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ORIGINAL ARTICLE

Tsg101 is upregulated in a subset of invasive human breast cancers and its targeted overexpression in transgenic mice reveals weak oncogenic properties for mammary cancer initiation

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Previous studies reported that the *Tumor Susceptibility Gene 101* (*TSG101*) is upregulated in selected human malignancies, and the expression of exogenous Tsg101 was suggested to transform immortalized fibroblasts in culture. To date, the potential oncogenic properties of Tsg101 have not been examined *in vivo* owing to the lack of appropriate model systems. In this study, we show that Tsg101 is highly expressed in a subset of invasive human breast cancers. Based on this observation, we generated the first transgenic mouse model with a targeted overexpression of Tsg101 in the developing mammary gland to test whether exogenous Tsg101 is capable of initiating tumorigenesis. Normal functionality of exogenous Tsg101 was tested by rescuing the survival of Tsg101-deficient mammary epithelial cells in conditional knockout mice. The overexpression of Tsg101 resulted in increased phosphorylation of the epidermal growth factor receptor and downstream activation of MAP kinases. Despite an increase in the activation of these signal transducers, the mammary gland of females expressing exogenous Tsg101 developed normally throughout the reproductive cycle. In aging females, the overexpression of Tsg101 seemed to increase the susceptibility of mammary epithelia toward malignant transformation. However, owing to the long latency of tumor formation and the sporadic occurrence of bona fide mammary cancers, we conclude that the Tsg101 protein has only weak oncogenic properties. Instead of cancer initiation, it is therefore likely that Tsg101 plays a more predominant role in the progression of a subset of spontaneously arising breast cancers.
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Introduction

The *Tumor Susceptibility Gene 101* (*Tsg101*) was originally identified in a screen for potential tumor suppressors using insertional mutagenesis in murine NIH3T3 cells (Li and Cohen, 1996). This gene is expressed ubiquitously in human and murine cell types (Li *et al*., 1997; Wagner *et al*., 1998), and its encoded protein mediates a variety of biochemical and biological functions. Some of these functions have been postulated from the predicted (and partially resolved) protein structure, the identification of Tsg101 binding proteins and the generation of Tsg101-deficient animal models. These suggested functions include a role in endosomal trafficking (Babst *et al*., 2000; Garrus *et al*., 2001), transcriptional regulation (Watanabe *et al*., 1998; Hittelman *et al*., 1999), proliferation and cell survival (Ruland *et al*., 2001; Krempler *et al*., 2002; Wagner *et al*., 2003).

Although some molecular functions of Tsg101, in particular its involvement in endosomal trafficking, have been studied in more detail, the biological role of Tsg101 as a tumor susceptibility gene remained controversial. Using a conventional antisense approach, Li and Cohen (1996) reported that a functional knockout or overexpression of Tsg101 resulted in instant neoplastic transformation of NIH3T3 cells. Following this initial observation, Li *et al*. as well as other research groups suggested that genomic deletions and aberrant splice variants occurred in sporadic forms of breast cancer and a variety of other human malignancies (Gayther *et al*., 1997; Lee and Feinberg, 1997; Li *et al*., 1997; Sun *et al*., 1997). Some of these claims, in particular the occurrence of genomic deletions, could subsequently not be verified (Steiner *et al*., 1997), and after revising the genomic architecture of the *Tsg101* locus, we determined that many aberrant splice variants were, in fact, alternative splice products that originated solely through exon skipping (Wagner *et al*., 1998). To experimentally address whether the loss of function of *Tsg101* is involved in neoplastic transformation *in vivo*, we generated mutant mice that lack *Tsg101* in all cells or specifically in mammary epithelial cells of adult females (Wagner *et al*., 2003). Surprisingly, neither haploinsufficiency of *Tsg101* (see also the report by Ruland *et al*.

(2001)) nor the deletion of both *Tsg101* alleles in mammary epithelial cells resulted in the development of cancer. More importantly, we demonstrated that Tsg101 remained essential for the proliferation and the survival of fully neoplastic cells (Carstens *et al*., 2004). In summary, these gene-targeting studies clearly demonstrated that a null mutation of *Tsg101* is neither an initiating event for tumorigenesis nor a process that promotes cancer progression.

