Cyclin D1-Dependent Induction of Luminal Inflammatory Breast Tumors by Activated Notch3

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Abstract

Accumulating evidence suggests that Notch3 (N3) is involved in breast cancer development, but its precise contributions are not well understood. Here, we report that pregnant mice expressing an activated intracellular form of N3 (N3IC) exhibit a cyclin D1-dependent expansion of premalignant CD24⁺ CD29⁺⁺⁺⁺ luminal progenitors with enhanced differentiation potential in vitro and in vivo. Parous mice developed luminal mammary tumors in a cyclin D1-dependent manner. Notably, mice expressing higher levels of N3IC exhibited tumors resembling inflammatory breast cancer that frequently metastasized. N3IC-induced tumors contained a large percentage of tumor-initiating cells, but these were reduced significantly in tumors derived from N3IC transgenic mice that were heterozygous for cyclin D1. After transplantation in the presence of normal mammary cells, N3IC-expressing tumor cells became less malignant, differentiating into CK6⁻ CK18⁺ CK5⁻ alveolar-like structures akin to expanded luminal progenitors from which they were likely derived. Taken together, our results argue that activated N3 signaling primarily affects luminal progenitors among mammary cell subsets, with more pronounced levels of activation influencing tumor type, and provide a novel model of inflammatory breast cancer.

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Introduction

The mammary gland is made of ducts and alveoli. The latter expand significantly during pregnancy and lactation. Ducts and alveoli are made of 2 major epithelial cell subsets: (i) the CK14⁻ outer basal myoepithelial and (ii) the CK8⁺ CK18⁺ inner luminal cells (1). Alveolar luminal cells produce milk proteins such as casein and whey acidic protein (WAP). Transplantation of mammary cell progenitors into cleared fat pads has been the technique of choice for many years to probe their differentiation potential (2). From these studies, it seems that the normal mammary glands develop from self-renewing multipotent stem cells capable of forming a whole mammary tree (ref. 3; for review see refs. 1, 4). These mammary stem cells (MaSC) generate bipotential CK5⁺ CK14⁻ multipotent progenitors capable of differentiation into committed basal and luminal progenitors, which differentiate into more mature basal (myoepithelial) or luminal cells, respectively (2, 5). However, a recent study, based on in vivo lineage tracing, has challenged these conclusions and shown that CK5⁺ CK14⁺ multipotent stem cells are present only during gestation and are absent in adult mice (6). They are rapidly replaced at birth by unipotent myoepithelial CK5⁺ CK14⁻ and unipotent luminal CK8⁺ CK14⁻ progenitor cells, which differentiate into mature basal or luminal cells, respectively, to form both ducts and alveoli (6).

The involvement of Notch members, int3/Notch4 (N4; ref. 7) and Notch1 (N1; ref. 8), in the development of breast tumors was initially found through mouse mammary tumor virus (MMTV) provirus insertion sites in mice infected with MMTV (for review, see ref. 9). In both the cases, the Notch gene was truncated and its intracellular (IC) domain was overexpressed. When expressed in mammary glands of Tg mice, N4IC (10) and N1IC (11) were oncogenic and so was Notch3IC (N3IC; ref. 11). In the last few years, evidence has accumulated that NOTCH family members are also involved in a certain proportion of human breast cancers. This is especially the case of N1 (12–18), but also of N3. Overexpression of N3, along with N1, is associated with poor overall survival (19). N3 signaling was also reported to play a crucial role in the proliferation of HER-2-overexpressing ductal carcinoma in situ (20) and of ErbB2-negative breast cancer cells (21), to favor osteolytic bone metastasis of some breast cancer cell lines (22) and to be required for the growth of aggressive inflammatory breast cancer cells (23). Moreover, N3 controls survival of human mammary stem/progenitor cells in hypoxic environment (24). Therefore, a certain percentage of human breast tumors may be driven by the activation of the N3 pathway.

In the present study, we investigated the effects of N3IC on the mammary stem/progenitor cells and the requirement of cyclin D1 for the development of N3IC-induced phenotypes in MMTV/N3IC Tg mice (11). In addition, we characterized N3IC-induced tumors and tumor-initiating cells. It seems that N3IC...
targets luminal progenitors for expansion during the pre-malignant stage, and that these latter cells eventually give rise to luminal tumors of the inflammatory type when N3^C levels are high in a cyclin D1-dependent manner.

Materials and Methods

Mice
The FVB cyclin D1 KO (25) and actin/GFP Tg mice were obtained from the Jackson Laboratory. MMTV/N3^C Tg mice (F85824) have been described (11). Additional founder mice (F221384, F221385, F221388, and F221390) were generated, as before (11). A line could be established from F221390 founder but not from the other founders that were infertile and/or developed tumors. Mice were all on the FVB background for at least 6 generations.

