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Effect of N-Cadherin Misexpression by the Mammary Epithelium in Mice

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Abstract N-cadherin is not typically expressed by epithelial cells. However, it is detected in breast cancers and increases tumor cell migration and invasion in vitro. To explore its misexpression, we generated transgenic mice with N-cadherin in the mammary epithelium. Mammary glands appeared normal and no tumors arose spontaneously. To investigate N-cadherin misexpression in mammary tumors, *neu* was overexpressed through breeding. Tumors developed in +/*neu* and N-cadherin/*neu* mice, although few tumors in bitransgenic mice expressed N-cadherin, and they did not differ from N-cadherin-negative tumors. J. Cell. Biochem. 95: 1093–1107, 2005.

Key words: N-cadherin; mammary gland; tumorigenesis

Cadherins form a family of cell surface, transmembrane proteins that mediate cell–cell recognition and adhesion. E-cadherin typically is expressed by epithelial cells, whereas N-cadherin is found in multiple cell types, including nerve, myocardial, and mesenchymal cells. A conserved intracellular cadherin domain binds β-catenin, a protein whose interaction with α-catenin links the cadherin/catenin complex to the actin cytoskeleton. Beta-catenin also exists unbound to cadherins and can be found in the nucleus where it is an essential player in the Wnt signaling pathway, regulating transcription.

Cadherins are important for both embryogenesis and the normal function of tissues in the adult. Expression of classical cadherins such as E-, P-, and N-cadherin is regulated in a spatial and temporal fashion in developing and adult organisms. E- and N-cadherin play such critical roles in embryonic development that loss of either one results in embryonic lethality [Larue et al., 1994; Radice et al., 1997b]. In contrast, loss of P-cadherin does not cause embryonic lethality, but does result in altered mammary gland development in the mouse [Radice et al., 1997a]; hair and eye abnormalities in humans [Sprecher et al., 2001].

Cadherins also have been implicated in cancer. E-cadherin is both a tumor and invasion suppressor. Selective loss of E-cadherin in the adult has been shown to play a role in tumorigenesis in the mouse [Perl et al., 1998] and to

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correlate with cancer development and malignant behavior in humans [see reviews Nollet et al., 1999; Berx and van Roy, 2001; Van Aken et al., 2001; Conacci-Sorrell et al., 2002; Hajra and Fearon, 2002; Christofori, 2003]. In addition to losing E-cadherin by multiple molecular mechanisms, tumor cells often express cadherins not typically found in their normal cellular counterparts [reviewed by Cavallaro et al., 2002]. P-cadherin is not found typically in luminal mammary epithelial cells, but is expressed by human breast carcinomas, and its expression correlates with poor prognosis [Peralta Soler et al., 1999]. In addition, N-cadherin typically is not found in normal epithelial cells but is detected in tumors in vivo [Islam et al., 1996; Peralta Soler et al., 1999], including breast cancers, although the physiological significance of this is not clear. In vitro, human mammary tumor cell lines misexpressing N-cadherin exhibit increased migration and invasion [Nieman et al., 1999; Hazan et al., 2000], and when injected into nude mice show increased metastasis [Hazan et al., 2000]. The influence of N-cadherin on stimulating cell migration and invasion involves its interaction with the fibroblast growth factor receptor (FGFR) [Nieman et al., 1999; Suyama et al., 2002].

Our current understanding of the roles that cadherins play in tumor formation and malignant cell behavior points to the possibility that N-cadherin may play conflicting roles in tumorigenesis. N-cadherin, like E-cadherin, may initially suppress tumorigenesis through its action as a cell–cell adhesion protein, even if it is expressed inappropriately. However, later in the process, after multiple molecular alterations have occurred, N-cadherin may enhance malignant behavior by increasing tumor cell migration and invasion through its interaction with the FGFR. To further study the role N-cadherin misexpression plays in mammary epithelial cell behavior, we generated a transgenic mouse with N-cadherin in the normal adult mammary epithelium, under control of the mouse mammary tumor virus (MMTV) promoter. Adult transgenic and wild type mice were compared with respect to mammary gland morphology and function. To examine the effect of N-cadherin misexpression on mammary tumor cell behavior, the *neu* oncogene, also under control of the MMTV promoter, was overexpressed in the N-cadherin transgenic mouse using a breeding strategy. Control +/*neu* and bitransgenic N-cadherin/*neu* mice were compared for age of mammary tumor onset, tumor size, tumor number per mouse, tumor histology, and the presence of non-mammary lesions.

Results presented here show no effect of N-cadherin misexpression in the adult mammary epithelium on the morphology or function of the normal mouse mammary gland; nor did tumors arise spontaneously due to N-cadherin misexpression. In response to *neu* overexpression, mammary tumors developed in both control and N-cadherin transgenic mice. Surprisingly, very few tumors in the bitransgenic mice expressed N-cadherin protein. N-cadherin-positive tumors did not differ significantly from N-cadherin-negative tumors in size, histology, or metastasis to the lung, suggesting that N-cadherin misexpression did not alter mammary tumor behavior in our mouse model.

MATERIALS AND METHODS

Generation of N-Cadherin Transgenic Mice

Transgenic mice expressing N-cadherin in the mammary epithelium were generated as follows. A cDNA encoding mouse N-cadherin (plasmid kindly provided by Dr. Masatoshi Takeichi, Kobe, Japan) was removed from the vector by MscI and XbaI digestion and ligated into pBS-MMTV-pA that had been digested with SmaI and SpeI. The pBS-MMTV-pA expression vector containing the MMTV promoter was kindly provided by Dr. Lewis A. Chodosh, University of Pennsylvania, Philadelphia, PA. A 9 kb MMTV/N-cadherin-containing fragment was isolated from the plasmid by digestion with NotI and XhoI, purified and injected into fertilized oocytes derived from FVB/N mice, and two-cell embryos were transferred into the oviducts of pseudopregnant CD-1 mice. Transgenic founder mice and transgenic progeny were identified by PCR using the following primer set: 5′-TGGAGAGACTTCTGAAACAGC-3′ and 5′-CCATTCATCAGTTCCATAGGTTG-3′. Transgenic animals were identified by the presence of a distinct 446 bp band. The transgenic line was maintained on the FVB/N background and all studies involved mice of this genetic background. All studies involving mice were conducted at the Lankenau Institute for Medical Research and were approved by the Institutional Animal Care and Use Committee.

Generation of N-Cadherin Transgenic Mice With Mammary Tumors

To induce mammary tumor formation in the N-cadherin transgenic mice, hemizygous FVB MMTV/N-cadherin mice were mated to commercially available homozygous FVB transgenic mice having unactivated rat *neu,* under the control of the MMTV promoter (Jackson Laboratory, Bar Harbor, ME) (strain name: FVB/N-TgN(MMTVneu)202Mul). Homozygous MMTV/*neu* transgenic mice develop focal mammary tumors by an average age of 29 weeks. All progeny resulting from this mating strategy were hemizygous for the MMTV/*neu* transgene, whereas approximately half carried the MMTV/N-cadherin transgene (i.e., N-cadherin/*neu*), while the rest lacked it (i.e., +/*neu*) and served as controls. All mice underwent two full-term pregnancies and lactations to fully activate the MMTV promoter in both transgenes. Nineteen +/*neu* and 17 N-cadherin/*neu* littermates or age-matched female mice were analyzed for the age of tumor onset, number of tumors per mouse, size of tumors, and presence of non-mammary lesions.