By generally defining *Tsg101* a tumor *susceptibility* gene, Li and Cohen (1996) proposed that not only the functional inhibition but also the overexpression of Tsg101 were involved in neoplastic transformation. Although the expression of Tsg101 appeared to be upregulated in selected human cancers (Lo *et al*., 2000; Liu *et al*., 2002; Zhu *et al*., 2003; and this report), the paradigm of Tsg101 as an oncogene has never been experimentally addressed using an appropriate *in vivo* model system. To test whether excess levels of Tsg101 are sufficient to trigger neoplastic transformation, we generated a transgenic mouse model that overexpresses wild-type Tsg101 in the developing mammary gland. Collectively, our results show that the overexpression of wild-type Tsg101 is only weakly oncogenic. With regard to the observed upregulation of Tsg101 specifically in invasive human breast cancers, we propose that Tsg101 might not be involved in cancer initiation but it might promote tumor progression.

Results and discussion

Tsg101 is upregulated in a subset of invasive human breast cancers

There are only very few published studies that utilized methods other than reverse transcriptase-polymerase chain reaction (RT–PCR) to determine the expression of Tsg101 in primary human carcinomas (Lo *et al*., 2000; Liu *et al*., 2002; Zhu *et al*., 2003). All these reports demonstrated that the Tsg101 protein was expressed, albeit at heterogeneous levels, in normal and neoplastic tissues. To examine whether Tsg101 is differentially expressed in normal breast epithelia versus benign and neoplastic lesions of the breast, we performed immunohistochemistry on 16 normal or benign cases and 16 invasive human breast cancer cases. Initially, we tested four different antibodies against Tsg101 from three different companies. We found that two of them (Novus Biologicals Inc., Littleton, CO, USA and Abgent Inc., San Diego, CA, USA) were useful for detecting Tsg101 expression by immunohistochemistry. The results obtained with the monoclonal antibody against Tsg101 from Novus Biologicals Inc. are shown in Figure 1. Tsg101 is moderately expressed in the epithelial compartment of the human breast. Stromal cells exhibited a much lower immunoreactivity against Tsg101 (Figure 1a). The expression of Tsg101 varied to some extent between benign cases (Figure 1b and c), but overall, the expression of Tsg101 appeared to be lower compared with invasive breast cancers (Figure 1d–f). Among the 16 invasive breast cancer cases, eight exhibited an elevated expression of Tsg101, seven had levels equivalent to normal breast tissue and one specimen did not show any significant Tsg101 immunoreactivity throughout the section containing neoplastic epithelial cells (Supplementary Table S1, see Supplementary Materials). Generally, the intensity of Tsg101 staining did not correlate with the size and type of the malignant lesion or the degree of its invasiveness. Among the eight cases with elevated Tsg101 expression, two had remarkably high immunostaining against Tsg101 in the vast majority of cancer cells within the entire section. The increase in the intensity of Tsg101 staining from differentiated structures (Figure 1e, white arrow) to highly invasive breast cancer cells (Figure 1e, black arrow) was noticeable within the same specimen, suggesting that the Tsg101 protein was more abundant in fully neoplastic cells. Intense staining of Tsg101 was

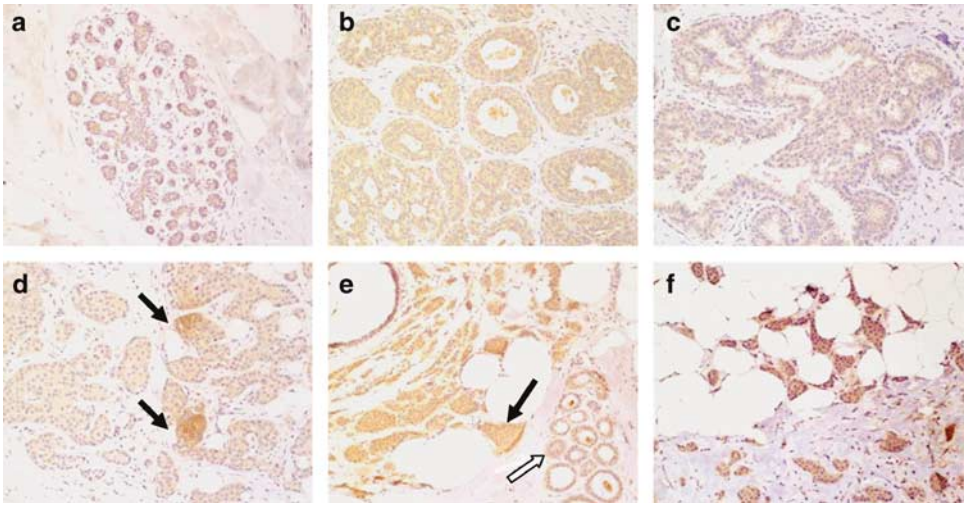

Figure 1 Tsg101 immunostaining in normal human breast tissue (**a**), benign lesions of the breast and hyperplasia (**b** and **c**) and invasive human breast cancers (**d–f**). Note that there is a difference in the intensity of Tsg101 staining between differentiated structures (**e**, white arrow) and the invasive cells (black arrow) within the same breast cancer specimen (magnification $\times 200$).

