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Notch-1 signaling promotes the cyclinD1-dependent generation of mammary tumor-initiating cells which can revert to bi-potential progenitors from which they arise

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Abstract

In a previous work, we reported that young transgenic (Tg) mice expressing the intracellular domain of Notch1 ($N1^{IC}$) showed expansion of lin^- $CD24^+$ $CD29^{high}$ mammary cells enriched for stem cells and later developed mammary tumors. Mammary tumor formation was abolished or greatly reduced in cyclin $D1^{-/-}$ or cyclin $D1^{+/-}$ $N1^{IC}$ Tg mice, respectively. Here, we studied the epithelial cell subsets present in $N1^{IC}$-induced tumors. $CD24^-$ $CD29^{int}$ and $CD24^+$ $CD29^{high}$ cells were found to be present at low numbers in tumors. The latter had the same properties as those expanded in young Tg females, and neither cell population showed tumor-initiating potential, nor were they required for maintenance of tumors after transplantation. $CD24^{int}$ $CD29^{int}$ cells were identified as tumor-initiating and mammosphere-forming cells and represent a large percentage tumor cells in this model. Their number was significantly lower in tumors from cyclin $D1^{+/-}$ $N1^{IC}$ Tg mice. Using cyclin D1 shRNA knockdown, we also show that $N1^{IC}$-induced tumor cells remain addicted to cyclin D1 for growth and survival. Interestingly, at lower levels of cyclin D1 or after transplantion in the presence of normal mammary cells, these $N1^{IC}$-expressing tumor cells reverted to a state of low malignancy and differentiate into duct-like structures. They seem to adopt the fate of bi-potential stem/progenitor cells similar to that of the expanded $CD24^+$ $CD29^{high}$ stem/progenitor cells from which they are likely to be derived. Our data indicate that decreasing cyclin D1 levels would be an efficient treatment for tumors induced by N1 signaling.

Keywords

Mammary tumors; Notch1; Cyclin D1 addiction; Tumor-initiating cells

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

INTRODUCTION

The mammary gland is made of two major epithelial cell subsets, the $CK14^+$ outer basal myoepithelial and the $CK8^+CK18^+$ inner luminal cells, each present in ducts and alveoli (1–3). Much of what is known about the hierarchy of the mammary cells comes from transplantation studies in mice. From these experiments, it appears that the normal mouse mammary gland develops from self-renewing multipotent stem cells capable of forming a whole mammary tree (4–7), reviewed in (1;2;8). These mammary stem cells (MaSC) give rise to bi-potential $CK5^+$ progenitors (basal and luminal) which differentiate into committed basal or luminal progenitors which further differentiate into more mature basal (myoepithelial) or luminal cells, respectively. However, a recent study has challenged these conclusions and showed that transplantation assays for mammary stem/progenitor cells can be misleading (9). This study revealed that $CK5^+CK14^+$ multipotent stem cells are present only during embryonic life and are rapidly replaced at birth by lineage-restricted unipotent myoepithelial $CK5^+CK14^+$ and by unipotent luminal $CK8^+$ stem cells.

It has been known for years that malignant tumors are made of heterogeneous cell populations (10;11) and that some cancer cells can revert to a non-malignant state under defined experimental conditions (12). Recently, it was found that normal mammary epithelial cells and their stroma can suppress the malignant phenotype of tumor cells (13;14). In many types of cancers, tumor cells form a hierarchy, some cells being more efficient than others at forming tumors after transplantation into new hosts (11;15–20). These tumor-initiating cells (also designated cancer stem cells) constitute, in most cases, a very small percentage of tumor cells, the bulk of the tumor mass being made of cells incapable of generating tumors upon transplantation and presumably derived from the self-renewing tumor-initiating cells. This cellular organization has been observed in a large number of human tumors (16;17;21), including in breast cancers (2) and in a few mouse mammary tumor models (22–27). However, not all human or mouse tumors fit this pattern and, in some tumors, the majority of tumor cells exhibit tumor-initiating potential (28–31). Moreover, the low tumor-initiating capacity of some tumor cells may simply reflect problems of the transplantation procedure itself rather than the biology of tumor cells, as discussed (11;18;19;32;33).

Despite their suggestive name of “cancer stem cells”, and the apparent hierarchy in some tumors, the origin of the tumor-initiating cells cannot necessarily be ascribed to stem or progenitors cells of the tissue in which they arise. Identifying the cell subset in which cancer originates is a major aim of current cancer research (34). Investigation on the cellular origin of breast cancer has been conducted. Mammary tumors of $p53^{+/-}$ mice seem to originate from bi-potent progenitors (24). In MMTV-Wnt Tg mice, both the stem cells and luminal progenitors were reported the targets of Wnt (25;27;35). The neu oncogene also appears to target luminal progenitors in MMTV/Neu Tg mice (23). In BRCA1-deficient patients or mice, basal tumors arise from luminal progenitors (36–38) and in ETV6-NTRK3 Tg mice, mammary tumors arise from committed alveolar or luminal progenitors (26).

The Notch pathway is deregulated in breast cancer (39). In ~ 5% of human breast cancers, Notch1 (N1) mutations leading to overexpression of N1 intracellular domain ($N1^{IC}$) were

recently identified (40). Enhanced Notch signaling is capable of sustaining human mammosphere formation *in vitro*, suggesting that it can affect early progenitors (41). Transduction of oncogenic $N1^{IC}$ in mouse mammary cells enriched for stem cells followed by their reimplantation into cleared mammary fat pads was reported to cause expansion of committed luminal progenitors (42). In contrast, our group found that bi-potential MaSC-enriched progenitors were expanded in unmanipulated virgin pre-malignant MMTV/$N1^{IC}$ Tg glands and thus appeared to be the target of $N1^{IC}$ (43). Moreover, we showed that this $N1^{IC}$-induced expansion was cyclin D1-dependent (43). However, although both of these mammary models of $N1^{IC}$ overexpression induce tumors, neither of these studies investigated the tumor-initiating cells, nor their relationship with the expanded pre-malignant mammary progenitor cells.