Immunohistochemistry

Ectopic N-cadherin protein was detected in the mammary glands and mammary tumors using immunofluorescence light microscopy. Inguinal mammary glands were dissected free of skin and muscle, gently stretched on a glass slide, fixed overnight in 4% formaldehyde, embedded in paraffin, and sectioned (5 μM). Mammary tumors were placed in OCT Embedding Medium, frozen in liquid nitrogen, sectioned (5 μM), and fixed in ice-cold methanol for 10 min. Frozen thin sections were prepared for tumors to ensure highest sensitivity of protein detection. In the case of paraffin embedded mammary tissue, heat-induced antigen retrieval using a vegetable steamer (Black and Decker HS2000) and an antigen unmasking solution (Vector Laboratories, Burlingame, CA) was employed as described [Peralta Soler et al., 1999]. Background signal was blocked by a combination of Blotto (5% non-fat dry milk in PBS) and 20 μg/ml Fab fragment of goat anti-mouse IgG (Zymed Laboratories, South San Francisco, CA). The primary monoclonal antibodies to N-cadherin were 13A9 or 3B9 (available from Zymed Laboratories), both of which were generated by us. The antibodies bind the intracellular domain and recognize N-cadherin across species. We also used primary antibodies to E-cadherin (clone 36, BD PharMingen Transduction Laboratories, San Diego, CA), β-catenin (5H10) (Zymed Laboratories), and Neu (c-erbB-2/HER-2/neu Ab-17, LABVISION Corp. NeoMarkers, Fremont, CA) in our studies. The second antibody was Cy3-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA). Cadherin and catenin staining was visualized using a Zeiss fluorescence microscope, and photographed using Polaroid film (ASA 3000).

Western Immunoblot Analysis

Thoracic mammary glands and mammary tumors were dissected out, placed on dry ice and stored at −80°C. To extract proteins, tissue was weighed and then homogenized in 10 mM Tris acetate buffer, pH 8.0, containing 0.5% NP40, 1 mM EDTA and 25 μl/ml of a commercial cocktail of protease inhibitors (Sigma; St. Louis, MO) at a ratio of 1 ml extraction buffer to 0.12 g of tissue. The homogenized tissue was agitated on ice for 15 min, centrifuged at 12,000 rpm in an Eppendorf microfuge for 20 min at 4°C to remove solids, and the supernate collected, aliquoted and stored at −80°C. Protein concentration was determined by the Lowry method. Immunoblot analysis was performed as described [Johnson et al., 1993]. Extracted proteins were separated on a 7.5% acrylamide SDS–PAGE gel, loading 300 μg protein per well. Prestained standards were used for determining molecular weights (BioRad, Hercules, CA). The separated proteins were transferred electrophoretically to nitrocellulose overnight and Western immunoblot analysis was performed as described [Johnson et al., 1993]. The N-cadherin antibodies were the same ones used for immunohistochemistry and the secondary antibody was alkaline phosphatase conjugated goat anti-mouse IgG (Southern Biotechnology Associates, Inc; Birmingham, AL). The signal was generated using 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro blue tetrazolium (NBT) (Sigma; St. Louis, MO). Monoclonal antibodies to E-cadherin (clone 36) and β-catenin (5H10) were from BD PharMingen Transduction Labs (SanDiego, CA), anti-Neu was from NeoMarkers (Fremont, CA), and anti-β-actin (AC-15) was from Sigma (St. Louis, MO). Immunoblotting for β-actin confirmed that a similar amount of protein was loaded for each

sample. All monoclonal antibodies used in this study are highly specific for their respective antigens.

Whole Mammary Gland Analysis (Whole Mount Preparation)

The method used to evaluate mammary gland development at the whole tissue level was as described [Faulkin and De Om, 1960]. Inguinal mammary glands were dissected out, stretched on a glass slide, and fixed overnight in 4% formaldehyde. The glands were dehydrated and defatted in acetone overnight and stored in xylene until stained. The glands were rehydrated in a sequence of decreasing ethanol concentration, treated with Harris stain, dehydrated in increasing ethanol concentration, and stored in xylene. The stained mammary parenchyma was observed distinctly against the translucent fat pads.

Histochemical Analysis of Mammary Glands and Tumors

Mammary glands were dissected out, fixed, and embedded in paraffin. Five-micron sections were cut, de-waxed, dehydrated in descending alcohol concentration, and stained with H&E. Tumors were dissected out of the gland, frozen in OCT, sectioned, and stained with H&E. Selected tumors were sent to a consulting veterinary pathologist, who was blinded to identity of the samples.

Tumor Analysis

Tumor onset was determined by recording the age when the first tumor was detected by visual observation and/or palpation of the mammary glands, which was done daily. The total number of tumors per mouse was determined following euthanasia by counting visible tumors in dissected and exposed mammary glands. The average number of tumors per mouse in the two experimental groups, with/without exogenous N-cadherin, was calculated by dividing the total number of tumors by the number of mice per group. All visible tumors were counted, even those too small to measure their size accurately. Size was determined for those tumors that were large enough to be measured accurately (i.e., >5 mm diameter) and did not disintegrate upon excision. After the tumors were removed from the surrounding mammary tissue, they were weighed and measured using a standard caliper. Tumor volume was computed using either of two equations, depending on the shape of the tumor: (1) $V_{ol} = Pi/6 \times a^2 \times b$ (elliptical solid; a = shorter dimension and b = longer dimension), or (2) $V_{ol} = Pi/6 \times d^3$ (circular solid; d = diameter). To calculate the average tumor size for the experimental groups, we first determined the average tumor size per mouse and then calculated an overall average for the group. Tumors large enough to be measured were divided into portions for immunoblot analysis and histological analysis, including H&E staining and immunohistochemistry. Following euthanasia, the lungs, liver, spleen, kidneys, intestines, bladder, and reproductive organs were screened visually for evidence of metastases. Tissues with lesions were processed for histological analysis. Selected tumors were sent for further analysis to a consulting veterinary pathologist who was uninvolved with the project and blinded to the identity of tumors.

Statistical Analysis

For statistical analyses of most parameters studied, for example, comparing tumor size and number of tumors/animal between bitransgenic N-cadherin/*neu* mice and control hemizygous +/*neu* mice, we used a two-tailed Student's *t*-test. To compare the age of tumor onset between the two experimental groups, we sought the help of a statistician who evaluated the data using the Wilcoxon Rank Sum test and STAT Xact software.

RESULTS

Generation of N-Cadherin Transgenic Mice

We generated transgenic mice that expressed N-cadherin in the mammary epithelium in order to evaluate the effect that N-cadherin misexpression has on normal mammary gland behavior and on mammary tumor cell behavior in vivo. N-cadherin is expressed inappropriately by epithelial derived tumors [Islam et al., 1996; Peralta Soler et al., 1999], including breast cancers, although the physiological significance of this is not fully understood. Our published in vitro studies with human mammary tumor cell lines, some of which express N-cadherin, indicated that N-cadherin misexpression may enhance tumor cell malignancy since it increases cell migration and invasion in vitro [Nieman et al., 1999]. In addition, studies from the Hazan laboratory showed that N-cadherin

misexpression increases the metastasis of human mammary tumor cells in nude mice [Hazan et al., 2000].