detected along the invasive front, where epithelial cells expanded into the adipose tissue (Figure 1f). Six cases with moderately elevated expression of Tsg101 exhibited intense staining in less than half of the malignant cells. Throughout those sections, we observed at least five to 10 focal regions, where cells exhibited more extensive immunoreactivity against Tsg101 (Figure 1d, arrows). In summary, the immunohistochemical analysis demonstrated that Tsg101 expression is quite heterogeneous among different cases of human breast cancer, and the staining is also variable between cancer cell populations within the same cancer specimen. Our observations are consistent, in part, with a previous report by Zhu *et al*. (2003) that shows that Tsg101 is expressed in cases of ductal carcinoma *in situ* and invasive carcinoma, whereas epithelium of normal ducts and lobules showed a much weaker staining. In contrast to this report, however, we observed more intensive Tsg101 staining in advanced breast cancer cells that invaded into the surrounding stroma, suggesting that Tsg101 might play a role in breast cancer progression.

Generation of transgenic mice overexpressing wild-type Tsg101 in the developing mammary gland

Li and Cohen (1996) suggested that the overexpression of Tsg101 causes neoplastic transformation of immortal fibroblasts in culture. To experimentally address whether Tsg101 has oncogenic properties *in vivo*, we generated transgenic mice that overexpress the full-length Tsg101 under regulatory elements of the *whey acidic protein* (*Wap*) gene (Figure 2a). The transgenic Tsg101 was fused to a hemagglutinin (HA) tag to distinguish the expression of exogenous Tsg101 from the endogenous protein. The pronuclear injection of the WAP-Tsg101 construct yielded five transgenic founder lines, and two out of five lines exhibited the correct temporal and spatial expression of Tsg101 in the developing mammary gland (Figure 2b). The highest expression of HA-tagged Tsg101 protein was achieved in line 4389 (Figure 2c), which was subsequently characterized in more detail and used for functional assays.

Next, we verified the mammary gland-specific expression of the WAP-Tsg101 construct in line 4389 using RT–PCR to ensure the correct spatial regulation of the transgene. As illustrated in Figure 3a, the expression of exogenous Tsg101 could not be detected in organs other than the mammary gland. Using Western blot analysis, we also confirmed that the transgenic Tsg101 precisely followed the temporal activation of the endogenous *Wap* locus, which is greatly upregulated during late pregnancy and lactation (Figure 3b). As shown in Figure 3d, the expression of exogenous Tsg101 resulted in significantly higher steady-state levels of total Tsg101 protein in transgenic females compared with their wild-type controls. Although the HA-tagged protein was virtually absent in the mammary gland of virgin females, exogenous Tsg101 was still present, albeit at much lower levels compared with lactation, during the postlactational involution period (Figure 3c). This observation suggested that at least one full-term pregnancy was required to express WAP-Tsg101 at detectable levels by Western blot.