In the present study, we studied some features of tumor-initiating cells in mammary tumors from MMTV/$N1^{IC}$ Tg mice, their relationship with the expanded pre-malignant $CD24^{+}CD29^{high}$ $CK5^{+}$ progenitors and their dependence on the presence of cyclin D1 for their phenotype. Our data show that these $N1^{IC}$-induced tumor cells remain addicted to the presence of cyclin D1 and are derived from bi-potential (luminal/basal) $CK5^{+}$ progenitor cells and prone to revert to a non-malignant state.

RESULTS

The $Lin^{-}CD24^{+}CD29^{high}$ MaSC-enriched cell subset, present in primary mammary tumors of MMTV/$N1^{IC}$ Tg mice, are not maintained upon transplantation of these tumors

As previously reported (43), the $Lin^{-}CD24^{+}CD29^{high}$ MaSC-enriched cell population (R4) was found to be expanded in pre-cancerous mammary tissues from 8 and 12 week-old MMTV/$N1^{IC}$ virgin Tg mice (Fig 1A), reaching to 1.2 x 10^{4} cells per gland at 12 and 40 weeks. We evaluated ten different primary tumors by FACS analysis for the presence of these cells. They represented ~ 2% of lin^{-} epithelial cells of primary tumors (Fig. 1A, C) or ~ 2 x 10^{5} cells for a ~ 1 g tumor of ~ 1.5 x 10^{7} lin^{-} epithelial cells. R4 cells from primary tumors proliferated less than R5 cells, but more than R1 and R2 cells (Fig. 1B). When unselected tumor cells (n = 10) were serially passaged into cleared mammary fat pads, the proportion of lin^{-} $CD24^{+}CD29^{high}$ cells progressively decreased, by as much as 176 folds at the second passage (Fig 1 A,C), and so did the $CD24^{-}CD29^{int}$(R1) cell subset (Fig. 1A, D). During transplantation, a $CD24^{int}CD29^{int}$ (R5) new tumor cell sub-population was progressively selected to represent ~ 90% of lin^{-} tumor cells at the second passage (Fig. 1A, E). Thus, the bi-potential $CD24^{+}CD29^{high}$ progenitor cells expand (~ 10 fold) during primary tumor formation but their presence does not appear to be required for the maintenance of the tumors after transplantation.

Mammary tumors were also transplanted sub-cutaneously. No $CD24^{+}CD29^{high}$ cell subset was present in the 10 subcutaneous tumors analyzed (data not shown), consistent with the stroma-dependency of tumor and normal stem cells for growth and differentiation, respectively (7). Moreover, subcutaneous tumors were significantly smaller than fat pad tumors in FVB or *nude* mice (Fig. 1F), indicating that host-derived mammary stromal cells contribute to their growth, possibly by favoring the growth and/or survival of tumor-initiating cells.

The Lin^{-}CD24^{+}CD29^{high} cell subset of N1^{IC}-induced mammary tumors does not represent a major source of tumor-initiating cells

We next evaluated the repopulating and tumor-initiating capacity of tumor-derived CD24^{+}CD29^{high} (R4) cells present in spontaneous primary mammary tumors of MMTV/N1^{IC} Tg mice (Fig 2A). For this, 250–1000 of these donor cells were transplanted into cleared fat pads or subcutaneous tissues of *nude* or FVB mice. These tumor-derived CD24^{+}CD29^{high} cells generated two types of outgrowths: either non-tumor glandular (Fig 2B) or tumor (Fig 2C) outgrowths. After transplantation of low (250–1000) number of cells, the majority of outgrowths in fat pads consisted of non-tumor glandular outgrowths in up to 60–90% of recipient mice (Fig 2 B, E). Such non-tumor glandular outgrowths were not generated after transplantation of the other R5 (see below) and R1 (Fig 2D) cell subsets selected from the same tumors. As expected, tumor outgrowths were also generated, after a long latency (6–10 months), in 20–40% of mice transplanted with high numbers of tumor-derived CD24^{+}CD29^{high} (R4) cells (Fig 2C, D). These tumor outgrowths arose at the same frequency in fat pads as in subcutaneous tissue (Fig 2D), although their size was consistently smaller in this latter location. Histologically and by cytokeratin staining, they were similar to the donor tumors (Fig 2A, C) and to those originating from pre-malignant CD24^{+}CD29^{high} cells (43). They also shared a similar expression profile of CD24 and CD29 with their donor tissues (Fig 2Ad, Cd). Tumor-derived R4 cells did not significantly transit to R5 phenotype in mixed transplantation (Fig. S1).

These results show that the tumor-derived CD24^{+}CD29^{high} population contain cells which have retained their progenitor bi-potential property and can develop into glandular structures. The same population also contains genuine tumor-initiating cells of low malignancy which do not appear to represent a major source of highly malignant tumor-initiating cells.

The tumor-initiating cells of N1^{IC}-induced mammary tumors can be abundant and show a CD24^{int}CD29^{int} phenotype

We next attempted to identify more abundant and more malignant tumor-initiating cells from these N1^{IC}-expressing tumors. Transplantation of very few unselected cells or even of a single cell into cleared mammary fat pads or subcutaneous tissues generated tumors (Fig S2A). We then tested the tumor-initiating potential of the other tumor-derived CD24^{int}CD29^{int} (R5) or CD24^{-}CD29^{int} (R1) cell subsets. These were sorted-purified (Fig 3A) and transplanted into the cleared fat pads or subcutaneous tissues of *nude* or FVB mice. The CD24^{-}CD29^{int} (R1) cells did not form any outgrowth, even when transplanted at high number (10^{4}) (Fig 2D). However, CD24^{int}CD29^{int} (R5) cells generated tumor outgrowths at high frequency, with small number of cells (250) after a short latency of 8-weeks (Fig S2B, 3B) and they had the same histological morphology (Fig 3C) and cytokeratin staining (Fig 3D) as the donor tumors, indicating they represent genuine tumor-initiating cells. We evaluated their frequency by transplanting them at decreasing numbers into cleared fat pads or subcutaneous tissues of *nude* or FVB mice (Fig 3B). Again, as few as one lin^{-}CD24^{int}CD29^{int} cell was able to generate a tumor (Fig 3B, E), histologically similar to the donor tumor. Cells expressing lower levels of CD24 and CD29 (R5a, R5b) had lower repopulating frequency (Fig 3F, G). We calculated the frequency of tumor-initiating cells to

be 1 in 57 [95% confidence interval (95% CI) 1/114-1/29}] for unselected $N1^{IC}$ tumor cells and of 1 in 27 (95% CI 1/52 -1/14) for cell-sorted purified (R5) cells**.** Together, a single unselected or selected cell from 6 out of 8 primary tumors tested could generate a tumor (Fig. 3 B,E and S2). Thus, the $N1^{IC}$-induced tumors contain a very high proportion of tumor-initiating cells which are $CD24^{int}CD29^{int}$.