To drive N-cadherin misexpression in the mammary epithelium, we used a modified MMTV promoter, which is highly active in the pregnant and lactating gland [Gunther et al., 2002]. The low to absent activity of this promoter before sexual maturity eliminated possible effects on embryonic development of the mammary gland. The mouse N-cadherin cDNA was placed under the control of a modified MMTV promoter (Fig. 1A) as described in the Methods section, and transgenic mice were generated on the FVB genetic background. This strain is not susceptible to developing mammary tumors spontaneously. The presence of the N-cadherin transgene was detected in founders by PCR analysis using primers specific for the construct (Fig. 1B).

Expression of N-Cadherin Protein in the Mammary Epithelium

The progeny of one founder exhibited especially high and homogenous expression of N-cadherin protein in the mammary epithelium, and therefore studies reported here were conducted using this line. Expression of N-cadherin protein in the mammary glands of F1 generation female progeny was evaluated by immunohistochemistry (Fig. 2) and Western immunoblot analysis (Fig. 3). We initially examined mammary glands from mice at day-2 of lactation, when the population of epithelial cells in the gland is rich and the MMTV promoter activity is high. The monoclonal antibodies to N-cadherin recognize both endogenous and exogenous N-cadherin; however endogenous N-cadherin was barely detectable in the mammary gland of wild type mice (Figs. 2 and 3) and therefore the exogenous N-cadherin was readily detected above background levels by both immunohistochemistry (Fig. 2) and immunoblot analysis (Fig. 3). We estimated from the immunohistochemistry that the population of mammary epithelial cells positive for N-cadherin was >85%, although we routinely observed cells that appeared negative for N-cadherin.

N-cadherin was localized to the plasma membrane of mammary epithelial cells and was detected at cell–cell borders in a pattern typical of a cadherin cell–cell adhesion protein

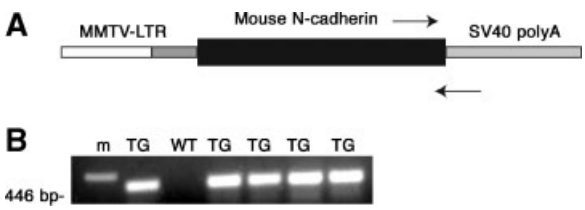

Fig. 1. Generation of MMTV/N-cadherin transgenic mice. **A**: Schematic of the transgenic construct. The construct contained the MMTV-LTR (open box), followed by the leader sequence of v-H-ras (dotted box), the mouse N-cadherin cDNA (closed box), and SV40 sequence carrying splicing and polyadenylation signal sequences (hatched box). Arrows note the relative positions of the oligonucleotide primers used to amplify the transgene allele. **B**: Identification of transgenic mice by PCR. N-cadherin transgenic mice were identified by the presence of a distinctive 446 bp product (marked by arrow). TG, transgenic; WT, wild type; m = 500 bp molecular weight marker.

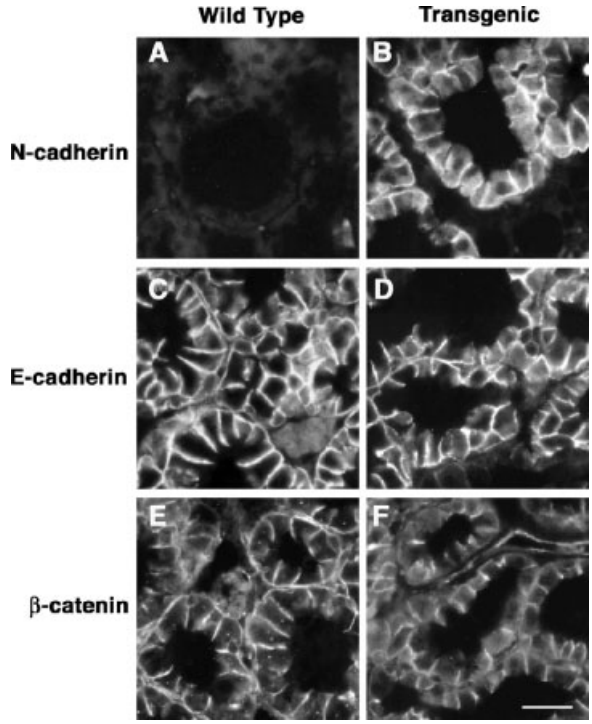

Fig. 2. Immunohistochemical detection of N-cadherin, E-cadherin, and β-catenin in mammary tissue. Mammary glands from day-2 lactating wild type and N-cadherin transgenic mice were isolated, fixed, paraffin-embedded, sectioned, and stained using monoclonal antibodies to N-cadherin, E-cadherin, or β-catenin. As shown in **panel B**, the N-cadherin antibody recognized the exogenous N-cadherin expressed at cell–cell borders in mammary epithelial cells, whereas no signal above background was observed in wild type tissue (**panel A**). **Panels C** and **D** show that E-cadherin was expressed at cell–cell borders in both wild type and transgenic tissue. **Panels E** and **F** show that β-catenin was expressed in both wild type and transgenic animals, and was found at cell–cell borders, but was not particularly noticeable in nuclei. Exposure times were similar. All pictures were taken at the same magnification. Magnification bar, 50 μm.

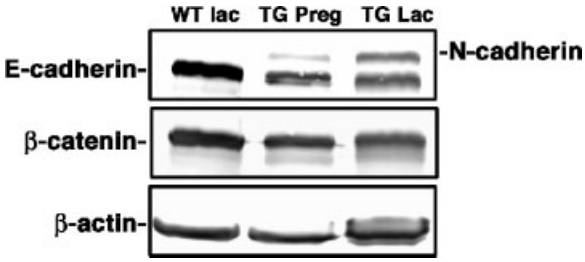

Fig. 3. Western immunoblot analysis of normal mammary glands from wild type and N-cadherin transgenic mice. Mammary glands from wild type (WT) and N-cadherin transgenic (TG) mice at day-18 pregnancy (Preg) or 2-day lactation (Lac) were extracted, separated by SDS–PAGE, and immunoblotted with antibodies to N-cadherin (135 kD), E-cadherin (120 kD), β-catenin (95 kD), or actin (45 kD) to ensure equal protein loading. Wild type mice exhibited a low to negative signal for N-cadherin band in wild type mice, whereas N-cadherin was readily detectable in mammary glands from pregnant and lactating transgenic mice. The E-cadherin signal was consistently lower in extracts of transgenic glands, compared to wild type glands.

(Fig. 2). Endogenous E-cadherin also was expressed by mammary epithelial cells and was present in both N-cadherin transgenic and wild type mice, as was β-catenin. By immunohistochemical analysis the E-cadherin was present at cell–cell borders. Although immunohistochemistry cannot provide quantitative analysis, qualitatively the E-cadherin appeared to be expressed similarly in both wild type and transgenic mice (Fig. 2), indicating that ectopic N-cadherin did not eliminate or radically affect endogenous E-cadherin expression, nor did it displace E-cadherin from cell–cell contacts. Beta-catenin staining showed no apparent difference in its pattern or level when comparing mammary glands from N-cadherin transgenic and wild type mice. The β-catenin staining was prominent at cell–cell contacts and was not noticeable in the nucleus where it might be transcriptionally active (Fig. 2).