Analysis of mammary cell subsets

Mammary cell subsets were prepared and analyzed by fluorescence-activated cell sorting (FACS), as described (26), according to the published procedure (27, 28). Briefly, tissues were digested with 300 U/mL of collagenase and 100 U/mL of hyaluronidase (Stem Cell Technology Inc.) for 1 hour at 37°C and sequentially resuspended in 0.25% trypsin-EGTA for 1 to 2 minutes, 0.1 mg/mL DNaseI for 5 minutes, and 0.64% NH4Cl for 3 minutes before filtration through a 70-μm mesh. Cells were labeled with primary antibodies as described (26). Acquisition was conducted on FACS scan or FACS Calibur (BD Biosciences), and data were analyzed using the CellQuest Pro (BD Biosciences) software. Cell sorting was carried out on a MoFlo cell sorter (Cytomation Inc.), and gates were set to exclude dead cells and Lin^- cells (CD31-, CD45-, and TER119 positive).

Apoptosis analysis

After immunostaining, cells were stained for 20 minutes with 7-aminoactinomycin D (7 AAD), washed once with 10 mL of 10 mmol/L HEPES (pH 7.4), 150 mmol/L NaCl, 1.8 mmol/L CaCl2, 1 mmol/L MgCl2, 5 mmol/L KCl buffer, and stained with Annexin V-FITC for 20 minutes, all at room temperature, before being subjected for FACS analysis.

Immunostaining

Cells and tissue sections were stained, as described before (26). Cells were permeabilized in 0.1% TritonX-100/PBS on ice for 30 minutes and then blocked with 5% mouse, rabbit, or goat serum in PBS for 60 minutes. The 3D structures were treated with 10% and 20% D-sucrose for 30 minutes of each before embedding. Sections were fixed in 10% formaldehyde PBS for 60 minutes at 4°C, and antigen retrieval was conducted by boiling them in 10 mmol/L citrate buffer (pH 6) for 30 minutes before incubation with primary antibodies overnight at 4°C and washing with PBS 3 times for 5 minutes at room temperature. Alexa-Fluor488, -594, and -633-conjugated secondary antibodies (Molecular Probes) were added for 60 minutes in the dark at room temperature. The slides were washed repeatedly in PBS and mounted with Vectashield containing 1.5 μg of 4′,6-diamidino-2-phenylindole (Vector Laboratories, Inc.). Digital fluorescence images were taken on an Axiovert S100TV microscope.

Mammary fat pad transplantation and analysis

Transplantation of mammary cell subsets was carried out as before (26). Single cell suspension was prepared by digesting small tumor pieces as described above for normal cells. Cells were washed twice in working medium before transplantation and labeling.

Establishment of tumor cell lines and tumor cell transplantation

Tumor cells were prepared as described above and were established and transplanted subcutaneously or into cleared mammary fat pads, as previously described (29).

Differentiation in vitro

Culture conditions for generation of 3D structures from mammary cells in Matrigel have been described before (26).

Statistical analysis

Comparison of the distribution of different cell populations was analyzed by Student t test as before (26). Analysis of the frequency of tumor-initiating cells was conducted as we did for N1^C-expressing cells (29).

Results

MaSC-enriched CD24^+ CD29^high cells (R4) from MMTV-N3^C Tg mice are rarely expanded and impaired in their repopulation activity

We previously reported a severe mammary developmental defect in MMTV-N3^C Tg mice (F85824). Males from this high expressor line were sterile and females could neither nurse nor feed their pups, and Hes1 expression was increased (11). We extended these results by generating another founder line (F221390) expressing N3^C at much lower levels (Fig. 1A). As expected, expression of a number of Notch targets (Hes1, Hes5, c-myc, and cyclin D1) was increased in different Tg mammary cell subsets (Supplementary Fig. S1). However, PTEN and p53 RNA levels were comparable in Tg and non-Tg mammary cell subsets (Supplementary Fig. S1). However, expression of a number of Notch targets (Hes1, Hes5, c-myc, and cyclin D1) was increased in different Tg mammary cell subsets (Supplementary Fig. S1). However, PTEN and p53 RNA levels were comparable in Tg and non-Tg cells from both founders (Supplementary Fig. S1), in contrast with their downregulation in MMTV-N1^C Tg mice (26). Males from this low expressor (F221390) N3^C Tg line were fertile and females could nurse and feed their pups.