Serial passages of $N1^{IC}$-induced tumors was found to enhance their tumor-initiating potential (Fig S3A) and Tg expression (Fig S3B). Moreover, $N1^{IC}$-derived tumor cells formed mammospheres at high frequency *in vitro* and this frequency increases with *in vivo* serial passages (Fig S3C, D). Since the capacity to form mammospheres *in vitro* has been reported to be a reliable assay for the presence of breast tumor-initiating cells (23;44–47), our results suggest that mammosphere formation may constitute a reliable surrogate assay for the frequency of $N1^{IC}$-expressing tumor-initiating cells.

In $N1^{IC}$-induced tumors, tumor cells remain dependent on cyclin D1 for survival and cyclin D1 deficiency decreases the frequency of tumor-initiating cells

We previously reported that the development of primary mammary tumors in MMTV/$N1^{IC}$ Tg mice was abolished in total absence of cyclin D1 (cyclin $D1^{-/-}$) and greatly reduced in heterozygote cyclin $D1^{+/-}$ Tg mice (43). We next determined whether $N1^{IC}$-induced tumors from wild-type cyclin $D1^{+/+}$ host remain sensitive to cyclin D1 deficiency. For this experiment, we transduced cylin $D1^{+/+}$ $N1^{IC}$-derived M212059 (date not shown) and M212057 tumor cells with retroviral vector encoding shRNA against mouse cyclin D1 ($shRNA^{cyclinD1}$) and a gene of selection (hygromycin). Cells transduced with $shRNA^{cyclinD1}$ had lower levels of cyclin D1 (Fig 4A), formed fewer hygromycin-resistant colonies (Fig 4B), and these colonies were smaller and grew at slower rate (Fig 4C) than those transduced with control vector. These $shRNA^{cyclinD1}$-expressing cells also exhibited lower survival and enhanced apoptosis after serum withdrawal, as assessed with annexinV/7AAD staining (Fig 4D) or TUNEL assay (Fig. 4E) indicating that cyclin D1 depletion sensitizes them to apoptosis.

We next tested whether cyclin D1 also had an impact on the tumor-initiating potential of tumor cells once the tumors were already formed. For this, we selected ten mammary tumors from ten independent cyclin $D1^{+/-}$ $N1^{IC}$ Tg mice and evaluated the repopulating capacity of their $CD24^{int}CD29^{int}$ cells (R5) by transplanting them subcutaneously or into cleared fat pads of normal mice. As compared to cyclin $D^{+/+}$ $N1^{IC}$-induced tumors, cyclin$^{+/-}$ $N1^{IC}$-expressing tumors were much smaller (5–10 folds) and contained significantly fewer tumor-initiating cells, after transplantation in nude (Fig. 4F) or FVB (Fig S5) mice. By limiting dilution analysis, we calculated the frequency to be 1 in 149570 (95% CI 1/1394840-1/16039), i.e. ~2500 folds lower than their frequency in wild-type $N1^{IC}$ Tg tumors. Consistent with this finding, cultures of 10,000 unselected or selected $CD24^{int}CD29^{int}$ (R5) $N1^{IC}$-expressing-cyclin $D1^{+/-}$ tumor cells formed smaller and lower numbers of mammospheres than those derived from wild-type $N1^{IC}$ tumor cells (Fig 4G, H).

To further study the tumorigenic potential of these cells, three tumor cell lines were derived from each cyclin $D1^{+/+}$ and cyclin $D1^{+/-}$ $N1^{IC}$ Tg mammospheres. Cells from these lines were reimplanted into cleared fat pads of FVB recipient mice. Most outgrowths generated

with wild-type cyclin $D1^{+/+}$ $N1^{IC}$ Tg cells were tumors (Fig 5A), expressing high levels of CK5 (Fig 5B). In contrast, cells from cyclin $D1^{+/-}$ $N1^{IC}$ Tg lines generated fewer outgrowths (Fig 5A), which were non-tumors (see below). Together, these results show that the tumor-initiating potential of $N1^{IC}$-induced tumors is dependent on cyclin D1.

Cyclin D1 deficiency in $N1^{IC}$-induced tumor cells and presence of normal mammary epithelial cells around them promote their differentiation

Further analysis of the structures generated from tumor cells was revealing. As expected from our previous work (43), the mammosphere structures generated *in vitro* from cyclin $D^{+/+}$ $N1^{IC}$ Tg cells were disorganized and showed enhanced number of CK5-positive cells (Fig 4I). In contrast, although few in number and small in size, mammospheres derived from $N1^{IC}$-expressing cyclin $D1^{+/-}$ tumor cells were made from polarized epithelial cells organized in ductal-like structures made of $CK18^+$ and $CK19^+$ cells surrounded by $CK5^+$ cells (Fig 4I). These resemble to structures obtained with non-Tg mammospheres (43). Similarly, after transplantation *in vivo*, cyclin $D^{+/-}$ $N1^{IC}$ Tg cells generated mostly duct-like structures with $CK18^+$ luminal cells surrounded by $CK5^+$ myoepithelial cells (Fig 5A, C), indicating that cyclin D1 deficiency prompted these cells to become less malignant and more differentiated.