Consistent with immunohistochemical analysis, both N-cadherin (135 kD) and E-cadherin (120 kD) were detected by immunoblot analysis in the glands of the N-cadherin transgenic mice, and E-cadherin was detected in glands from wild type mice (Fig. 3). In immunoblots, the E-cadherin level appeared somewhat reduced in N-cadherin transgenic mice compared to wild type mice, suggesting that exogenous N-cadherin has some inhibitory effect on the level of endogenous E-cadherin. This might be due to limiting β-catenin, since β-catenin (95 kD) was detected at similar levels in wild type and N-cadherin transgenic mice (Fig. 3). A decrease in endogenous cadherin due to expression of an inappropriate exogenous cadherin is not necessarily the norm. The Radice laboratory did not observe a decrease in endogenous N-cadherin in the mouse heart forced to express exogenous E-cadherin [Ferreira-Cornwall et al., 2002]; nor did the Wheelock and Johnson laboratory always see a decrease in E-cadherin in epithelial cells forced to express N-cadherin [Nieman et al., 1999]. We should point out that comparing E-cadherin (or exogenous N-cadherin) levels in mammary tissue among mice within, or between experimental groups is not straightforward. The mammary gland is comprised of multiple cell types, whereas only the epithelium expresses E-cadherin (and exogenous N-cadherin). Moreover, the epithelial fraction of the mammary gland with the physiological state of the female.

Morphology and Function of Mammary Glands

To evaluate the effect of N-cadherin misexpression on the mammary gland, we observed and compared the morphology and function of glands from sexually mature transgenic mice and wild type littermates. Because the MMTV promoter is inactive or weakly active before sexual maturity, we did not examine mammary glands prior to sexual maturity. To analyze adult mammary gland morphology, two methods were used. First, we prepared "whole mounts" of mammary glands in order to examine them at a whole tissue level. Second, we fixed, paraffin-embedded, sectioned, and stained mammary glands for light microscopic analysis.

Whole mount analysis revealed similar ductal system architecture in transgenic and wild type mammary glands at mid- and late-stage of pregnancy (data not shown), indicating no gross differences in mammary gland structure. In addition, no significant or consistent differences were seen in mammary gland morphology of wild type versus N-cadherin transgenic mice by microscopic analysis. Figure 4 shows representative H&E stained sections of mammary glands from day-18 pregnant wild type and N-cadherin transgenic mice. Despite examining mammary glands from multiple N-cadherin transgenic and wild type mice, we failed to detect any gross or microscopic architectural or morphological differences beyond the animal-

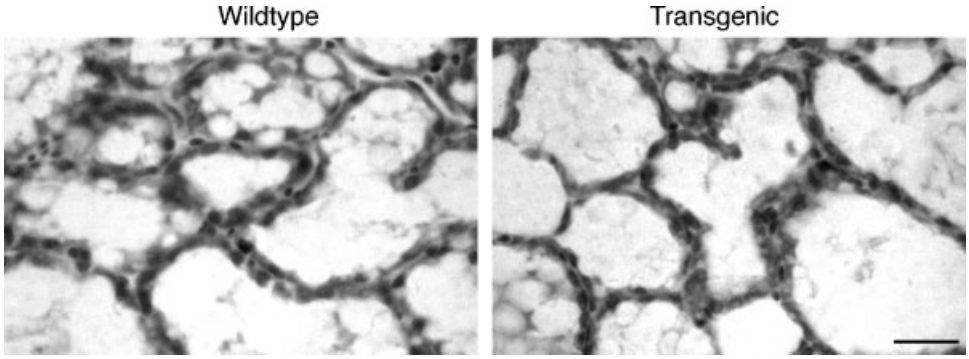

Fig. 4. Hematoxylin and Eosin (H & E) stained mammary gland from day-18 pregnant wild type and N-cadherin transgenic mice. Shown is the ductal structure, with fat in between. No differences were noted between the two genotypes that were beyond mouse-to-mouse variation within the wild type group. Pictures were taken at the same magnification. Magnification bar, 100 μm.

to-animal variability observed within each group. In addition, mammary gland function did not appear to be affected by N-cadherin misexpression. Throughout our studies we routinely noted pup size and litter number as we expanded the colony for analysis, and observed no consistent differences between transgenic and wild type mice.

No differences in the mammary glands were observed with age when comparing the transgenic and wild type mice. To look for differences that might occur with age, and for the possible spontaneous formation of mammary tumors due to N-cadherin misexpression, we examined and compared N-cadherin transgenic and wild type female mice 19–21 months of age. All females underwent two full pregnancies and lactations, so that N-cadherin misexpression was maximally stimulated for ~12 weeks during their lifespan. We did not observe any differences in the gross or microscopic architecture or morphology of mammary glands of N-cadherin transgenic versus wild type mice, despite the fact that N-cadherin could be detected at higher than endogenous levels in the mammary glands of non-pregnant, non-lactating aged transgenic mice (data not shown). In addition, no mammary tumors formed in either transgenic or wild type aged mice.

Analysis of Mammary Tumors in Mice With/Without Exogenous N-Cadherin

The results described above indicated that N-cadherin misexpression in the adult mammary epithelium has no significant effect on the structure or function of the normal mammary gland, nor does it induce tumor formation. However, to determine if N-cadherin misexpression alters tumor cell behavior in the mice, we induced mammary tumors in the N-cadherin transgenic mice by mating them to transgenic mice that develop mammary tumors in response to an oncogene. We chose the MMTV/*neu* mouse because of the high incidence of mammary tumors in this model and because *neu* is the mouse homolog of *HER2*, which is amplified in a substantial portion of human breast cancers.

Homozygous FVB mice overexpressing *neu,* which like N-cadherin was under control of the MMTV promoter (i.e., MMTV/*neu*), were purchased from Jackson Laboratory and mated to hemizygous MMTV/N-cadherin transgenic mice. All of the progeny from this mating strategy were hemizygous for the *neu* transgene, whereas approximately half carried the N-cadherin transgene (i.e., N-cadherin/*neu*), and the other half lacked it (i.e., +/*neu*). A PCR-based strategy was used to distinguish animals with or without the N-cadherin transgene. Similar numbers of littermate or age-matched female mice from the two groups (i.e., bitransgenic N-cadherin/*neu* and control +/*neu*) were selected for tumor analysis. All mice in both groups underwent two full-term pregnancies and lactations to fully activate the MMTV promoters in both transgenes. We started with 20 mice in each group but eliminated from the study any mouse that did not successfully undergo two pregnancies and lactations. We ended up with 19 mice in the control +/*neu* group and 17 mice in the bitransgenic N-cadherin/*neu* group.

