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SHORT REPORTS

Parity-induced mammary epithelial cells facilitate tumorigenesis in MMTV-neu transgenic mice

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Using a Cre-lox-based genetic labeling technique, we have recently discovered a parity-induced mammary epithelial subtype that is abundant in nonlactating and nonpregnant, parous females. These mammary epithelial cells serve as alveolar progenitors in subsequent pregnancies, and transplantation studies revealed that they possess features of multipotent progenitors such as self-renewal and the capability to contribute to ductal and alveolar morphogenesis. Here, we report that these cells are the cellular targets for transformation in MMTV-neu transgenic mice that exhibit accelerated mammary tumorigenesis in multiparous animals. The selective ablation of this epithelial subtype reduces the onset of tumorigenesis in multiparous MMTV-neu transgenics. There is, however, experimental evidence to suggest that parity-induced mammary epithelial cells may not be the only cellular targets in other MMTV-promoter-based transgenic strains. In particular, the heterogeneous MMTV-wnt1 lesions predominantly express the ductal differentiation marker Nkcc1 that is absent in MMTV-neu-derived tumors. Our observations support the idea that tumors originate from distinctly different epithelial subtypes in selected MMTV-promoter-driven cancer models and that diverse oncogenes might exert discrete effects on particular mammary epithelial subtypes.
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It is a well-established fact that a full-term pregnancy early in life is associated with a long-term risk reduction for developing breast cancer. A woman who has her first child after the age of 35 has approximately twice the risk of developing breast cancer as a woman who has a child before age 20 (see current NCI Cancer Fact Sheet on Pregnancy and Breast Cancer Risk). Despite this long-term reduction in breast cancer risk in parous women, epidemiologists agreed at a recent NCI-sponsored workshop on 'Early Reproductive Events and Breast Cancer' (http://nci.nih.gov/cancerinfo/ere) that each gestation increases temporarily the likelihood for developing breast cancer. This transient increase in breast cancer risk lasts only for a few years after a full-term pregnancy.

Pregnancy has a very similar dual effect on the etiology of mammary cancer in animal models. Parous rats and mice have a greatly reduced susceptibility to chemically-induced mammary tumorigenesis compared to their nulliparous siblings (Russo and Russo, 1996; Medina and Smith, 1999). This difference is thought to reflect either systemic changes resulting from pregnancy, such as reduced levels of circulating hormones, or more probably the alteration of the mammary tissue itself. The mechanism(s) for this protective effect has not been defined. One widely accepted explanation, offered by Russo and Russo (1996), is that the protection is afforded by the pregnancy-induced differentiation of the target structures for chemically-induced carcinogenesis, that is, terminal end buds and duct termini. In contrast to chemically induced cancer models, most transgenic strains that express oncogenes under steroid and peptide hormone-responsive promoters exhibit pregnancy-associated mammary cancers or accelerated tumorigenesis in nonpregnant, parous females. Transgenic models are therefore inappropriate to recapitulate the protective effects of pregnancy on breast cancer, but they might be suitable to model the transient increase in breast cancer risk following a full-term pregnancy reported in humans. To date, the cellular basis and underlying molecular mechanisms for the tumor-promoting effects of pregnancy in humans and transgenic mice have not been identified.

We have recently discovered a new mammary epithelial subtype, which is abundant in nonlactating and nonpregnant, parous mice (Wagner *et al*., 2002). This epithelial subpopulation originates from differen-