In view of the propensity of $N1^{IC}$-expressing cells to adopt a more differentiated phenotype when cyclin D1 levels were decreased, we tested their fate after their transplantation into cleared fat pads in the presence of normal mammary epithelial cells. It has been shown that some tumor cell lines adopt a mammary cell fate in such conditions (13;14). Total or sorted-purified R5 GFP-positive tumor cells (Fig. 6A) were transplanted in the presence of normal mammary epithelial cells at different ratios. At 5:1 ratio of tumor/normal cells, all (5 out of 5) transplanted fat pads formed tumors (Fig 6B). However, at ratio of 1:1 or 1:5, all (5/5) fat pads contained GFP-positive duct-like structures (Fig 6B–D), made of $CK5^+$ basal outer and $CK18^+$ luminal inner cells (Fig 6E). In contrast, only tumors developed in the presence of $N1^{IC}$-expressing pre-malignant mammary cells (Fig. S5). These results strongly suggest that $N1^{IC}$ tumors cells have adopted the fate of bi-potential (basal/luminal) progenitor cells, in the presence of normal cells.

DISCUSSION

In the present study, we characterized different populations of epithelial cells from mammary tumors of MMTV/$N1^{IC}$ Tg mice and studied their origin and the impact of cyclin D1 deficiency on their malignancy.

Hierarchy of $N1^{IC}$-induced primary tumors: abundance of tumor-initiating cells and low percentage of poorly malignant progenitor and mature cells

The most abundant cell subsets in $N1^{IC}$-induced tumors are the $CD24^{int}CD29^{int}$ (R5) cells. They constitute ~60% of all lin^- epithelial cells of primary tumors and were enriched (to over 80%) upon serial transplantation of tumor cells. Such serial transplantation also enriched the percentage of tumor-initiating and mammosphere-forming cells and shortened the latency of tumor appearance. Some of these tumors were found to harbor a very high

percentage of tumor-initiating cells, as few as 20 or even 1 unselected tumor cell being able to initiate the development of tumor after transplantation. Only a few tumor types have previously been found to harbor such a high percentage of tumor-initiating cells (29;31).

In addition, primary $N1^{IC}$-induced tumors contain small populations of $CD24^{-}CD29^{int}$ (R1) and of $CD24^{+}CD29^{high}$ (R4) cells which were absent in secondary tumors. By all criteria analyzed, this latter R4 population is indistinguishable from the previously characterized $CD24^{+}CD29^{high}$ (R4) MaSC-enriched cell subset present in pre-cancerous mammary glands of young virgin females from this Tg line (43). Both the tumor-derived and pre-cancerous R4 cell subsets share the same CD24/29 profiles, the same capacity to differentiate into duct-like structures, the same basal/luminal bi-potential differentiation ability, the same ability to induce tumors at low frequency after a long latent period (low tumor-initiating capacity) and the same dependence on mammary stroma, i.e. the same inability to grow sub-cutaneously after transplantation. Upon serial tumor transplantation into cleared fat pads, the percentage of the R1 and R4 cell populations rapidly diminishes, indicating that these cells are not required for tumor maintenance. We estimated the number of the expanded $CD24^{+}CD29^{high}$ R4 cell population in young virgin (12-week-old) Tg mice to be ~1.2×10^4 per gland (i.e. ~20% of all lin^{-} epithelial cells). Since we now find that they constitute about 2% of the total lin^{-} epithelial cells in primary tumors, their number appears to have increased within primary tumors. Thus, in this type of cancer, a bi-potential progenitor cell population (R4) and a more mature cell subset (R1), with low or no tumor initiating capacity, respectively, are mixed with, and expanded along the majority of tumor-initiating (R5) cells. This expansion is apparently totally dependent on the primary tumor environment, and specifically the primary stroma. Indeed, these poorly malignant R1 and R4 cell populations are not maintained upon tumor cell transplantation, strongly suggesting that the tumor-initiating cells themselves (R5) and the secondary stroma do not produce the factors necessary for their expansion and/or survival.

In primary tumors, the R1 and R4 cell subsets likely originate from the malignant tumor-initiating (R5) cells, provided that it is postulated that, once generated, they only survive and proliferate in the presence of primary, but not secondary, tumor cell environment. This model is consistent with our observation that tumor-initiating (R5) cells have the ability to generate $CK5^{+}CK8^{+}$ duct-like structures very efficiently when transplanted in the presence of normal mammary epithelial cells (Fig 6), thus behaving as bi-potential progenitor cells. As an alternative, but less likely origin, these tumor-derived $CD24^{+}CD29^{high}$ (R4) cells may simply represent further expansion of the $CD24^{+}CD29^{high}$ (R4) cells already present in pre-cancerous glands in which tumors arise, and which show all the features of bi-potential progenitor cells (43). Again this expansion would be dependent on the presence of the primary, but not secondary, tumor environment. In fact, it is possible that these two mechanisms participate in the expansion of the R1 and R4 populations in primary tumors, since these two scenarios proposed for their origin are not mutually exclusive. In both cases, the unique characteristics of the primary tumor environment are evident, but the cellular and molecular basis of this phenomenon remains to be elucidated. It is interesting to note that, in $N1^{IC}$-expressing tumors, tumor-initiating cells represent the majority of tumor cells, whereas cells endowed with more stem (progenitor) cell characteristics (R4) show much lower tumor-initiating potential. Such difference may reflect different tissues, different

transplantation protocols or other technical issues, as discussed (11;18;32;33) or may be related to properties of the Notch1 oncogenic pathway itself and the cell subset it targets (see below).

$N1^{IC}$-induced tumor cells remain addicted to cyclin D1 which is needed to generate high number of tumor-initiating cells

We previously reported that tumor formation in MMTV/$N1^{IC}$ Tg mice was totally or greatly impaired when these mice were bred on cyclin $D1^{-/-}$ or cyclin $D1^{+/-}$ background, respectively (43). We show here that $N1^{IC}$-induced tumor cells remain highly dependent on the presence of cyclin D1 for their growth. The few tumors arising in heterozygote cyclin $D1^{+/-}$ $N1^{IC}$ Tg mice were poorly malignant and contained a huge decrease (~2500 folds) of tumor-initiating cells relative to the number detected in wild-type cyclin $D1^{+/+}$ $N1^{IC}$-expressing tumors, suggesting that the low number of tumor-initiating cells prevented tumor formation. Moreover, we found that malignant tumor cells from wild-type cyclin $D1^{+/+}$ $N1^{IC}$ Tg mice remained totally addicted to this molecule. Indeed, partial shRNA-mediated decrease of cyclin D1 was sufficient to significantly increase their apoptosis and dramatically impaired their growth and their mammosphere-forming potential. Most interestingly, a partial decrease of cyclin D1 in these malignant cyclin $D1^{+/+}$ tumor cells provided them with the ability to form duct-like differentiated structures whose morphology and cytokeratin staining profile are very similar to those observed previously after transplantation of low malignant pre-cancerous $CD24^{+}CD29^{high}$ (R4) cells (43). These results strongly suggests that, upon lowering cyclin D1 levels, these tumor cells revert to a state similar to that of the $CD24^{+}CD29^{high}$ (R4) progenitor cells, consistent with the notion that the R5 tumor cells originate from these R4 progenitor cells (see below).