For each mouse in both experimental groups we recorded the age of tumor onset. Following

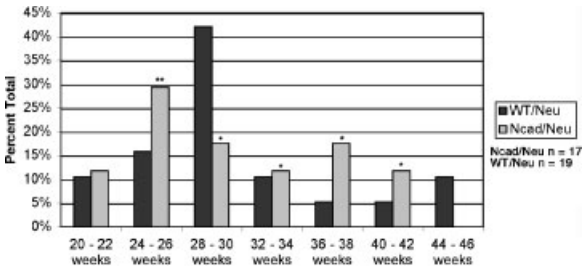

Fig. 5. Age of onset of mammary tumors in bitransgenic MMTV/N-cadherin/*neu* versus control MMTV/+/*neu* mice. Tumor onset was determined by noting the age when the first tumor was detected by visual or manual inspection. The number (n) of mice was 19 for the control group (WT/Neu) and 17 for the bitransgenic group (Ncad/Neu). Stars denote bitransgenic mice that were later identified as having N-cadherin-positive tumors. Although the highest incidence of tumors was a couple weeks earlier in the bitransgenic group compared to the wild type group, this result was not statistically significant.

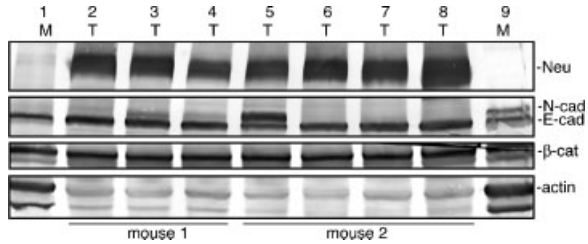

Fig. 6. Immunoblot analysis of tumors from bitransgenic MMTV/N-cadherin/*neu* mice. Tumors were extracted and analyzed for the expression of Neu (185 kD), N-cadherin (135 kD), E-cadherin (120 kD), β-catenin (95 kD), and actin (45 kD) to ensure similar protein loading. Extracts of mammary tissue (M) from +/*neu* (**lane 1**) and N-cadherin/*neu* (**lane 9**) mice were included as controls. None of the mice were pregnant or lactating when the mammary tissue and tumors were collected, although all had previously undergone two full-term pregnancies and lactations. Three tumors (T) were taken from one bitransgenic mouse (**lanes 2–4**) and four were from a second mouse (**lanes 5–8**). Equal amounts of protein were loaded for all samples. Note that the Neu signal was intense in all tumors, but was not detected in the mammary glands from the control +/*neu* and N-cadherin/*neu* bitransgenic mice. One tumor had a strong N-cadherin signal (lane 5), one moderate (lane 3), and one low (lane 4). In four tumors, N-cadherin was not detected (lanes 2, and 6–8). E-cadherin was present in all samples, and the level appeared slightly reduced when N-cadherin expression was high (lane 5). β-catenin levels appeared comparable in the tumors.

euthanasia of the mice due to high tumor burden, we recorded the total number of tumors per mouse, the size of measurable tumors, and the presence of any lesion(s) outside the mammary gland. Tumor onset was defined as the week of age that the first tumor was observed visually or palpated manually. All animals in both groups eventually developed at least one tumor. Figure 5 shows that the highest incidence of tumor onset in the control +/*neu* group was seen at 28–30 weeks of age, and that by 46 weeks of age all animals had tumors. The highest incidence of tumor onset in the bitransgenic N-cadherin/*neu* group was slightly earlier, occurring at 24–26 weeks of age, and all mice had tumors by 42 weeks of age. The difference in tumor onset was not statistically significant between the two groups, as determined by the Wilcoxon Rank Sum test.

To examine exogenous N-cadherin expression in tumors from the bitransgenic N-cadherin/*neu* mice, we performed immunoblot analysis on every tumor (i.e., 41 tumors from 17 mice). Surprisingly, this analysis revealed that the majority (~80%) of the tumors lacked a signal that was above background from endogenous N-cadherin, although six tumors showed a moderate-to-strong signal above background (Fig. 6). All tumors expressed E-cadherin and β-catenin. The level of E-cadherin in N-cadherin-positive tumors, like that in the N-cadherin-expressing mammary epithelium (Fig. 3), was somewhat reduced in comparison to N-cadherin-negative tumors, as determined by immunoblot analysis (Fig. 6). As expected, since mammary tumors form only in mice bearing the *neu* transgene, both N-cadherin-positive and -negative tumors expressed high levels of Neu Protein (Fig. 6).

To examine the cellular localization of N-cadherin in tumors, immunohistochemistry was performed on five of the six N-cadherin-positive tumors. One tumor was too small to divide for both immunoblot and immunohistochemical analysis. We also performed immunohistochemisty on several tumors negative for N-cadherin by immunoblot analysis. Immunohistochemistry on tumors positive for N-cadherin by immunoblot analysis revealed either heterogenous or homogenous staining, with obvious surface membrane staining (Fig. 7). Tumors negative for N-cadherin by immunoblot analysis were negative by immunohistochemistry, or had only a few N-cadherin-positive cells, even when N-cadherin staining could be detected in adjacent normal epithelium. Tumors from both bitransgenic and control mice expressed E-cadherin and β-catenin (Fig. 7). The decrease in E-cadherin expression in N-cadherin-positive tumors seen by immunoblot analysis was not apparent by immunohistochemistry. Beta-catenin staining was similar in tumors from both groups and was

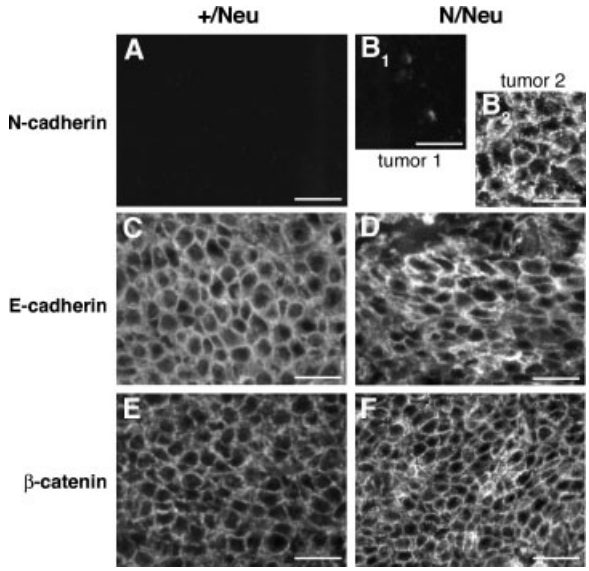

Fig. 7. Immunohistochemical analysis of mammary tumors from control MMTV/+/*neu* and bitransgenic MMTV/N-cadherin/*neu* mice. Tumors from control (+/Neu) and bitransgenic (N/Neu) mice were analyzed by immunohistochemical analysis for N-cadherin (**A**, **B_1**, **B_2**), E-cadherin (**C** and **D**) and β-catenin (**E** and **F**). Representative examples of the results are shown. Note that the tumor from the control MMTV/+/neu (+/Neu) mice was negative for N-cadherin (A), and positive for both E-cadherin (C) and β-catenin (E), as expected. Panel B_1 shows a N-cadherin-negative tumor from a bitransgenic N-cadherin/*neu* (N/Neu) mouse. Panel B_2 shows an N-cadherin-positive tumor from a bitransgenic mouse. Panels D and F show E-cadherin and β-catenin staining, respectively, for the N-cadherin-positive tumor. The level and membrane localization of E-cadherin and β-catenin appeared similar for control and bitransgenic mice.

not noticeably present in the nucleus. The low percentage of N-cadherin-positive tumors in the N-cadherin/*neu* group was surprising because exogenous N-cadherin was detected in the majority of the mammary epithelial cells (Fig. 2), from which the tumors presumably arose.