We used both Tg lines to study the effect of N3^C overexpression on the CD24^+ CD29^high (R4) mammary cell subset enriched for stem cells (Fig. 1B). The percentage of this cell population was not significantly different in Tg and non-Tg virgin females from both founders (Fig. 1C), except for 6 out of 36 females from the outbred CD1 F85824 Tg line, which showed R4 expansion and enhanced Tg expression (Supplementary Fig. S2). The function of the R4 cell subset was assessed in vivo by measuring their repopulation activity after transplantation into cleared fat pads. Both non-Tg and N3^C Tg CD24^+ CD29^high cells of both founders generated a tree-like structure that could be serially passaged 3 times without apparent loss of repopulation activity (Fig. 1D), strongly suggesting that, in most of the mice, the multipotent stem cell pool was not much affected by the expression of the N3^C transgene, in contrast with the inhibitory effect of N1^C on this population (26). However, at mid gestation, the N3^C...
Tg-derived transplanted gland-like structures showed reduced arborization and fewer and poorly developed alveoli compared with non-Tg ones (Fig. 1E), as previously reported in these Tg mice (11). But these showed close to normal CK14/CK18 staining (Fig. 1F). A similar phenotype of poorly differentiated structures could also be observed in 3D cultures of CD24<sup>+</sup>CD29<sup>high</sup> Tg cells, in comparison with the more developed alveoli-like outgrowths generated with non-Tg cells (Fig. 1G). A large proportion of cells within these <i>in vitro</i> generated Tg structures were positive for CK18, and a smaller percentage was stained with CK5 and CK14 (Fig. 1H). Interestingly, almost all Tg cells were positive for CK6, a marker for luminal progenitors, whereas non-Tg structures showed less CK6 staining (Fig. 1H). These results suggest that N3IC favors the expansion of luminal progenitors from CD24<sup>+</sup>CD29<sup>high</sup> MaSC-enriched progenitor cells in these <i>in vitro</i> conditions.

**Pregnancy-dependent expansion of mammary CD24<sup>+</sup>CD29<sup>high</sup> cells (R3) enriched for luminal progenitors in MMTV/N3IC Tg mice**

In contrast with the (R4) MaSC cell subset, the CD24<sup>+</sup>CD29<sup>high</sup> (R3) cell population enriched for luminal progenitors (27, 28), was significantly expanded in pregnant, but not in virgin, N3IC Tg mice of both founders (Fig. 2A and B). Direct <i>ex vivo</i> labeling of these cell-sorted purified R3 cells showed that a higher percentage of Tg than non-Tg cells were CK6<sup>+</sup>(21.8 ± 2.2 vs. 8.7 ± 1.7%; Supplementary Fig. S3), strongly suggesting that they were luminal progenitors. In addition, a higher proportion of Tg cells were positive for estrogen receptor (Supplementary Fig. S3). CD24<sup>+</sup>CD29<sup>high</sup> (R3) N3IC Tg cells showed higher bromodeoxyuridine (BrdUrd) incorporation, including CD61<sup>+</sup> cells (Fig. 2C and D) and reduced apoptosis, especially during early involution.
Cancer Research

Figure 2. Pregnancy- and cyclin D1-dependent expansion of mammary CD24+ CD29low (R3) cells enriched for luminal progenitors in MMTV/N3IC Tg mice. A, representative FACS profiles of the Lin− mammary cell subsets from 14.5 days pregnant Tg and non-Tg FVB females bred on wild-type or cyclin D1+/− heterozygote (CDN1+/−) background. Note the expansion of R3 cells in wild-type Tg mice. B, percentage of R3 cells subset in Tg, cyclin D1+/− Tg (F221390), and non-Tg females. C, evaluation of R3 cell proliferation. Virgin or 14.5 days pregnant (P14.5) Tg and non-Tg mice were given BrdUrd orally. After 14 days, R3 cells were purified by cell sorting and BrdUrd incorporation evaluated by labeling with anti-BrdUrd Ab. Cells were also stained with 7AAD and Annexin V. FACS profiles were obtained by gating on CD24+ CD29low (R3) 7AAD-positive (dead) or 7AAD/Annexin V-double-positive (apoptotic) cell subset. D, 3D structures generated from Tg and non-Tg R3 cells in vitro. E, percentages of apoptotic and dead R3 cells after weaning. F, 3D structures generated from Tg and non-Tg R3 cells in vitro. Phase contrast, hematoxylin and eosin (H&E) staining, and immunostaining with the indicated cytokeratins are shown. G, outgrowths of R3-derived cells from virgin Tg and non-Tg mice were transplanted into cleared fat pads of non-Tg FVB mice and evaluated 8 weeks later in 14.5-day pregnant recipients. Wholemount, hematoxylin and eosin staining, and immunostaining are shown. Note the total absence of outgrowth from non-Tg R3 cells, as expected, and the almost total absence of CK14 and CK5 staining.