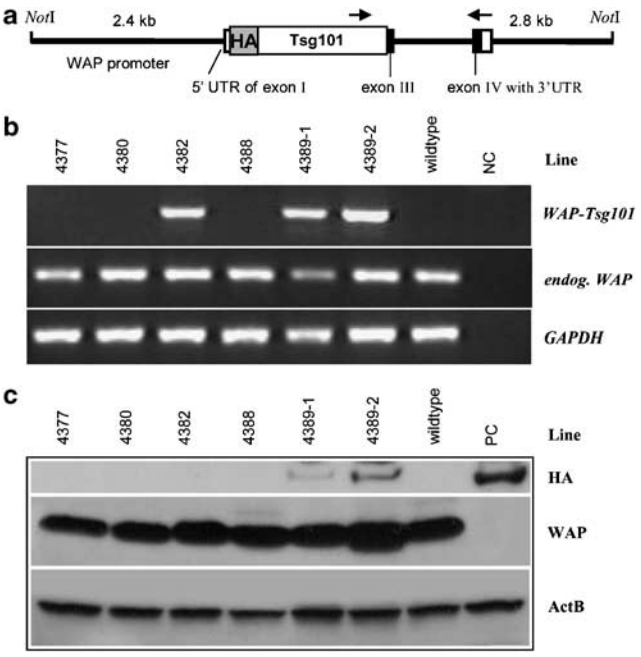

Figure 2 Generation of transgenic lines expressing Tsg101 under the *whey acidic protein* gene promoter (WAP-Tsg101). (**a**) Structure of the WAP-Tsg101 transgene. (**b** and **c**) Verification of transgene expression using RT–PCR and Western blot analyses. RNA and protein extracts were isolated from mammary gland biopsies of lactating females of each transgenic founder line and a non-transgenic (wild type) control female. The location of the WAP-Tsg101-specific RT–PCR primers are illustrated as arrows in (**a**). Expression of GAPDH and beta-actin (ActB) were used as loading controls. Endogenous *Wap* gene expression was used to verify proper development of the mammary glands.

The overexpression of Tsg101 in mammary epithelial cells does not cause phenotypic abnormalities during mammogenesis

Previously, we observed that the overexpression of Tsg101 appeared to be highly toxic to proliferating mammary epithelial cells in culture, and an HC11 cell line that stably overexpresses Tsg101 could not be established (Wagner, Dierisseau and Hennighausen unpublished). Similar observations were made by other investigators (Zhong *et al*., 1998). Using a Northern blot assay on normal mouse tissues, we found that endogenous *Tsg101* mRNA was abundant throughout mammary gland development, and the amount of *Tsg101* transcripts was only marginally increased during late pregnancy and lactation (Wagner *et al*., 1998). Based on our previous findings, we anticipated that the forced overexpression of exogenous Tsg101 under the *Wap* gene promoter would lead to a lactation defect in postpartum dams owing to increased cell death. Surprisingly, lactating transgenic females were able to nurse and support their litters comparable with wild-type control dams. In addition, the comparative analysis of mammary gland whole mounts at pregnancy day 16 and lactation day 1 and 4 (i.e. the interval of maximal