To look for differences in tumor behavior between the bitransgenic N-cadherin/*neu* and control +/*neu* mice, we compared the tumor size. We looked at the data in two ways. First, we pooled all of the bitransgenic N-cadherin/*neu* mice and compared them to the control mice. In addition, we divided the bitransgenic mice into two subgroups: (1) mice having a tumor with moderate-to-high N-cadherin expression and (2) mice having tumors with background N-cadherin expression, as determined by immunohistochemistry and/or immunoblot analysis. Six bitransgenic mice each had one tumor with moderate-to-high N-cadherin expression. N-cadherin-positive and -negative tumors were found in the same mice. We observed no significant difference in average tumor size comparing control +/*neu* mice to the pooled N-cadherin/*neu* mice (Fig. 8; Student's *t*-test), indicating that N-cadherin expression in the surrounding mammary epithelium did not affect tumor size; nor did we see any significant difference in average tumor size when comparing N-cadherin-positive and -negative tumors (Fig. 8; *t*-test), suggesting that N-cadherin expression by a tumor did not alter its size.

We also analyzed the number of tumors per mouse, initially comparing the pooled bitransgenic group to the control group. No significant difference was noted (Fig. 9; *t*-test). The pooled N-cadherin/*neu* group (17 mice) had an average of 2.4 tumors per mouse and the control group (19 mice) had an average of 2.9 tumors per mouse. However, when we subdivided the bitransgenic group into mice with N-cadherin-positive tumors (6) and mice without N-cadherin-positive tumors (11), we observed a difference. Bitransgenic mice with N-cadherin-positive tumors had an average of 4.3 tumors per mouse, whereas bitransgenic mice without an N-cadherin-positive tumor only had an average of 1.4 tumors per mouse (Fig. 9). The difference between the bitransgenic subgroups was significant (Student's *t*-test; $P < 0.001$). In addition, the bitransgenic subgroup lacking N-cadherin-positive tumors had significantly fewer tumors per mouse than the control +/*neu* group (Student's *t*-test; $P = 0.007$). The number of tumors per mouse in the bitransgenic subgroup with N-cadherin-positive tumors did not differ significantly from the control (Student's *t*-test; $P = 0.12$).

The observation of reduced numbers of tumors per mouse in the bitransgenic N-cadherin/*neu* sub-group lacking N-cadherin-positive tumors (11 out of 17 mice) was puzzling. We considered several explanations for this result, including: (1) the MMTV promoter driving the *neu* transgene was less active and thus Neu levels reduced, (2) changes in E-cadherin due to exogenous N-cadherin expression affected tumor formation, and (3) N-cadherin expression in the normal epithelium acted as a tumor suppressor. To address the first possibility, we examined by immunoblot analysis the level of Neu protein in normal mammary glands from tumor-bearing mice of three groups: +/*neu* control and N-cadherin/*neu*

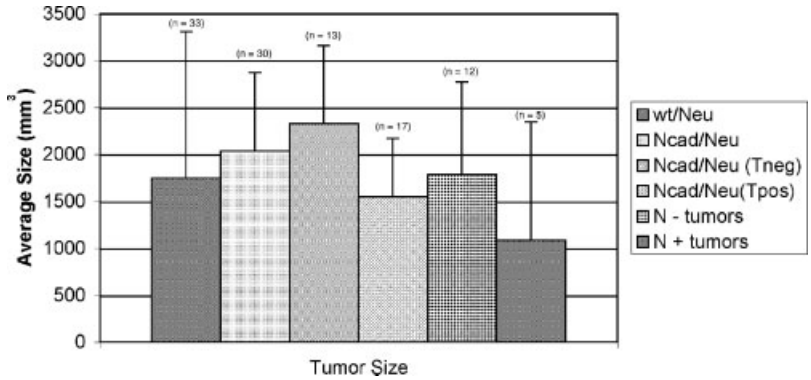

Fig. 8. Average tumor size in bitransgenic MMTV/N-cadherin/*neu* versus control MMTV/+/*neu* mice. Tumor size was measured with calipers after euthanasia of the mouse and tumor dissection (see Methods). Only tumors whose size could be measured accurately (>5 mm diameter) or that did not disintegrate upon dissection were included in this study, that is, n = 33 tumors for the control group and n = 30 tumors for the bitransgenic group. For the purpose of analysis, we divided the data into several categories: tumors from control mice (+/Neu); tumors from all bitransgenic mice regardless of whether or not the mouse had an N-cadherin-positive tumor (Ncad/Neu); tumors from bitransgenic mice with only N-cadherin-negative tumors (Ncad/Neu(Tneg); tumors from mice with N-cadherin-positive tumors (Ncad/Neu(Tpos). For bitransgenic mice having N-cadherin-positive tumors, we compared the size of their N-cadherin-negative tumors (Ncad-tumors) versus their N-cadherin-positive tumors (Ncad + tumors). Lines in bars indicate standard deviation. We noted no significant difference among any of the groups, regardless of how the data were analyzed.

bitransgenic with/without N-cadherin-positive tumors. All mice had undergone two full-term pregnancies and lactations, but were neither pregnant nor lactating when the mammary glands were collected.

Figure 10 shows that in control group the level of Neu varied from a low or undetectable level (lanes 2–4) to a high level (lane 5). This may be due to differences in the activity of the MMTV promoter in response to what stage of the estrus cycle the female was in. Alternatively, a high Neu signal may reflect the presence of a tumor not visible by eye, since tumors express high levels of Neu (Figs. 6 and 10, lane 1). The levels of Neu also varied from low or undetectable to high among the bitransgenic animals (lanes 6–13). However, a trend was noted in the case of the bitransgenic mice. When Neu was high, N-cadherin was low (Fig. 10, lanes 6, 7, 10), and when N-cadherin was high, Neu was low (lanes 8, 9, 12, 13). This inverse relationship between Neu and N-cadherin protein levels suggest there might be competition between the two MMTV-driven

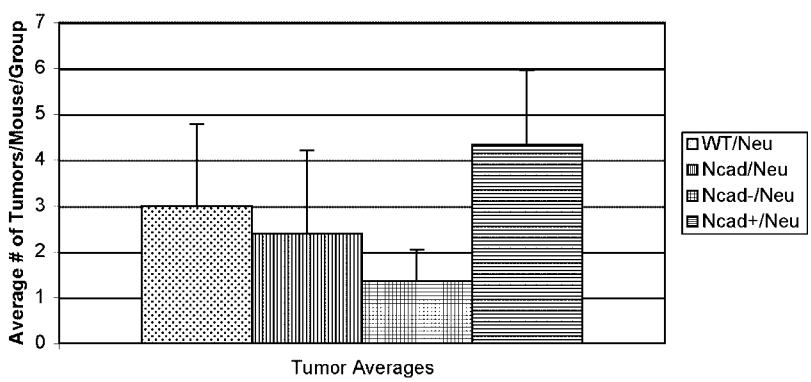

Fig. 9. Average number of tumors per mouse in bitransgenic N-cadherin/*neu* versus control +/*neu* groups. The number of tumors per mouse was determined by visual inspection following mouse euthanasia and mammary gland dissection. All visible tumors were counted, even those too small to measure their size accurately (i.e., <5 mm). Fifty-six tumors were detected in the 19 control +/*neu* mice (WT/Neu) and 41 tumors were detected in the 17 bitransgenic N-cadherin/*neu* mice (Ncad/Neu). The data for the bitransgenic mice were further analyzed by dividing the group into the 11 mice with only N-cadherin-negative tumors (Ncad−/Neu) and the 6 mice with N-cadherin-positive tumors (Ncad+/Neu). Mice with N-cadherin-positive tumors also had N-cadherin negative tumors. Lines in bars represent standard deviation. Note that the number of tumors per mouse is significantly lower in bitransgenic mice with only N-cadherin-negative tumors compared to bitransgenic mice with N-cadherin-positive tumors (Student's *t*-test; $P < 0.001$), and to control mice ($P = 0.007$).