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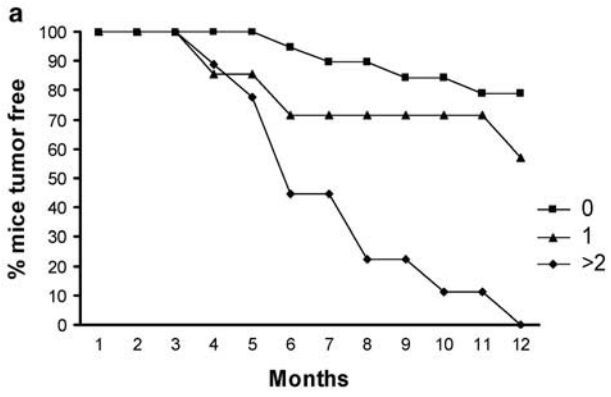
tiating cells during pregnancy. These cells permanently reside at the terminal end of mammary ducts after post-lactational remodeling. We utilized the Cre-lox technology to visualize these cells and to study their unique growth properties. In brief, the expression of the *whey acidic protein* (*Wap*) gene is generally used to monitor an advanced differentiation status of alveolar cells during the second half of pregnancy. The transient upregulation of Cre recombinase expressed by the *Wap* gene promoter (Wagner *et al*., 1997) permanently activates a Rosa-LacZ reporter transgene (Soriano, 1999) due to the Cre-mediated excision of a floxed transcriptional *Stop* sequence between *Rosa* promoter and the *β-galactosidase* (*LacZ*) coding sequence. The ubiquitously expressed Rosa-LacZ reporter transgene remains active in cells that change their fate during development whether they still express Cre recombinase or not. Consequently, the activation of the reporter transgene permanently labels pregnancy-hormone-responsive cells with an advanced differentiation profile that are apoptosis resistant during the post-lactational involution stage. A standard X-gal-staining technique can be utilized to visualize these β-galactosidase-expressing cells. Nulliparous Wap-Cre, Rosa-LacZ double transgenic females exhibit less than 1% of X-gal-positive cells. The amount of labeled cells increases to approximately 20% in nonpregnant, parous mice, and we therefore named this epithelial subtype the parity-induced mammary epithelial cell population (PI-MECs) (Wagner *et al*., 2002). Using immunohistochemistry, we were unable to detect expression of endogenous Wap protein and nuclear staining of Cre recombinase in PI-MECs (Oh and Wagner, unpublished observations). These observations suggest that regulatory elements of the *Wap* gene (endogenous and transgene, respectively) are not constitutively active in these cells. We reported earlier that PI-MECs serve as alveolar progenitors in subsequent pregnancies, and transplantation studies revealed that these cells possess two main features of multipotent progenitors: (a) self-renewal and (b) contribution to ductal and alveolar morphogenesis.

The identification and visualization of PI-MECs permitted us to more closely examine the role of this epithelial subtype as a potential mediator of the effects of pregnancy on mammary tumorigenesis. The unique growth properties of PI-MECs (i.e. responsiveness to pregnancy hormones, survival during involution, and ability to self-renew) make this epithelial subtype an ideal target for pregnancy-induced mammary tumorigenesis. We therefore hypothesized that PI-MECs might be the cellular basis for the development of mammary neoplasia in animal models that exhibit accelerated tumorigenesis in parous females. To address this issue, we bred mice expressing the unactivated Her2/neu (ErbB2) oncogene under transcriptional regulation of the MMTV-LTR (Guy *et al*., 1992) into the Wap-Cre, Rosa-LacZ double transgenic mice. MMTV-neu transgenics (FVB/N-TgN(MMTVneu)202Mul/J) are optimal for the experimental design, since this animal model exhibits a relatively long latency of tumorigenesis (T_{50} of 205 days), which makes it possible to generate single and multiparous females many weeks or months before the first tumor becomes palpable. It has been shown previously that the genetic background, in particular C57/Bl6, is able to further extend the mean latency of tumorigenesis in virgin females of this strain (Rowse *et al*., 1998). MMTV-neu mice generate ER-negative lesions that exhibit histopathological features similar to a subset of human breast cancers (Cardiff *et al*., 2000). More importantly, the overexpression of Her2/neu has been observed in a significant subset of pregnancy-associated breast cancers in humans (Reed *et al*., 2003). We maintained triple transgenic MMTV-neu, Wap-Cre, and Rosa-LacZ females, as well as their single transgenic (MMTV-neu) and double transgenic (MMTV-neu, Rosa-LacZ) littermate controls in a mixed genetic background (50% FVB, 25% 129/Svev, 25% C57/Bl6). Nulliparous ($N=15$), primiparous ($N=8$), and multiparous mice ($N=9$) were monitored weekly for tumor formation over a time course of 12 months (Figure 1a). Only 20% of the nulliparous females developed palpable lesions within that period, but all of them succumbed to mammary cancer after a latency period of 14–18 months. In contrast, pregnancy shortened the overall onset of the disease, and all multiparous littermate controls developed mammary tumors in less than 1 year. In conclusion, pregnancy clearly promotes tumorigenesis in this model.