(Fig. 2E), two characteristics that are likely to contribute to their expansion.

These R3 cell-sorted purified cells were further studied by incubating them in methylcellulose. In these 3D culture conditions, non-Tg R3 cells only accumulate in clumps and rarely form elaborate structures (Fig. 2F). In contrast, CD24+ CD29low cells (R3) from N3IC− Tg mice had the potential to generate cavitated, polarized, and well-organized structures (Fig. 2F and Supplementary Fig. S3B). A very large proportion of cells within these structures were CK6−, CK18−, and CK19 positive (Fig. 2F), but negative for CK5, CK14, and smooth muscle actin (SMA; Fig. S3C), strongly suggesting their luminal nature. The same R3 cells were also analyzed in vivo after transplantation into cleared fat pads of non-Tg FVB mice. As expected, non-Tg R3 cells produced no growth, whereas Tg R3 gave rise to lobular-like outgrowths (Fig. 2G).

Together, these results indicate that N3IC− induces expansion of luminal progenitor cells during pregnancy.

Cyclin D1 deficiency abrogates the N3IC−-induced expansion of CD24+ CD29low (R3) mammary cell subset

We previously showed that cyclin D1 deficiency prevents N1IC−-induced expansion of CD24+ CD29high+ MaSC-enriched cell population (26). To determine whether cyclin D1 deficiency would have the same effect on the N3IC−-mediated expansion of the CD24+ CD29low (R3) population, MMTV/N3IC− Tg mice...
(F221390) were bred on the cyclin D1 KO background and pregnant Tg females analyzed. The expansion of R3 cell subset (Fig. 2A and B), as well as their defects of proliferation and survival (Fig. S4A–C) were abrogated in cyclin D1+/−/− N3IC Tg mice. In addition, deficiency of cyclin D1 inhibited their proliferation in 3D cultures in vitro (Supplementary Fig. S4D) and prevented their growth in vivo, after transplantation into cleared fat pads (Supplementary Fig. S4E). These results indicate that N3IC is mediating the expansion of R3 cells through cyclin D1.

N3IC-induced mammary tumors are pregnancy dependent and express luminal markers

We previously reported the development of mammary tumors in parous mice from the high expressor (F85824) CD1 N3IC Tg line (11). Parous, but not virgin, FVB Tg females from both founder lines also developed mammary tumors at very high frequency and with only minor latency differences (Fig. 3A), despite major differences in N3IC expression levels at the premalignant stage. Tumors from 4 different founders were positive for luminal markers (CK6, CK19), but negative for CK5, K14, K18, and SMA (Fig. 3B). However, tumors from high (F85824) and low (F221390) Tg expressors differed macroscopically and histologically. Grossly, F221390 tumors were white and firm, whereas F85824 tumors were hyper-vascularized, soft, and necrotic (Fig. 3C and D). Histologically, F85824 tumors show mononuclear cell infiltration (Fig. 3E), large areas of necrosis (Fig. 3F), intratumoral vascular formations (Fig. 3G), and increased number of Mac-1–positive myeloid (Fig. 3H) and CD31-positive endothelial

![Image](https://example.com/image.png)

**Figure 3.** N3IC-induced mammary tumors are pregnancy dependent and express luminal markers. A, incidence of mammary tumors in virgin or parous MMTV/N3IC Tg mice from F221390 and F85824 founders. B, representative immunostaining of N3IC-induced tumors with antibodies against CK5, CK6, and CK19. Note that most tumor cells are negative for CK5. C–L, macroscopic (C and D), histologic (C, bottom; E–G, J, and K) and immunofluorescence (H, I, and L) evaluation of N3IC-induced local tumors from mice of 2 founder lines. Note mononuclear cell infiltration (E) positive for Mac1 (J), large areas of necrosis (F), large intratumoral vascular formations (honeycomb like; G and I), and intravascular tumor cells mixed with red blood cells (J and K) in tumors from F85824 high expresser founder. Accumulation of K19–positive tumor cells within CD31–positive vessels was confirmed by immunofluorescence (L). M and O, representative macroscopic (top) and histologic (bottom) pictures of metastases in the indicated organs of N3IC Tg mice from the F85824 and F221388 founders. P, intravascular lung metastases shown by hematoxylin and eosin staining (left) or by immunofluorescence (right). Note the presence of K19–positive tumor cells surrounded by CD31–positive endothelial cells. Q, lung and liver metastases staining positive for β-casein.
were pooled from primary (B and C) or serially transplanted (D) tumors. Results represent number of tumors over number of transplanted sites in 14 out of 25 Tg mice studied (50%) from 2 N3IC-founders (F85824, F221388), tumors were observed in lungs and draining lymph nodes, in liver (2 Tg mice; Fig. 3M–O), and in lung vessels (Fig. 3P), suggesting a metastatic process. To ascertain the origin of these tumors, they were labeled with tissue-specific markers: cassein for mammary cells as well as SP-C and CC10 for lung cells. One lung tumor out of 12 stained positive for CC10 (not shown), indicating a primary lung tumor. Four lung tumors (out of 12) and 1 liver tumor (out of 2) were positive for cassein (Fig. 3Q), indicating they were genuine mammary metastases. The remaining 7 tested lung tumors were negative for the 3 markers and are likely to be too undifferentiated for tissue typing. Together, these results indicate that expression of N3IC in different subsets of mammary epithelial cells induce the development of luminal tumors only in parous females. In high expressor Tg mice, these tumors show features of aggressive inflammatory breast cancers.