$N1^{IC}$-induced tumor-initiating cells originate from expanded bi-potent stem/progenitor cells

We previously showed that the R4 $CD24^{+}CD29^{high}$ MaCS-enriched progenitor cells are already expanded in young 8 week-old virgin female from MMTV/$N1^{IC}$ Tg mice, and express high levels of CK5 (43). Our results indicate that the R5 $CD24^{int}CD29^{int}$ tumor-initiating cells are very likely generated from these pre-malignant, expanded R4 MaCS-enriched progenitor cells, most likely after sustaining new genetic or epigenetic insult(s) (second-hit) which allow them to become more aggressive. We previously provided evidence that the R4 cells are unstable and had a transformed phenotype *in vitro* and exhibited a low tumorigenic potential *in vivo* and that their expansion predicts tumor formation (43). We now show that tumor-initiating R5 cells have lost their dependence on mammary stroma and that their malignant state is reversible upon decreased cyclin D1 expression, or by transplantation in the presence of normal mammary epithelial cells. Under these experimental manipulations, the R5 tumor cells take the fate of progenitors having the same characteristics as the R4 bi-potential, poorly malignant $CD24^{+}CD29^{high}$ cells present in young pre-cancerous Tg mice and revert to a less malignant phenotype. Together, these results argue strongly for a precursor/product relationship between these two tumor cell populations. Thus, spontaneous R4 to R5 transition is the most likely scenario leading to tumor formation.

Our experiments did not directly address the origin of these expanded $CK5^+$ $CD24^+$ $CD29^{high}$ (R4) bi-potential progenitor cells. It was recently shown that mouse mammary glands contain multipotent $CK5^+CK14^+$ stem cells only before birth, but harbor only unipotent $CK5^+CK14^+$ myoepithelial and unipotent $CK8^+$ luminal stem cells after birth (9). Because of their basal and luminal differentiation potential, it is very likely that the $CK5^+CD24^+CD29^{high}$ (R4) cells, found to be expanded in young MMTV/$N1^{IC}$ Tg mice, represent genuine multipotent $CK5^+CK14^+$ stem cells targeted by $N1^{IC}$ for expansion. The expression of the MMTV promoter around birth (Fig S6) would be consistent with such a scenario. However, we cannot rule out that $N1^{IC}$ could mainly affect unipotent progenitors and induce them to expand and to express a bi-potential program. Additional experiments are needed to approach this important question.

In conclusion, we provide evidence that the malignant $CD24^{int}CD29^{int}$ (R5) tumor-initiating cells of $N1^{IC}$-induced tumors are likely derived from the low malignant $CD24^+CD29^{high}$ (R4) cells, described previously in pre-malignant glands of young virgin females as bi-potential progenitor cells. We also show that these R5 cells remain dependent on cyclin D1 for their malignant phenotype and can revert to a state of low malignancy and stem/progenitor cells similar to the one from which they were derived. Since the Notch1-cyclin D1 pathway is highly conserved, its inhibition may offer an interesting opportunity for treatment of N1-induced mammary tumors, especially that only partial downregulation of cyclin D1 is sufficient to significantly impair tumor development.

MATERIALS AND METHODS

Mice

MMTV/$Notch1^{IC}$ Tg mice have been described previously (48).

FACS analysis and Cell Sorting

Both procedures were carried out as previously described (43).

Tumor Transplantation and Analysis

The fat pad transplantation technique has been described (7;43).

Establishment of transplantable tumor cell line

Primary tumors from different donors were collected and single cell suspensions prepared by limiting dilution, as described in supplementals.

TUNEL assay

This assay was performed as previously described (48).

Immunostaining

Cells or tissue sections were stained, as described before (43).

Cycling D1 depletion with shRNA

Retrovirus vectors harboring shRNA against cyclin D1 were obtained from Sigma. Viral stocks were prepared and tumor cells infected and hygromycin-resistant clones were selected, as described in supplementals.

Cell proliferation with bromodeoxyuridine (BrdU) in vivo

Labeling in vivo with BrdU was performed as before (43).

Statistics

Comparison of the distribution of different cell populations was analyzed by Student t-test. Analysis of the tumor-forming frequency was calculated using WEHI web interface based on the limdil function in the statmod package.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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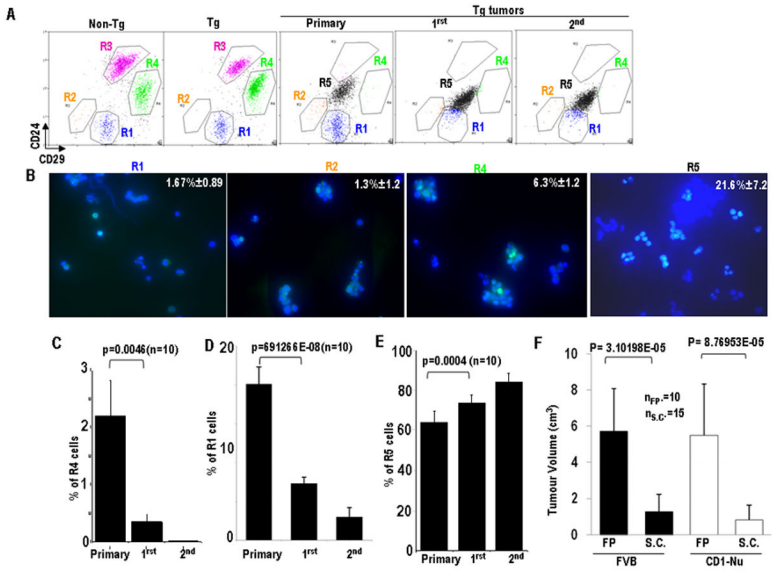