Before determining whether PI-MECs are mediators of the tumor-promoting effects of pregnancy, we verified that the introduction of the MMTV-neu transgene did not lead to an abnormal expression of the Wap-Cre transgene and subsequent activation of the Rosa-LacZ reporter. Nulliparous, triple transgenic females ($N=4$) did not exhibit an increased number of X-gal-positive cells (Figure 1b), confirming that, like in wild-type mice, the activation of the Wap-Cre transgene is mainly linked to alveolar differentiation during pregnancy in Her2/neu-overexpressing mice. Mammary glands of parous littermates exhibited a considerable number of labeled PI-MECs (Figure 1c). The whole mount analysis of involuted triple transgenic mice also revealed that PI-MECs contributed significantly to the formation of multiple dysplastic foci. Consequently, the vast majority of solid tumors and invasive adenocarcinomas (27 of 28 tumors stained for active β-galactosidase activity) were comprised of X-gal-positive epithelial cell (Figure 2a and b). One primary tumor was X-gal negative, which might suggest that this lesion had a different cellular origin. It is, however, more reasonable to propose that these cells are the progeny of PI-MECs that lack activation of the *Wap* promoter due to the mosaic expression pattern of Wap-Cre transgene, which was reported previously (Wagner *et al*., 2003). Three animals with X-gal-positive mammary tumors were examined for pulmonary metastasis to determine whether transformed PI-MECs are capable of spreading to distant sites. Indeed, cells expressing β-galactosidase could be detected in pulmonary metastases of triple transgenic parous females (Figure 2c). The *Wap* gene promoter is not active in normal pulmonary epithelia and therefore expression of the Rosa-LacZ transgene was not detected



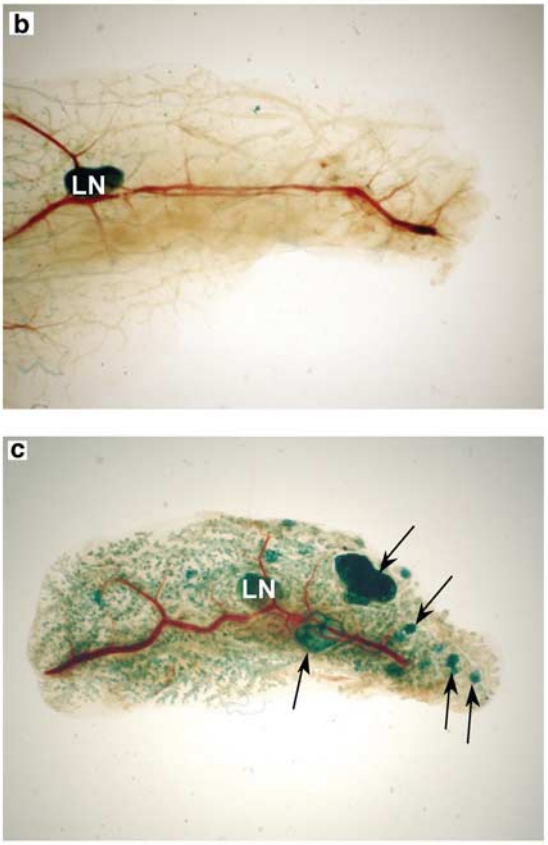


Figure 1 (**a**) Tumor development in nulliparous (0), primiparous (1), and multiparous (>2) triple transgenic females carrying the MMTV-neu oncogene in addition to the Wap-Cre and Rosa-LacZ transgenes. Mice were monitored twice weekly for a period of 12 months and killed when a tumor became palpable. The data were plotted as percentage of tumor-free animals against the time in months. (**b**, **c**) X-gal staining of a #4 mammary gland whole mount from a nulliparous (**b**) and multiparous (**c**) MMTV-neu, Wap-Cre, Rosa-LacZ triple transgenic female 37 weeks of age (magnification $\times 4$). Arrows in panel c indicate the location of multiple dysplastic foci and larger cancerous lesions in parous triple transgenic females; LN, lymph node

in the lung of double transgenic Wap-Cre and Rosa-LacZ littermate controls (Figure 2d).