N3IC-induced tumors contain high percentage of tumor-initiating and mammosphere-forming cells

Notch signaling has been shown to favour the formation of mammospheres in vitro and to enhance tumor-initiating cells in vivo (30). We determined the frequency of tumor-initiating cells in N3IC-induced tumors by transplanting unselected or cell-sorted selected (Fig. 4A) Lin- mammary tumor epithelial cells subcutaneously or into cleared fat pads of nude or syngeneic FVB (not shown) non-Tg mice. Transplantation of as few as one unselected cell could induce tumor formation (Fig. 4B). Similarly, transplantation of cell-sorted

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<td>10^6</td>
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Figure 4. N3IC-induced tumors contain high percentage of tumor-initiating and mammosphere-forming cells. A, representative profile of Lin- tumor cells stained for CD24 and CD29. B–D, frequency of tumor formation by unselected (B and D) or by cell-sorted (as shown in A; B and C) tumor cells freshly prepared from primary (B and C) or serially transplanted (D) tumors. Results represent number of tumors over number of transplanted sites in nude mice. They were pooled from in vitro 2 groups of tumors from low (L1, L2) or high (H1, H2) expressor line (B and D). Results on individual tumors of these groups are shown in Supplementary Fig. S5 and S6. S.C, subcutaneously; FP, cleared fat pads; F, frequency; L, latency. E, characterization of 3D mammospheres generated in vitro from N3IC-induced tumor cells. Representative phase contrast appearance (left) and histology revealed by hematoxylin and eosin staining (right). Bottom, quantification of mammosphere-forming potential after primary and serial transfer. F, immunofluorescence of mammospheres with the indicated antibodies. Note that no cells are CK5 positive and only a small percentage is SMA<sup>hi</sup> and CK14<sup>hi</sup> (arrows).
purified cells from R5 and, to a lesser extent, from R3 sub-populations could induce tumors, but not those from R1, R2, or R4 subsets (Fig. 4B and C). These properties were maintained after serial transplantation in nude (Fig. 4D) and FVB (not shown) mice. We evaluated the frequency of tumor-initiating cells, in groups receiving decreasing numbers of tumor cells, to be 1/545 to 797 for unselected and 1/175 to 1/282 for R5 selected tumor cells.

We also assessed the capacity of tumor cells to form mammospheres in vitro, an assay that is thought to be a reliable for detecting the presence of breast tumor-initiating cells (31, 32). N3IC-induced tumor cells from both founders formed mammospheres, having rudimentary structures with some cavitations (Fig. 4E) at very high efficiency, even after serial transplantation (Fig. 4F). Most of the mammospheres were CK6- and CK19 positive and a large percentage were CK18+, but very few cells staining for CK5, CK14, and SMA (Fig. 4F), consistent with a luminal origin. Thus, N3IC-induced tumors contain abundant tumor-initiating cells showing luminal features.

Tumor formation is prevented in cyclin D1-deficient MMTV/N3IC Tg mice, and N3IC-induced tumor cells remain dependent on cyclin D1 for their growth/survival

The required presence of cyclin D1 for the development of N1IC--induced mammary tumors (26) and for the N3IC--induced expansion of CD24+/CD29low (R3) cells (see above) prompted us to test whether cyclin D1 was also necessary for tumor formation by N3IC. MMTV/N3IC Tg mice were bred on the cyclin D1 gene-deficient background, and parous females were observed for tumor development for up to 16 months. All wild-type (cyclin D1+/−) Tg females developed mammary tumors, whereas their heterozygote cyclin D1+/− and homozygote cyclin D1−/− Tg female littermates showed a much lower incidence or remained free of tumors, respectively (Fig. 5A). These results indicate that cyclin D1 is necessary for executing the oncogenic function of N3IC.