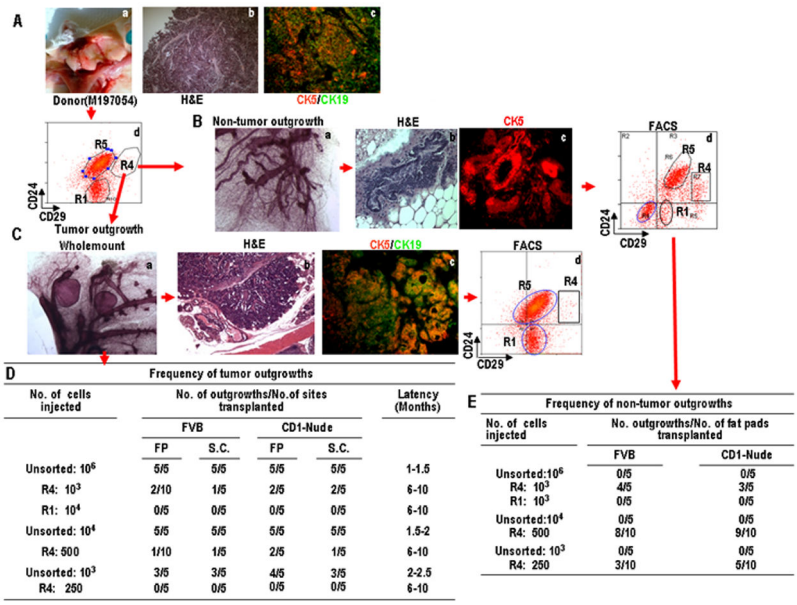
Figure 1. Changes in cellular subsets during transplantation of N1^{IC}-induced mammary tumors

A) Representative FACS dot plots showing profiles of the Lin$^-$ mammary cell subsets stained for CD24 and CD29. Cells were from pre-cancerous (8 week-old virgin) glands, primary tumors or tumors generated after the first (1st) or second (2nd) transplantation into cleared mammary fat pads.

B) Proliferation of the cell-sorted purified tumor cell subsets from two primary tumors assessed by IHC with anti-BrdU Ab.

C–E) Percentage of CD24$^+$CD29^{high} (R4) (**B**), CD24$^-$ CD29^{low} (R1) (**C**) and CD24^{Int}CD29^{Int} (R5) (**D**) Lin$^-$ cell subsets in primary (n=10) and transplanted (n=5) tumors relative to total Lin$^-$ cells.

F) Tumor volume after transplantation of tumor cells (1 x 10^4) sub-cutaneously (s.c.) or into cleared fat pads of non-Tg normal FVB or *nude* mice.



D Frequency of tumor outgrowths

No. of cells injected	No. of outgrowths/No.of sites transplanted				Latency (Months)
	FVB		CD1-Nude		
	FP	S.C.	FP	S.C.	
Unsorted: 10^6	5/5	5/5	5/5	5/5	1-1.5
R4: 10^3	2/10	1/5	2/5	2/5	6-10
R1: 10^4	0/5	0/5	0/5	0/5	6-10
Unsorted: 10^4	5/5	5/5	5/5	5/5	1.5-2
R4: 500	1/10	1/5	2/5	1/5	6-10
Unsorted: 10^3	3/5	3/5	4/5	3/5	2-2.5
R4: 250	0/5	0/5	0/5	0/5	6-10

E Frequency of non-tumor outgrowths

No. of cells injected	No. outgrowths/No. of fat pads transplanted	
	FVB	CD1-Nude
Unsorted:10^6	0/5	0/5
R4: 10^3	4/5	3/5
R1: 10^3	0/5	0/5
Unsorted:10^4	0/5	0/5
R4: 500	8/10	9/10
Unsorted: 10^3	0/5	0/5
R4: 250	3/10	5/10

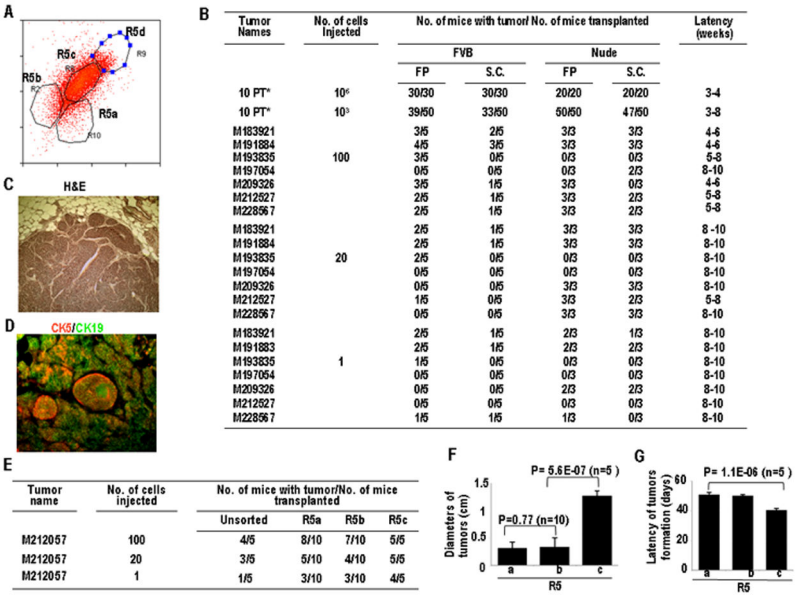
Figure 2. The CD24$^+$CD29^{high} (R4) cell subset present in primary N1^{IC}-induced tumors exhibits low tumor-initiating activity and has conserved some differentiation potential

Characterization of the CD4$^+$CD29^{high} (R4) cells from primary tumors (n=5) was performed after transplantation.

A) Representative donor primary tumor (a) stained with H&E (10 x) (b) or analyzed by IHC with anti-CK5/CK19 Ab (10x) (c) and by FACS (d). Transplantation of the R4 cells into cleared fat pads gave rise to either non-tumor (**B**) or tumor (**C**) outgrowths, both analyzed 6–10 months post-transplantation.