The X-gal staining solution did not homogeneously penetrate a solid lesion, and therefore the intensity of the staining greatly diminishes towards the center of the

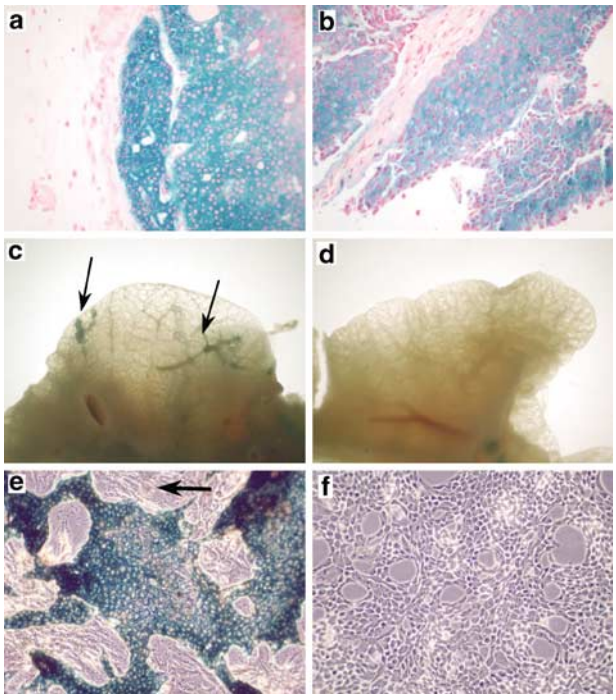


Figure 2 (**a**, **b**) Histological sections of X-gal-stained solid tumors from parous mice carrying the MMTV-neu oncogene in addition to the Wap-Cre and Rosa-LacZ transgenes. Slides were counterstained with Nuclear Fast Red (Vector Laboratories) (magnification $\times 200$). (**c**, **d**) Pulmonary metastases of X-gal-labeled mammary cancer cells. Fragments of the lung from parous triple transgenic females (**c**) and their double transgenic Wap-Cre, Rosa-LacZ littermate controls (**d**) were stained as whole mounts and examined under a stereoscope. Arrows in panel c indicate the location of pulmonary metastases (magnification $\times 10$). (**e**, **f**) X-gal staining of primary mammary cancer cells derived from parous triple transgenic females (MMTV-neu, Wap-Cre, Rosa-LacZ) and their single transgenic controls (MMTV-neu). Note that tumor-associated fibroblasts (panel E, arrow) do not express β-galactosidase (magnification $\times 200$)

specimen (Figure 2a). Owing to this technical limitation, the X-gal staining of neoplastic cells *in situ* does not allow a quantitative analysis of the relative contribution of PI-MECs in a primary tumor. We derived primary mammary epithelial cell lines from five parous and three nulliparous triple transgenic females as well as two control mice carrying only the MMTV-neu transgene. All neoplastic cells derived from parous triple transgenic mice stained positive for active β-galactosidase in culture (Figure 2e). Only tumor-associated fibroblasts present in two of the five primary cultures were X-gal negative (Figure 2e, arrow). No background staining was observed in tumor cells from single transgenic controls (Figure 2f). Upon examination, tumorigenic cells from nulliparous triple transgenic females also exhibited an activation of the Rosa-LacZ reporter (data not shown), and it was therefore reasonable to assume that: (a) the progression of tumorigenesis triggers partial differentiation and Wap-Cre expression, or (b) tumorigenesis in nulliparous females occurs primarily from the relatively small population (<1%) of hormone-responsive epithelial cells expressing the *Wap* gene. To address

