tumor development in RT2;+/– and RT2;–/– mice prevents metastatic dissemination.

The data indicate that loss of NCAM expression is one rate-limiting event in the induction of metastatic spread of β tumor cells. Based on this, future research will have to elucidate the molecular nature of the functional contribution of NCAM to the malignant behavior of tumor cells: Does loss of NCAM expression result in reduced cell–cell adhesion or disturbed cell–matrix interactions, or does it affect an unknown function of NCAM? In addition to its role in cell adhesion, NCAM may be involved in the transduction of signals from the extracellular environment to the interior of a cell. In neurons, NCAM as well as L1 and N-cadherin can bind and stimulate fibroblast growth factor receptors[36,37]. NCAM has also been found to associate with other signal transducing molecules, including focal adhesion kinase and the src-related tyrosine kinase fyn (ref. 38). Thus, unraveling the function of the various NCAM isoforms and their potential signaling capacities may lead to a better understanding of how tumor cells control their metastatic phenotypes.

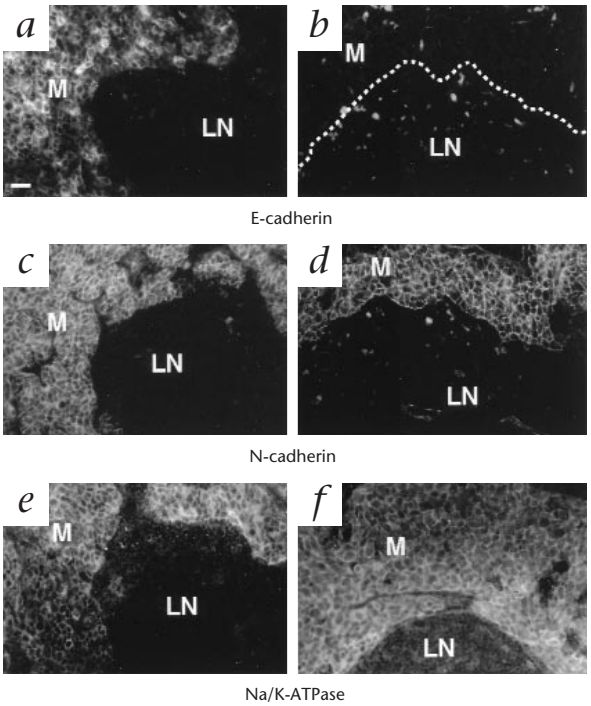

Fig. 3 Expression of epithelial markers in β-cell metastasis. Adjacent histological sections from a β-cell metastasis of an RT2;+/– mouse with relatively benign cellular morphology (***a***, ***c*** and ***e***) and malignant cellular morphology (***b***, ***d*** and ***f***) immunofluorescently stained using antibodies against E-cadherin (a and b), N-cadherin (c and d) and Na/K-ATPase (e and f). E-cadherin is expressed in metastatic β tumor cells with a benign cellular phenotype but not in those with a malignant phenotype. M, metastatic tumor cells; LN, lymph node cells. The dotted line in (b) demarcates the border between metastatic tumor cells and lymph node cells. Scale bar (in *a*) represents 25 μm.

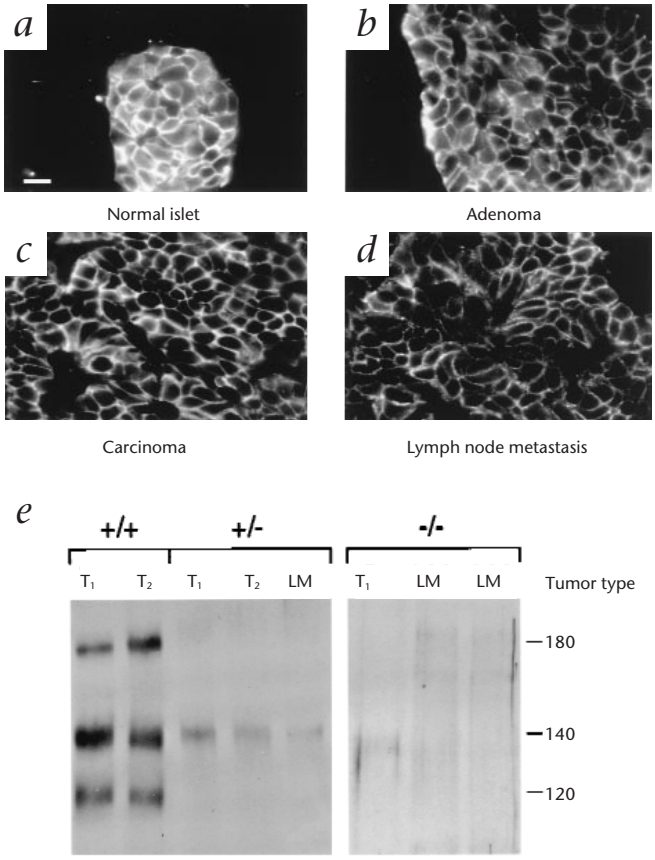

Fig. 4 Additional downregulation of NCAM expression during β-cell tumorigenesis. ***a***–***d***, Analysis (by immunofluorescence) of NCAM expression in the different stages of β-cell tumorigenesis in RT2;+/– mice. NCAM is expressed at relatively high levels in normal islets of Langerhans (a); it is also expressed by the tumor cells of adenomas (b), carcinomas (c) and lymph node metastasis (d), albeit at reduced levels. Scale bar (in *a*) represents 25 μm. ***e***, Quantitative immunoblotting analysis of NCAM expression in primary tumors and metastases of RT2;+/+, RT2;+/– and RT2;–/– mice. NCAM genotypes and tumor types are indicated above the lanes. The overall expression of NCAM in +/– tumors is less than half that in +/+ tumors. T_1, primary tumors with a diameter of less than 3 mm; T_2, primary tumors with a diameter of more than 4 mm; LM, lymph node metastases. Right margin, molecular masses of NCAM isoforms, in kDa.