B, C) Representative characteristics of non-tumor **(B)** and tumor **(C)** outgrowths after transplantation of R4 cells. Wholemounts (0.8 x) (a), histology with H&E staining (10 x) (b), IHC with the indicated anti-CK Ab (10 and 20 x) (c) and FACS profiles (d) of the structures are shown.

D, E) Frequency of tumor (**D**) or non-tumor (**E**) outgrowths of one primary tumor shown in Ca and Ba, respectively. Data were pooled. Similar experiments with 4 additional tumors gave similar results. FP, fat pad; s.c., sub-cutaneously.



Tumor Names	No. of cells Injected	No. of mice with tumor/ No. of mice transplanted				Latency (weeks)
		FVB		Nude		
		FP	S.C.	FP	S.C.	
10 PT*	10^6	30/30	30/30	20/20	20/20	3-4
10 PT*	10^3	39/50	33/50	50/50	47/50	3-8
M183921	100	3/5	2/5	3/3	3/3	4-6
M191884		4/5	3/5	3/3	3/3	4-6
M193835		3/5	0/5	0/3	0/3	5-8
M197054		0/5	0/5	0/3	2/3	8-10
M209326		3/5	1/5	3/3	0/3	4-6
M212527		2/5	1/5	3/3	2/3	5-8
M228567		2/5	1/5	3/3	2/3	5-8
M183921	20	2/5	1/5	3/3	3/3	8 -10
M191884		2/5	1/5	3/3	3/3	8-10
M193835		2/5	0/5	0/3	0/3	8-10
M197054		0/5	0/5	0/3	0/3	8-10
M209326		0/5	0/5	3/3	3/3	8-10
M212527		1/5	0/5	3/3	2/3	5-8
M228567		0/5	0/5	3/3	3/3	8-10
M183921	1	2/5	1/5	2/3	1/3	8-10
M191883		2/5	1/5	2/3	2/3	8-10
M193835		1/5	0/5	0/3	0/3	8-10
M197054		0/5	0/5	0/3	0/3	8-10
M209326		0/5	0/5	2/3	2/3	8-10
M212527		0/5	0/5	0/3	0/3	8-10
M228567		1/5	1/5	1/3	0/3	8-10

Tumor name	No. of cells injected	No. of mice with tumor/No. of mice transplanted			
		Unsorted	R5a	R5b	R5c
M212057	100	4/5	8/10	7/10	5/5
M212057	20	3/5	5/10	4/10	5/5
M212057	1	1/5	3/10	3/10	4/5

Figure 3. The $CD24^{Int}CD29^{Int}$ (R5) sub-population represents frequent and genuine tumor-initiating cells of $N1^{IC}$-induced mammary tumors

A) Representative FACS profile of a donor tumor. Cells were labeled as described in Fig. 1A.

B) Frequency of tumor-initiating cells within the $CD24^{Int}CD29^{Int}$ (R5) tumor cell subset of 10 primary tumors. The R5 cells were purified by cell sorting and transplanted at the indicated numbers sub-cutaneously (s.c.) or into cleared fat pads of 3 week-old syngeneic FVB or *nude* mice. (*) Data from these individual tumors (Group 1, Fig S5) were pooled.

C, D) Representative transplanted R5-generated tumors stained with H&E (10 x) (**C**) or processed for IHC with anti-CK5/CK19 Ab (20 x) (**D**). Note similar histology and CK staining of donor (Fig 2A) and transplanted tumors.

E) Frequency of tumor-initiating cells present in the R5a, R5b and R5c cell subsets of M212057 secondary tumor shown in A. The sorted-purified cell subsets were transplanted at the indicated numbers sub-cutaneously into 3 week-old *nude* mice and tumors evaluated 12 weeks later.

F, G) Latency of tumor formation (**G**) and diameter of tumors (**F**) generated by transplanted R5a, R5b and R5c cells (100 cells).

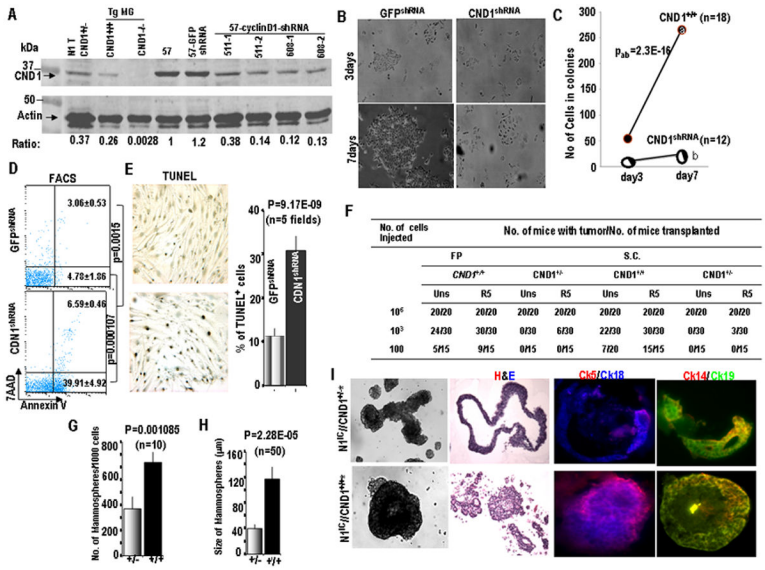


Figure 4. Cyclin D1 deficiency decreases the number of tumor-initiating and mammosphere-forming cells of N1^{IC} tumor cells

Tumor cells were obtained from the M212057 tumor cell line (57) originating in a cyclin D1$^{+/+}$ MMTV/N1^{IC} Tg mouse and transduced with shRNA against cyclin D1 (**A–E**) or from the few tumors growing in cyclin D1$^{+/-}$ MMTV/N1^{IC} Tg mice (**F–I**). As control, a tumor (T) sample from cyclin D1$^{+/-}$ N1^{IC} Tg mice.

A) Western blot analysis of cyclin D1 expression in N1^{IC}-expressing M212057 tumor cells (57) transduced with two distinct shRNA against cyclin D1 (shRNA^{cyclinD1}) (511 and 608) or GFP as control. Mammary glands (MG) from cyclin D1$^{+/+}$ and cyclin D1$^{-/-}$ are included. Filter was also probed for actin expression and ratio cyclin D1/actin obtained and normalized.