We next tested whether the N3IC still requires cyclin D1 once tumor cells have already acquired their full tumor-initiating potential. For this, we derived 2 cell lines from rare tumors arising in cyclin D1−/− N3IC Tg mice. These lines, as expected, showed lower expression of cyclin D1 (Fig. 5B), grew at a slower rate (Fig. 5C), formed fewer mammospheres (Fig. 5D), and were less tumorigenic (Fig. 5E) than tumor lines derived from cyclin D1+/− N3IC Tg mice. However, re-expression of cyclin D1 in cyclin D1−/− N3IC tumor cells (Fig. 5F) restored their growth (Fig. 5G) and their tumorigenic potential in vivo (Fig. 5H). In addition, we selected wild-type cyclin D1+/− N3IC tumor cell lines from both founders (M223035, M246634, M243043, M243044) and transduced them with retroviral vector encoding a gene of selection (puromycin) and short hairpin (shRNA) against mouse cyclin D1 (shRNAcyclinD1) previously shown to lower the cyclin D1 protein expression (26). Control vector (no shRNA or shRNA against GFP) gave rise to abundant puromycin-resistant colonies (Fig. 5I). In contrast, no puromycin-resistant colonies could be recovered after infection with shRNAcyclinD1-containing virus of these 4 cell lines, in 2 independent experiments (Fig. 5I). This was unlikely caused by technical problems because in the same experiment carried out the same day, with the same shRNAcyclinD1 virus stock, puromycin-resistant colonies of N1IC-expressing tumor cells could be recovered (26). These results indicate that the presence of cyclin D1 is required for growth and/or survival of mammary tumor cells already transformed by N3IC.

N3IC-induced tumor cells lose their malignancy and differentiate in the presence of normal mammary cells in vivo

It has been shown that some tumor cell lines adopt a mammary cell fate after their transplantation into cleared fat pads in the presence of normal mammary epithelial cells (33, 34). We found that N1IC-induced tumor cells exhibit this property (26) and seem to adopt the fate of the bipotential (basal/luminal) progenitor cells from which they are derived (26). To determine whether N3IC-induced tumor cells will also adopt the fate of R3 progenitor cells from which they seem to be derived, we tested their fate in such conditions. GFP-positive cells from tumor cell lines established from tumors of double (actin/GFP X MMTV/N3IC) Tg mouse were transplanted into cleared mammary fat pads, in the presence of normal mammary epithelial cells at different ratios. At 5:1 and 1:1 ratio of tumor/normal cells, transplanted fat pads contained only GFP-positive tumors or GFP-positive tumors and glandular outgrowths, respectively (Fig. 6A). At 1:5 ratio of the tumor/normal cells, only one tumor was detected and most GFP+ outgrowths were glandular (Fig. 6A–C). These latter had a lobular-like, cavitated differentiated structure (Fig. 6D and I), which stained positive for CK18 and CK6, but negative for CK5 (Fig. 6E), and was distinct from tumor outgrowths (Fig. 6F). These results strongly suggest that N3IC-induced tumor cells have differentiated along a luminal path and acquired a nonmalignant phenotype in the presence of normal cells.

Discussion

N3IC induces expansion of alveoli-restricted luminal progenitors

We found that, in the great majority of mice, N3IC overexpression in mammary glands had little impact on the percentage of CD24+/CD29high (R4) mammary cell subset, enriched for multipotent stem cells (27, 28), including their in vivo repopulation potential, suggesting an intact function. This contrasts with the significant expansion of this population induced by low levels of N1IC in MMTV/N1IC Tg mice (26). However, an expansion of CD24−/CD29low/R4 cells was observed at low frequency in mice from the very high expresser N3IC Tg line, indicating that N3IC has also the potential to expand stem cells when very highly expressed.