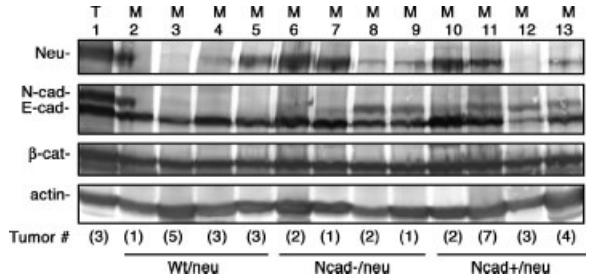

Fig. 10. Western immunoblot analysis of mammary glands from tumor-bearing +/*neu* and N-cadherin/*neu* mice. Mammary glands without tumors visible to the eye were collected from tumor-bearing, non-pregnant, non-lactating control +/*neu* mice (**lanes 2–5**) and bitransgenic N-cadherin/*neu* mice without (**lanes 6–9**) or with (**lanes 10–13**) N-cadherin-positive tumors. Each mammary gland was from a different mouse and the number of tumor/mouse is indicated in parentheses. The tumor extract added as a control for Neu (**lane 1**) was from the same mouse as the mammary gland extract in **lane 12**. Extracts were resolved by SDS–PAGE, transblotted to nitrocellulose, and probed for Neu, N-cadherin, E-cadherin, β-catenin, and actin as a loading control. There was some leakage of the tumor extract from **lane 1** into the mammary gland sample in **lane 2**. In addition, the background was high because the nitrocellulose was probed sequentially without stripping using antibodies to (1st) E-cadherin, β-catenin, and actin, (2nd) N-cadherin, and (3rd) Neu, and the data recorded after each probing. The levels of Neu in the mammary glands varied considerably among mice within a group. No trend was seen between the different groups of mice and no correlation was apparent between the level of Neu in the non-tumor-bearing mammary gland and the number of tumors in the mouse. N-cadherin was not detected in control mice (**lanes 2–5**), as expected. Its level varied among the bitransgenic mice, with no compelling trend comparing mice with or without an N-cadherin positive tumor. However, there was a trend for Neu to be lower when N-cadherin was higher (compare **lanes 8**, **9**, **11–13** to **lanes 6**, **7**, and **10**). E-cadherin levels varied some, with slightly reduced E-cadherin correlating with high N-cadherin (compare **lanes 8** and **9** to **6** and **7**). Beta-catenin levels were similar.

transgenes at the transcriptional or post-transcriptional level. Since tumors only form in response to Neu, and all tumors exhibited a high level of Neu (Fig. 6), tumors may be more likely to arise from epithelial cells with high Neu and low or no N-cadherin, resulting in few N-cadherin-positive tumors and even reduced numbers of tumors. However, in the bitransgenic mice, most mammary glands (Fig. 10) and some tumors (Fig. 6) expressed both Neu and N-cadherin, perhaps suggesting that additional factors besides Neu levels might be involved in the reduced number of tumors per mouse seen in the bitransgenic group with only N-cadherin-negative tumors.

We also examined E-cadherin in the mammary glands from the three groups of mice to determine if changes in its levels might explain the reduced number of tumors in bitransgenic mice lacking N-cadherin-positive tumors. E-cadherin was detected in all mammary glands and exhibited far less variability than either Neu or N-cadherin. Again we saw a trend that when N-cadherin expression was high, E-cadherin was somewhat reduced. Given that E-cadherin is a tumor suppressor we might predict that reduced E-cadherin would lead to increased tumors. However, there was no apparent relationship between E-cadherin levels in the normal mammary gland and the number of tumors that developed in the mouse. Thus, E-cadherin did not appear to contribute to the reduced number of tumors in the bitransgenic with only N-cadherin-negative tumors (lanes 6–9). The third possibility to explain the reduced tumors in these mice, that is, the N-cadherin expression by the mammary epithelium plays a tumor suppressor role, and that tumors only arise from N-cadherin-negative cells, could not be tested directly.

The tumor-bearing mice were euthanized at 6–14 months of age when the tumor burden affected their health and/or quality of life. The average age for euthanasia of the control +/*neu* group was 9.7 months, and for the pooled bitransgenic N-cadherin/*neu* group 9.1 months. Subdividing the bitransgenic group, the average age of euthanasia was 9.5 months for mice with N-cadherin-positive tumors and 8.8 months for mice with only N-cadherin-negative tumors. Using a *t*-test to compare the two groups of mice the presence of N-cadherin in the mammary epithelium or in the tumor did not appear to significantly affect the age at which the tumor burden necessitated euthanasia. Following euthanasia we analyzed bitransgenic and control mice for lesions outside the mammary gland by visual inspection of major organs. We detected lesions only in the lung. Five out of 19 (26%) control mice had a lung lesion. Three out of 17 (18%) mice in the bitransgenic MMTV/N-cadherin/*neu* group had a lesion. Subdividing the bitransgenic group into mice with/without N-cadherin-positive tumors revealed that 2 out of 6 (33%) of the mice with N-cadherin-positive tumors had a lung lesion, whereas only 1 out of 11 (9%) of the mice with N-cadherin-negative tumors bore a lesion. The low percentage of lung lesions in the bitransgenic subgroup without an N-cadherin-positive tumor likely reflects the reduced number of tumors per mouse seen in

this group (Fig. 9). The data presented here do not support an increase in metastasis due to N-cadherin misexpression in our mouse model. It is possible that to see N-cadherin-enhanced metastasis in this system, elevated FGFR, or perhaps increased matrix metalloproteinase levels is also required. It is also possible that the number of mice with N-cadherin-positive tumors was too low to draw conclusions with high confidence. However, we were unable to generate additional mice with N-cadherin positive tumors because of the challenge this represented for our available resources. Only approximately half of the progeny from mating MMTV/*neu* to MMTV/N-cadherin transgenic mice are bitransgenic, only approximately half of the bitransgenic are female, the mammary tumors take 6–10 months to develop, and only about a third of the females develop N-cadherin-positive tumors.

Histological sections of N-cadherin-positive and -negative tumors from bitransgenic N-cadherin/*neu* mice were compared to each other, and to tumor sections from control +/*neu* mice. The sections were analyzed by a consulting veterinary pathologist blind to the identity of the specimens. We were interested in knowing if N-cadherin misexpression altered any pathological characteristic of the tumors. Several histologic parameters were assessed, including overall tumor morphology, nuclear and cellular pleomorphism, cellular mitotic index, and severity of necrosis. No significant differences in histological features were noted between N-cadherin-positive and -negative tumors from the N-cadherin/*neu* mice, nor were there any differences between tumors from bitransgenic mice versus control +/*neu* mice. Thus, N-cadherin expression in mouse mammary tumors induced by *neu* did not result in any histologic change.