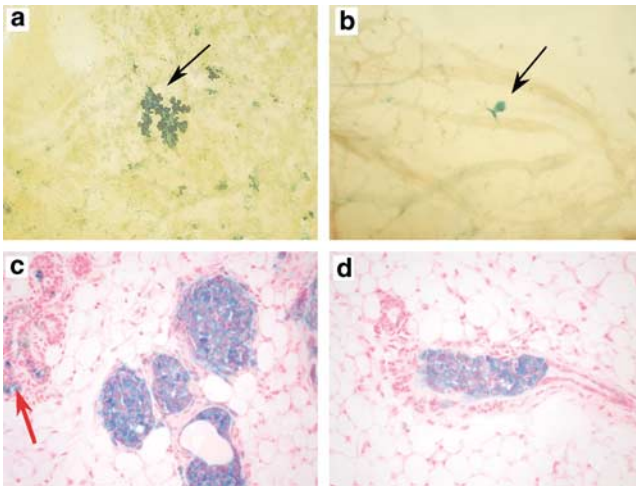


Figure 3 (**a**, **b**) X-gal staining of mammary whole mounts from parous (**a**) and nulliparous (**b**) females carrying the MMTV-neu oncogene in addition to the Wap-Cre and Rosa-LacZ transgenes. Arrows indicate the location of very early neoplastic lesions comprised of X-gal positive mammary epithelial cells (magnification $\times 20$. (**c**, **d**) Histological sections through X-gal-positive early neoplastic lesions. Note the migration of neoplastic cells into the luminal cavity of subtending ducts. The red arrow in panel c indicates the location of X-gal-positive, untransformed PI-MECs in close proximity to the primary lesion (magnification $\times 200$)

both issues, we investigated the activation of the Rosa-LacZ reporter gene in microscopically small lesions within the fourth inguinal glands of parous and nulliparous triple transgenic females 37 weeks of age (Figure 3a and b). The tiniest lesions that we were able to identify were X-gal positive in both parous and nulliparous tissue. This observation implied that Wap-Cre expression occurred before and not after the transformation event. The uniformity of the blue staining within these lesions might be another indication for the correctness of this assumption. In addition, we examined expression of the endogenous *Wap* gene and the Wap-Cre transgene in microscopic lesions using immunohistochemistry. Like in untransformed PI-MECs (see earlier), we were unable to detect Wap protein expression in these dysplastic lesions, and Cre recombinase was not present in the nuclei of neoplastic, X-gal-positive cells (data not shown). In summary, we have several lines of evidence to suggest that the Wap-Cre activation occurred before and not after cellular transformation: (a) microscopic lesions contain X-gal-positive cells in both parous and nulliparous mice, (b) neoplastic PI-MECs lack WAP protein expression as determined by immunofluorescence, and (c) constitutive Wap-Cre expression, that is, nuclear localization of Cre recombinase, was not observed in transformed PI-MECs using immunohistochemistry.

In both nulliparous and parous glands, neoplastic cells migrate away from the lobular unit into the luminal space of subtending ducts, giving the appearance that these cells are of ductal origin (Figure 3c and d). The Cre-lox-based technique to genetically label hormone-responsive cells expressing alveolar differentiation marker (i.e. *Wap*) might be an experimental verification of the theory by Wellings *et al*. (1975) that ductal carcinoma *in situ* (DCIS) and other types of 'ductal' lesions arise from terminal duct lobular units (TDLUs). The transient, estrus cycle-dependent activation of the endogenous *Wap* gene and a *Wap* promoter-driven reporter transgene in individual cells within terminal ducts or lobular units of nulliparous glands has been demonstrated earlier (Kordon *et al*., 1995; Robinson *et al*., 1995) and is therefore not an artifact caused by a 'leaky' Wap-Cre transgene. In conclusion, the results of this study suggest that tumorigenesis in parous and nulliparous MMTV-neu transgenics originate from a hormone-responsive, differentiating epithelial subtype within lobular units. The *de novo* generation and amplification of a large number of hormone-responsive and apoptosis-resistant epithelial cells (i.e. PI-MECs) during the first and subsequent reproductive cycles might account for the significantly increased cancer susceptibility of parous MMTV-neu transgenic females. If this assumption is correct, then the elimination of this epithelial subtype should abolish or greatly reduce tumorigenesis in this animal model.