In contrast with N1IC (26), N3IC overexpression was found to result in a very significant expansion on another cell subset, the CD24+/CD29high (R3) cell subpopulation, positive for CK6/CK18 and reported to contain luminal progenitors (27, 28). This occurs most likely through increased proliferation and enhanced survival documented in this cell subset. This expanded cell population was only observed in pregnant Tg
females, and may represent parity-induced mammary epithelial cells (PI-MEC). PI-MECs have been shown, through WAP/cre lineage tracing in vivo, to be absent in virgin females, to expand during the first normal pregnancy, and to contribute to lobuloalveolar development (35). Transplantation studies revealed that the PI-MEC cells contain multipotent progenitors able to self-renew, to contribute to ductal and alveolar development, and to generate both basal and luminal cells (35–37). However, more recent in vivo lineage-tracing experiments have challenged this latter interpretation and led to the conclusion that, after birth, the mammary glands do not harbour multipotent progenitors but rather contain only unipotent basal and unipotent luminal progenitor cells (6). In view of these latter results, N3<sup>K</sup> seems to mainly affect unipotent luminal progenitors, more specifically the alveoli-restricted unipotent luminal PI-MEC progenitors. The location of the N3<sup>K</sup>-induced initial hyperplastic nodules at the extremity of ducts, where in vivo tagged PI-MEC cells were observed (38), is consistent with this conclusion. However, N3<sup>K</sup>-expressing premalignant CD24<sup>+ </sup>CD29<sup>−</sup> (R3) cells have acquired new properties that are not shared by the normal R3 cells. Upon culture in vitro, they gave rise to more elaborate cavitatory structures than wild-type cells, both containing mainly CK6<sup>−</sup>, CK18<sup>− </sup>CK19<sup>− </sup>luminal cells, and almost no CK5<sup>−</sup> (basal) cells. In vivo, after their transplantation into cleared mammary fat pads, Tg CD24<sup>+</sup> CD29<sup>−</sup> (R3) Tg cells generated alveolar-like structures, whereas no outgrowth was apparent in glands transplanted with non-Tg R3 cells, as previously reported.

Figure 5. Tumor formation in MMTV/N3<sup>K</sup> Tg mice and growth/survival of N3<sup>K</sup>-induced tumor cells are cyclin D1 dependent. A, decreased incidence of mammary tumors in heterozygote (+/−) and homozygote (−/−) parous cyclin D1-deficient N3<sup>K</sup> Tg mice (F221390). B, Western blot analysis of cyclin D1 expression in tumor cells from cyclin D1<sup>+/−</sup>, or cyclin D1<sup>−/−</sup> N3<sup>K</sup> Tg mice. C, proliferation of tumor cells from established lines generated from cyclin D1<sup>+/−</sup> [M234718 (F221390), M234718 (F85824)] or cyclin D1<sup>−/−</sup> [M260306, M260306 (F221390)] N3<sup>K</sup> Tg tumors. Results of each group were pooled. D, frequency of mammosphere-forming cells among cyclin D1<sup>+/−</sup> (M260306, M260307) and control cyclin D1<sup>−/−</sup> (M234718, 223682, 223364) primary tumor cells. E, numbers of dishes (2/line) pooled from each group. F, frequency of tumor formation after transplantation of freshly prepared primary tumor cells. G, colony formation of tumor cells (M223682) established in vitro. H, rescue of cyclin D1 deficiency in tumor cells. Cells from cyclin D1<sup>−/−</sup> N3<sup>K</sup> Tg tumors (M260306) were transduced with retroviral vector encoding cyclin D1 or vector alone. Colonies expressing different levels of cyclin D1 as assessed by Western blot analyses (F) were assayed for growth (G). In addition, pools of transduced clones were injected (1 × 10<sup>6</sup> cells) into cleared mammary fat pads and tumor growth assessed 6 weeks later (H). I, colony formation of tumor cells (M223364) established from tumors arising in N3<sup>K</sup> Tg mice (F85824), in the presence of shRNA against cyclin D1 (shRNA<sub>cyclinD1</sub>) or against GFP (shRNA<sub>GFP</sub>) as control. Note that only single cells but no colonies are observed in shRNA<sub>cyclinD1</sub> transduced cells. Representative cell line of 4 tested.
for normal R3 cells (27). Thus, N3IC most likely targets unipotent luminal PI-MEC progenitors, and provide them with enhanced differentiation potential. It is also possible that another luminal progenitor, not yet characterized but distinct from PI-MEC, could represent N3IC target cells, as recently reported for N4IC in Tg mice (39). We cannot rule out either, although less likely, that N3IC would target basal progenitors and reprogram them and change their fate in vivo, allowing them to express luminal markers. Additional experiments will be needed to rule these possibilities out.

Consistent with the enhanced differentiation potential of Tg luminal progenitors shown here, we previously showed that N3IC most likely targets unipotent luminal PI-MEC progenitors, and provide them with enhanced differentiation potential. It is also possible that another luminal progenitor, not yet characterized but distinct from PI-MEC, could represent N3IC target cells, as recently reported for N4IC in Tg mice (39). We cannot rule out either, although less likely, that N3IC would target basal progenitors and reprogram them and change their fate in vivo, allowing them to express luminal markers. Additional experiments will be needed to rule these possibilities out.