DISCUSSION

Cadherins are known to play important roles in embryonic development, in the maintenance of tissue architecture and function in adult organisms, and in modulating cell behavior. Multiple studies have documented abnormal embryogenesis by cadherin deletion or overexpression, or by mutant dominant negative constructs [Detrick et al., 1990; Fugimori et al., 1990; Kintner, 1992; Heasman et al., 1994; Levine et al., 1994; Lee and Gumbiner, 1995]. Other studies have shown how adult tissues are affected by cadherin perturbation achieved through similar strategies [Hermiston and Gordon, 1995; Hermiston et al., 1996; Delmas et al., 1999; Kawaguchi et al., 2001; Boussadia et al., 2002; Dahl et al., 2002; Ferreira-Cornwall et al., 2002]. Fewer studies have examined how expression of a cadherin not typically found in a particular cell type affects cell or tissue behavior, although this sort of cadherin misexpression occurs in tumors [Palcios et al., 1995; Islam et al., 1996; Peralta Soler et al., 1999; Tomita et al., 2000].

Cadherins have been implicated in cancer, and E-cadherin is thought to be both a tumor and invasion suppressor. Most tumors arise from epithelial cells, which normally express E-cadherin, but typically lack other classical cadherins, such as P- and N-cadherin. Relevant to studies presented here, luminal mammary epithelial cells usually express only E-cadherin [Daniel et al., 1995]. However, P-cadherin is detected in human breast cancers and is a marker of poor prognosis [Peralta Soler et al., 1999; Gamallo et al., 2001]. In addition, N-cadherin is detected in human tumors, including breast cancer [Islam et al., 1996; Peralta Soler et al., 1999], although the physiological significance of this is not fully understood. We [Nieman et al., 1999] and Hazan et al. [2000] have shown that N-cadherin misexpression by human mammary tumor cells induces cell migration and invasion in vitro, regardless of their E-cadherin expression. In addition, the Hazan laboratory [Hazan et al., 2000] has observed enhanced metastasis when N-cadherin-expressing human mammary tumor cells were injected into nude mice, compared to tumor cells expressing only E-cadherin. Enhanced tumor cell invasion and migration appears to involve an interaction between N-cadherin and the FGFR [Nieman et al., 1999; Suyama et al., 2002].

To extend our understanding of the effect N-cadherin misexpression has on the behavior of mammary epithelial cells; we generated transgenic mice that inappropriately express N-cadherin in the adult mammary epithelium, under control of the MMTV promoter. Using these mice we studied the effect on both normal mammary gland behavior and the behavior of mammary tumors induced by overexpression of the *neu* oncogene, also under control of the MMTV promoter. The expression of N-cadherin

protein in the mammary epithelium did not affect the cellular distribution of endogenous E-cadherin or β-catenin, although the level of E-cadherin expression appeared slightly reduced compared to wild type mice. Macroscopic and microscopic examination of mammary glands revealed no significant differences in tissue architecture between wild type and N-cadherin transgenic mice. Mammary gland function also appeared normal in the N-cadherin transgenic mice, and no mammary tumors formed in any of the mice. In short, our observations indicate that there is no noticeable effect on morphology or function of the mammary gland. This was somewhat surprising to us, considering the extensive tissue remodeling that accompanies pregnancy and lactation, and the important role cadherins play in tissue architecture.

To examine the effect of N-cadherin misexpression on tumor cells, we induced mammary tumors in the N-cadherin transgenic mice by overexpressing the *neu* oncogene through a mating strategy. Expression of both *neu* and N-cadherin was under the control of the MMTV promoter, which maximized the probability that both proteins were expressed at the same time in the same epithelial cells. Mammary tumors formed in both hemizygous control +/*neu* and bitransgenic N-cadherin/*neu* mice, and the tumors arose at similar ages. Several parameters were analyzed, including tumor size, tumor number per mouse, presence of lesions outside the mammary gland, and expression of N-cadherin protein in the tumors of bitransgenic mice. Unexpectedly, we found that only 15% of tumors in N-cadherin/*neu* mice had moderate-to-strong N-cadherin expression, while the majority of tumors were negative. Moreover, N-cadherin-positive tumors were found in only about a third of the N-cadherin/*neu* mice, with N-cadherin-positive and -negative tumors occurring in the same mouse. This observation complicated analysis of our data because we had to consider the possibility that the tumors might be affected by N-cadherin expression in surrounding normal epithelium or by N-cadherin expressed in the tumor itself.

Several parameters did not appear to differ when comparing tumors from +/*neu* versus N-cadherin/*neu* mice, whether or not N-cadherin expression in the tumors was considered. Tumor size was similar; tumor morphology and histologic characteristics were indistinguishable. Moreover, the frequency of lung lesions did not appear to be enhanced in mice bearing N-cadherin-positive tumors. The lack of enhanced metastasis due to N-cadherin misexpression was not predicted, since our in vitro data with human breast cancer cell lines indicated that N-cadherin enhanced tumor cell migration and invasion. However, the number of mice with N-cadherin tumors was small and it is possible we failed to detect a significant change in metastasis over the background variation. It is also possible that additional molecular changes are needed for *neu*-induced tumors in mice to metastasize in response to N-cadherin misexpression. The tumors were well encapsulated and perhaps increased matrix metalloproteinase activity is needed to observe the N-cadherin effect on invasion and migration. Alternatively, an elevated expression of FGFR in the tumor cells might be required for N-cadherin to stimulate tumor cell migration and invasion.

We were puzzled that few tumors in bitransgenic mice expressed N-cadherin and that most bitransgenic mice had fewer tumors per mouse than control mice. Both of these observations can perhaps be explained by our finding that the levels of N-cadherin and Neu proteins varied inversely in the mammary glands of the bitransgenic mice, that is, mammary glands with high N-cadherin protein tended to have low Neu. This may result from competition between the two transgenes at either the transcription and/or translational level. Since Neu is required for tumor formation and tumors consistently exhibited high levels of Neu, tumors may preferentially arise from epithelial cells where Neu expression is favored over N-cadherin, resulting predominantly in tumors lacking N-cadherin, which is what we observed. Moreover, if N-cadherin expression is favored over Neu in mammary epithelial cells, then tumor formation might be suppressed, resulting in fewer tumors, which is what we observed.

In summary, our studies indicate that N-cadherin misexpression in the adult mouse mammary epithelium does not alter morphology or function of the normal mammary gland. In addition, N-cadherin does not induce spontaneous tumor formation, which was predicted since there is no evidence that cadherins act as oncogenes. When mammary tumors were induced by *neu* overexpression in order to determine if N-cadherin misexpression affected tumor behavior, most tumors were negative for

N-cadherin. For the few N-cadherin-positive tumors that did arise, no differences were detected in tumor behavior or histologic characteristics, compared to N-cadherin-negative tumors, such as would have been predicted by our published data with human mammary tumor cell lines. Taken together, our previous in vitro work and the in vivo results presented here emphasize that the role cadherins play in tumorigenesis and tumor cell behavior is complex and should not be viewed oversimplistically.

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