We generated multiparous females carrying the MMTV-neu transgene in addition to the conditional knockout of the *Tsg101* gene (Wap-Cre, $Tsg101^{fl/fl}$) to experimentally address the question whether the ablation of PI-MECs results in reduced mammary tumorigenesis in parous MMTV-neu mice. The conditional knockout of the tumor susceptibility gene 101 (*Tsg101*) causes cell cycle arrest and cell death *in vitro* and *in vivo* (Krempler *et al*., 2002; Wagner *et al*., 2003). *Tsg101* is, however, not a tumor suppressor gene as previously reported. On the contrary, we can demonstrate that this gene is indispensable for the survival of normal, immortalized, and fully transformed cell lines (Carstens *et al*., 2004, submitted). The Wap-Cre-mediated deletion of this gene impaired mammary lobulogenesis during pregnancy and lactation due to increased cell death (Wagner *et al*., 2003). According to our experimental design, the Wap-Cre-mediated deletion of *Tsg101* should therefore significantly reduce the number of untransformed, hormone-responsive cells that are the proposed cellular targets for the neoplastic transformation in MMTV-neu mice. Owing to the mosaic expression pattern of the Wap-Cre transgene, we did not expect to completely abolish lobulogenesis and tumorigenesis. Only two out of 10 (20%) multiparous MMTV-neu, Wap-Cre, $Tsg101^{fl/fl}$ animals developed small lesions after 12 months of age. Eight mice did not even develop microscopically small lesions in any of the inguinal mammary glands, as determined by whole mount analysis (data not shown). In contrast, nine out of 10 (90%) multiparous littermate controls (MMTV-neu, $Tsg101^{fl/fl}$ or MMTV-neu, Wap-Cre, $Tsg101^{fl/wt}$) developed multifocal lesions in one or both #4 inguinal glands. It should be noted that, in all cases, these small lesions did not progress into larger tumors within the 12-months latency period. As discussed earlier, a delay in the formation of large, solid tumors was expected, since the *Tsg101* floxed allele was maintained in a 129SvJ/

C57Bl6 mixed background. Despite this experimental variation, the Wap-Cre-mediated deletion of *Tsg101* clearly reduced the onset of mammary neoplasia in MMTV-neu mice compared to their littermate controls that have the same genetic background, suggesting that restraining the genesis and survival of differentiating alveolar cells during pregnancy, and therefore PI-MECs in parous mice, eliminates the cellular basis for transformation in this model.

Results obtained from the genetic labeling studies using a Cre-activated reporter transgene and the targeted ablation of differentiating subtypes demonstrate that tumorigenesis in MMTV-neu transgenic mice originates predominantly in epithelial cells expressing *alveolar*-type differentiation markers (i.e. hormone-responsive cells capable of activating the *Wap* gene promoter). Based on this assumption, MMTV-neu tumors should exhibit very little expression of Nkcc1 (Slc12a2), which is a sodium, potassium, and chloride transporter that is specifically upregulated in *ductal* but not alveolar epithelial subtypes (Shillingford *et al*., 2002). As predicted, Nkcc1 was not present in MMTV-neu-derived tumors as determined by immunohistochemistry (Figure 4a; T = tumor). Ductal epithelial cells adjacent to the neoplasm stained positive for this cell type-specific marker (Figure 4a, arrow). This observation might be another indication for the correctness of the assumption stated above. It is logical to hypothesize that the expression of the Her2/neu oncogene is relatively high in hormone-responsive PI-MECs compared to other epithelial subtypes, since the *Wap* gene promoter and the *MMTV-LTR* are synchronously upregulated during pregnancy. This raises the possibility that other MMTV-promoter-driven oncogenes lead to the transformation of the same epithelial subtype. Our studies revealed that PI-MECs are also the cellular targets for transformation in mice expressing the polyoma virus middle T oncogene (MMTV-PyVMT). Tumorigenic mammary epithelial cell cultures derived from parous MMTV-PyVMT females exhibited a uniform Wap-Cre-mediated activation of the reporter transgene (Wagner and Seagall, unpublished). Both, the Her2/neu and PyVMT oncogenes act in a cell autonomous fashion. Since a variety of oncogenes such as TGFα and Wnt1 act as autocrine as well as paracrine growth factors (Brisken *et al*., 2000; Kisseberth and Sandgren, 2004), it would be incorrect to assume that PI-MECs are the sole targets for cellular transformation in all MMTV-LTR-based tumor models. In addition, it was recently suggested that the MMTV-wnt1 oncogene targets undifferentiated progenitors or mammary stem cells (Li *et al*., 2003; Liu *et al*., 2004). This could be a reason why MMTV-wnt1 mice exhibit a greater variety of histopathologically distinct lesions compared to the MMTV-neu model (Cardiff *et al*., 2000). We can demonstrate here that, unlike MMTV-neu-derived tumors, various subtypes of MMTV-wnt1 tumors express the ductal differentiation marker Nkcc1 as determined by immunohistochemistry (Figure 4b–d). Some MMTV-wnt1-derived tumors were comprised of different epithelial subtypes where only a fraction of the cells maintained the expression of Nkcc1 (Figure 4d). Whether this observation is the result of the suggested paracrine function of Wnt1 and/or the transformation of pluripotent progenitors that differentiate into various subtypes remains to be examined. The involvement of PI-MECs in a subset of MMTV-wnt1-derived tumors is currently being investigated in parous MMTV-wnt1, Wap-Cre, and Rosa-LacZ triple transgenic females.