N3IC-induced mammary tumors originate from expanded luminal progenitors and need cyclin D1 for their development and maintenance

We reported before that the premalignant luminal cells lining the expanded alveoli of mammary glands from N3IC Tg mice were hyperplastic and highly proliferating (11). They most likely originated from the expanded CD24+ CD29low R3 luminal progenitors observed here. Because both of these cell populations are proliferating, they may represent the precursors to mammary tumors, most likely after having accumulated additional spontaneous mutations ("second hit"). The fact that premalignant changes and tumor formation are both restricted to parous females suggests a correlation between these two phenotypes. In addition, the CK staining of tumor cells, especially the CK6 positivity, is reminiscent of that of the expanded CK6+ CD24+ CD29low cells. Also, when GFP+ N3IC-tumor cells revert to nonmalignant cells after transplantation in the presence of normal mammary cells, the nonmalignant GFP+ mammary structures derived from GFP+ malignant cells have conserved some features of premalignant progenitors, especially CK6 positivity. Finally, partial cyclin D1 deficiency in parous heterozygote cyclin D1+/− Tg mice not only prevents the N3IC-induced expansion of
CD24^+CD29^low luminal progenitor cells, but also significantly inhibits tumor formation, indicating that both phenotypes positively correlate and strongly suggesting that progenitor cell expansion is required for tumor formation. Thus, in MMTV/N3^IC^ Tg mice, tumors are very likely originating from the expanded, premalignant CD24^+CD29^low luminal cell progenitors.

We found a strong dependence of N3^IC^-induced tumor formation on cyclin D1. A partial deficiency (cyclin D1^+/−), which does not seem to affect normal gland development at any stage (25, 42), has a major negative impact on the N3^IC^ oncogenic pathway. Indeed, the execution of the N3^IC^ program does not tolerate even a partial depletion of cyclin D1, including after full transformation has occurred. This property offers a very attractive therapeutic opportunity for N3-induced breast tumors, especially that cyclin D1 is also a direct target of N3 in ing after full transformation has occurred. This property offers a very attractive therapeutic opportunity for N3-induced breast tumors, especially that cyclin D1 is also a direct target of N3 in

N3^IC^-induced mammary tumors show features of inflammatory breast cancer, including metastases

Mammary tumors arose at high frequency in parous N3^IC^ Tg females, including those from the high expressor F85224 Tg line, which cannot lactate, indicating that lactation is not essential for their development. Unexpectedly, tumors developed after similar latencies and frequencies in both N3^IC^ Tg founder lines studied, despite very large difference of N3^IC^ transgene expression between these lines. Moreover, tumors from both Tg lines contain a very high proportion of tumor-initiating cells. These observations suggest that only moderate levels of constitutive N3^IC^ signaling are sufficient for transformation of luminal progenitors. However, tumors from mice of the high expresser line (F85824) were a lot more aggressive than those of the low expresser founder. In particular, they showed numerous vascular tumor emboli, a feature described in very aggressive human "inflammatory" breast cancer, including those overexpressing N3 (23, 44, 45).

Consistent with this apparent intravasation of tumor cells, mice of the F85824 line developed lung metastasis at fairly high frequency, although we could prove the mammary origin of only 4 out of 12 of them. Tumor emboli and lung metastasis were also observed in additional founder females from which a line could not be established, indicating that these phenotypes are very unlikely related to the transgene integration site, but rather to high N3^IC^ expression. Therefore, N3^IC^-induced mouse tumors from the high expressor line share a number of characteristics with human inflammatory breast cancer: high N3 expression, erythematous skin invasion, vascular tumor emboli, inflammatory cell infiltration, high necrosis, stem cell features, and pulmonary metastasis confined to vessels (23, 44, 46–49).

Conclusion

There is accumulating evidence that N3 is involved in human breast cancer (see Introduction). Its expression correlates with Her2 levels in human breast tumors (20). Its over-expression was found to be associated with poor overall survival (19). N3 signaling was reported to play an important role in the proliferation of ErbB2-negative breast tumor cells (21) and to be required for growth of aggressive inflammatory breast cancer cells (23). N3 nuclear immunoreactivity was detected in the majority of the human tumors of this latter tumor type (23). The MMTV/N3^IC^ Tg mice seem to represent a novel model for understanding the cellular and molecular mechanisms of these N3-expressing human tumors, especially for the luminal subtype (50) of inflammatory breast cancers.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: H. Ling, P. Jolicoeur
Development of methodology: H. Ling
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): H. Ling, J.R. Sylvestre
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): H. Ling, J.R. Sylvestre, P. Jolicoeur
Writing, review, and/or revision of the manuscript: H. Ling, P. Jolicoeur
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): H. Ling, J.R. Sylvestre, P. Jolicoeur
Study supervision: J.R. Sylvestre, P. Jolicoeur

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Activated Notch3 and Mammary Tumors


Cyclin D1-Dependent Induction of Luminal Inflammatory Breast Tumors by Activated Notch3

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