Methods

Transgenic mice. The generation and phenotypic characterization of Rip1Tag2 transgenic mice[22–27] and NCAM-deficient mice[15] have been described. *NCAM*$^{-/-}$ mice were bred with C57/Bl6 mice for several generations, to increase the C57/Bl6 background, then bred with Rip1Tag2 transgenic mice (in C57/Bl6 background). Rip1/*NCAM 120* transgenic mice were generated according to standard procedures[39]. The transgene was constructed by inserting a 3-kb cDNA fragment encoding mouse NCAM 120 into the plasmid pRip1Tag (ref. 22) from which the fragment encoding the SV40 early region was removed by digestion with *Xba*I and *Hin*dIII. Genotypes were confirmed by Southern blot and PCR analysis. Expression of the *NCAM 120* transgene was determined by RT–PCR using transgene-specific primers and by immunoblotting and immunohistochemical analyses. A founder line expressing the transgene in pancreatic β cells starting approximately 4 weeks after birth was subsequently backcrossed to C57/Bl6 mice for at least two generations before being bred with Rip1Tag2 and *NCAM* knockout mice.

Glucose (5% weight/volume) was added to the drinking water of all tumor-bearing mice starting when they were 10 weeks of age, to counteract hypoglycemia that resulted from insulinoma development. Mice were autopsied between 12 and 18 weeks of age.

Histopathological analysis. Mice were killed by cervical dislocation. For immunohistochemical analysis, pancreata were removed and fixed in HBS-Ca^{2+} containing 4% paraformaldehyde for 2 h at 4 °C. The biopsied tissues were incubated overnight at 4 °C in HBS-Ca^{2+}/30% sucrose, embedded in OCT (Tissue Tek, Torrance, California), and 'snap frozen' in liquid nitrogen. Sections 10 μm in thickness were cut, mounted on silane coated glass slides (Sigma) and immunostained as described[27,33]. Specific antibody staining was visualized using either the ABC-Vector horse radish peroxidase kit according to the manufacturer's recommendations (Vector Laboratories, Burlington, California) or fluorescence-labeled secondary antibodies. Antibodies were polyclonal rabbit antibody against human NCAM (1:100 dilution; a gift from E. Bock, Copenhagen, Denmark), monoclonal rat antibody against mouse E-cadherin (1:150 dilution; Zymed, San Diego, California), monoclonal rat antibody against mouse N-cadherin (1:400 dilution; a gift from M. Takeichi, Kyoto, Japan), polyclonal guinea pig antibody against insulin (1:150 dilution; DAKO, Carpinteria, California) and rabbit polyclonal serum against mouse Na/K ATPase (1:500 dilution; a gift from W.J. Nelson, Stanford, California). Biotin-conjugated donkey anti-rat and anti-rabbit IgG (1:500 dilution) and Cy3-streptavidin (1:750 dilution) were purchased from Jackson ImmunoResearch Laboratories (West Grove, Pennsylvania). Actin was visualized with rhodamine-conjugated phalloidin, diluted as recommended by the manufacturer (Molecular Probes, Eugene, Oregon).

Immunoblotting. Islets of Langerhans were isolated by collagenase perfusion as described[22]. The different tumor stages were microdissected from pancreas of Rip1Tag2 transgenic mice of different ages. Primary tumor cells were partially purified by a sedimentation procedure, and established β tumor cell lines were cultured as described[28]. After being washed with PBS, isolated tumor stages or tumor cells were lysed in lysis buffer (0.5% Triton X-100, 10 mM Tris-HCl, pH 8, 100 mM NaCl and 2.5 mM EDTA) and sonicated for 1 min (Transsonic 570/H; Elma, Singer, Germany). Wheat germ agglutinin (Pharmacia) was used to enrich for membrane proteins of interest. Each sample was incubated with 50 μl of wheat germ agglutinin agarose beads in lysis buffer for 2 h at room temperature. Beads were washed three times with lysis buffer, resuspended in 40 μl sample solution and heated for 5 min to 95 °C. Proteins were resolved by 8% SDS–PAGE and transferred to nitrocellulose membranes. Equal loading of proteins was confirmed by Ponceau S staining of the membranes, before they were probed with polyclonal rabbit antibody against human NCAM (1:100 dilution; a gift from E. Bock, Copenhagen, Denmark) using incubation and washing buffers adjusted to a pH of 10.2. Immunostained proteins were visualized using the ECL chemoluminescence detection system (Amersham) according to the manufacturer's recommendations.

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