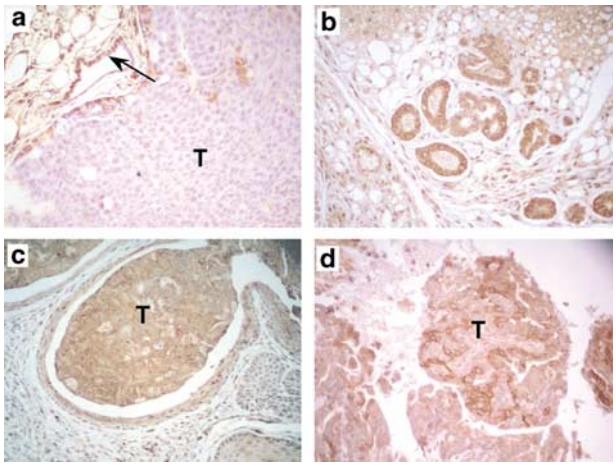


Figure 4 Immunohistochemical analysis of Nkcc1 expression in primary mammary tumors of MMTV-neu (**a**) and MMTV-wnt1 (**b**–**d**) mice (magnification $\times 200$). Note that the expression of Nkcc1 is confined to untransformed ductal epithelial cells (**a**, arrow). This ductal marker is not present in adjacent, Her2/neu overexpressing tumor cells (**a**, T = tumor). In contrast, Nkcc1 is expressed in hyperplastic ducts (**b**) and histopathologically distinct tumors (**c**, **d**) in MMTV-wnt1 mice

Our combined studies presented here support the idea that tumors originate from distinctly different epithelial subtypes in selected MMTV-promoter-driven cancer models expressing diverse types of oncogenes. In MMTV-neu and MMTV-PyVMT mice, PI-MECs located at the terminal duct lobular unit are the prime targets for neoplastic transformation. We are currently developing a GFP-based reporter system that allows the selection, clonal amplification, and transplantation of limiting dilutions of viable, transformed PI-MECs to verify again that these cells are the cancer-initiating cells in these models. More importantly, this method will also allow addressing the question whether tumors arise from pluripotent PI-MECs or their progeny (i.e. duct-only or alveolar-only bipolar progenitors) in these cancer models. The MMTV-promoter-driven expression of Wnt1, on the other hand, might preferentially target undifferentiated progenitors. The consideration of distinctly different 'cancer stem cells' in different models increases the complexity of mechanistic studies that intend to dissect signaling pathways leading to cancer. It is therefore of utmost importance to verify the presence and normal growth properties of the cancer-initiating epithelial subtypes when particular knockout models with developmental defects (for example cyclin D1 mutants that exhibit impaired proliferation of alveolar precursors) are crossed into these diverse MMTV-promoter-based transgenic tumor models.

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