B, D) Effects of shRNA^{cyclinD1} on morphology (**B**), growth (**C**) and apoptosis after serum withdrawal for 72 hours, as evaluated by FACS (**D**) or TUNEL assay **(E).**

F) Frequency of tumor formation after transplantation of tumor cells from primary cyclin D1$^{+/-}$ (n = 10, Group 3) and cyclin D1$^{+/+}$ (n =10, Group 2) N1^{IC} Tg tumors into *nude* mice. Results with the 10 tumors were pooled. Results from cyclin D1$^{+/+}$ N1^{IC} Tg cells are also presented in Fig 3B. Uns, unselected; R5, sorted.

G–H) Frequency of mammosphere-forming cells among the cyclin D1$^{+/-}$ and control cyclin D1$^{+/+}$ from 10primary unsorted tumor cells in each group (**F**) and size (**G**) of these structures.

I) Representating morphology and cytokeratine staining pattern of mammospheres generated from 6 cyclin D1$^{+/+}$ (M197054, M197872, M197874, M209600, M212057, M212059) or cyclin D1$^{+/-}$ (M228747, M248174, M248176) N1^{IC}-expressing primary tumors.

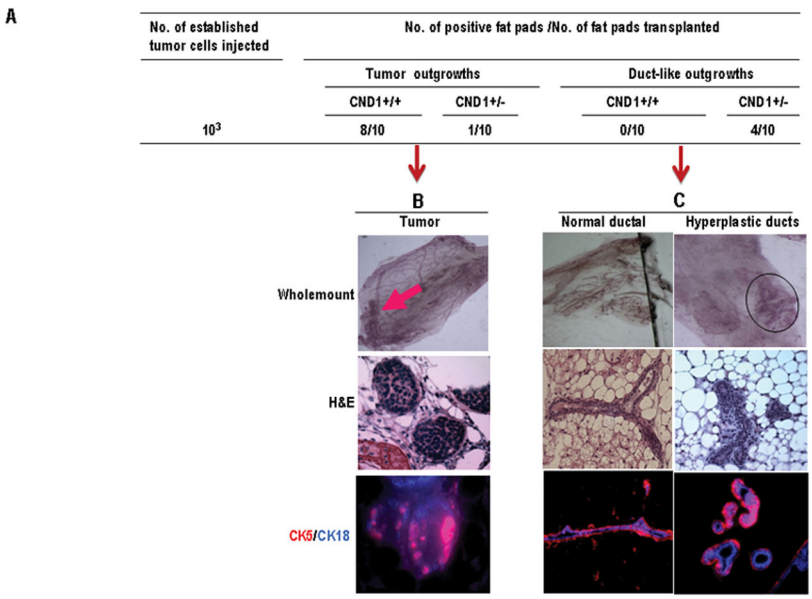
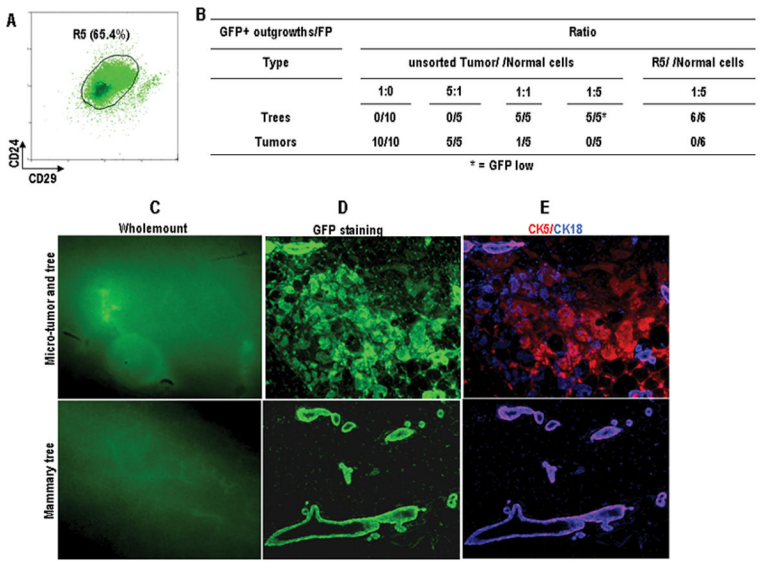


Figure 5. Cyclin D1 deficiency promotes differentiation of N1^{IC} tumor cells

A) Frequency of outgrowth structures generated after transplantation of established tumor cells from cyclin D1$^{+/+}$ (line M197874, M197872, M197054)) or cyclin D1$^{+/-}$ (line M228747, M248174, M248176) N1^{IC} Tg tumors.

B, C) Characteristics of tumor (**B**) and non-tumor (**C**) outgrowths obtained after transplantation of cyclin D1$^{+/-}$ N1^{IC}-expressing cells. Wholemount, histology with H&E staining and IHC with anti-CK5/CK18 Ab of the outgrowths are shown.



GFP+ outgrowths/FP		Ratio			
Type	unsorted Tumor/ /Normal cells				R5/ /Normal cells
	1:0	5:1	1:1	1:5	1:5
Trees	0/10	0/5	5/5	5/5*	6/6
Tumors	10/10	5/5	1/5	0/5	0/6

* = GFP low

Figure 6. Presence of normal mammary cells promotes the differentiation of N1^{IC}-expressing tumor cells

Constant number (1x10^3) of total or sorted-purified R5 GFP-positive tumor cells **(A)** from two distinct tumors (M247066, M247067) in double (actin/GFP x MMTV/N1^{IC}) cyclin D1$^{+/+}$ Tg mouse were mixed with normal mammary cells at different ratios and transplanted into cleared fat pads of normal FVB mice. Outgrowths were counted and analyzed 8 weeks after transplantation.

A) FACS profile of GFP-positive N1^{IC} tumor cells used.

B) Frequency of GFP+ outgrowths being either tumors or mammary trees.

C–E) Morphology (wholemounts) (**B**), GFP staining (**C**) and IHC with the indicated anti-CK antibodies (**E**) of tumors and mammary trees are shown.