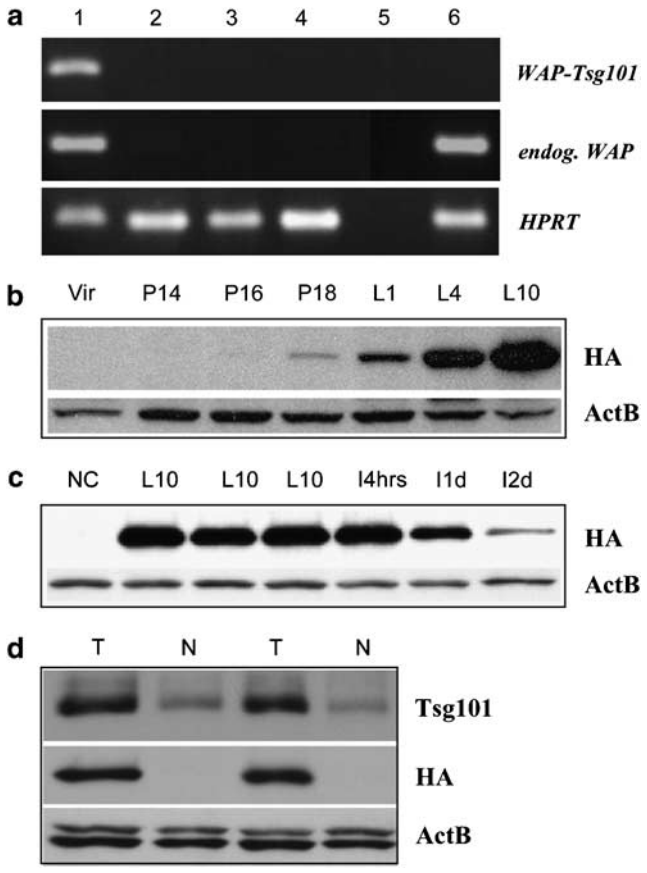

Figure 3 Temporal and spatial regulation of WAP-Tsg101 transgene expression. (**a**) RT–PCR to determine the tissue specificity of the WAP-Tsg101 construct (1, lactating mammary gland; 2, liver; 3, salivary gland; 4, brain; 5, no RT control; 6, lactating mammary gland of a wild type, i.e. non-transgenic, female). RNA was extracted from tissues of female mice at day 9 of lactation. Expression of the *HPRT* gene severed as loading control. (**b** and **c**) Expression profile of exogenous Tsg101 protein in the developing mammary gland of virgin (Vir), late pregnant (P, days 14–18), and lactating (L, days 1, 4 and 10) females and during postlactational involution (I, 4 h, days 1 and 2). Beta-actin (ActB) was used as a loading control. (**d**) Comparison of total Tsg101 levels between two WAP-Tsg101 transgenic females (T) and their wild-type controls (N) at day 10 of lactation with suckling pups (lanes 1 and 2) and pups removed for 4 h (lanes 3 and 4).

upregulation of Tsg101) did not reveal any abnormalities on the morphological level (Supplementary Figure S1). As HA-tagged Tsg101 was highly expressed throughout lactation in these females (Figure 3b and c), we can exclude the possibility that Tsg101-overexpressing cells were negatively selected. In conclusion, unlike in cultured HC11 cells, the forced overexpression of the HA-tagged Tsg101 protein did not affect the survival of mammary epithelial cells *in vivo*.

Expression of exogenous, HA-tagged Tsg101 promotes the survival of mammary epithelial cells lacking the endogenous Tsg101 gene

We previously demonstrated that the conditional deletion of *Tsg101* from cultured fibroblasts resulted in growth arrest and cell death (Krempler *et al*., 2002). Fibroblasts lacking both endogenous *Tsg101* alleles resumed normal proliferation when infected with a retroviral vector expressing the murine *Tsg101* cDNA. This study verified that exogenous Tsg101 carrying an N-terminal HA-tag functions equivalent to the wild-type protein in cultured fibroblasts. Unlike in HC11 cells, the overexpression of Tsg101 in epithelial cells of the developing mammary gland did not cause any adverse effects (see previous paragraph). Therefore, we needed to confirm that the HA-tagged protein, which has been shown to be functional *in vitro*, is also working properly (a) *in vivo* and (b) in mammary epithelial cells that, unlike fibroblasts, are of ectodermal origin. This can be demonstrated decisively by rescuing the loss of Tsg101 function in the developing mammary gland of Tsg101 conditional knockout females that also carry the WAP-Tsg101 transgene. We previously demonstrated that the WAP-Cre-mediated conditional knockout of Tsg101 resulted in a variable degree of phenotypic abnormalities in postpartum mammary glands. This phenomenon is based on a mosaic expression pattern of the WAP-Cre transgene. In animals with incomplete recombination, dying Tsg101-deficient alveolar cells could be quickly replaced by their adjacent alveolar precursors that did not express Cre recombinase (Wagner *et al*., 2003). Therefore, a subset of Tsg101 conditional knockout mice was able to lactate, and the recombined *Tsg101* knockout allele was virtually absent in the mammary gland of these animals. Hence, the idea behind the rescue of the mammary gland-specific Tsg101 conditional knockout through expression of exogenous Tsg101 was to test whether the presence of the WAP-Tsg101 transgene was able to alleviate the negative selection pressure against the *Tsg101* knockout allele. As shown in Figure 4a, the Tsg101 knockout allele was barely detectable by PCR in the mammary gland of lactating dams carrying two *Tsg101* floxed alleles and only the WAP-Cre transgene (Figure 4a, lane 2). On the other hand, the expression of exogenous Tsg101 in conditional knockout females (*WAP-Tsg101 WAP-Cre* $Tsg101^{fl/fl}$) was able to rescue the survival of mammary epithelial cells carrying the recombined *Tsg101* knockout allele (Figure 4a, lanes 3 and 4). Although terminal ducts and alveolar structures within the mammary gland of conditional mutants exhibited the previously reported mosaic phenotype at day 10 of lactation (Figure 4b, left panel), the overexpression of exogenous Tsg101 in the Tsg101-deficient background greatly supported the expansion of secretory alveolar lobules, and a mosaic phenotype was not observed (Figure 4b, right panel). The results of this genetic study clearly demonstrate that the HA-tagged, exogenous Tsg101 protein expressed from the *Wap* gene promoter is fully functional in mammary epithelial cells *in vivo*.

Tsg101-overexpression results in increased signaling though MAP kinases

As a component of the endosomal sorting complex required for transport (ESCRT-1), Tsg101 recognizes ubiquitinated target proteins, such as internalized cell surface receptors, and targets them for lysosomal

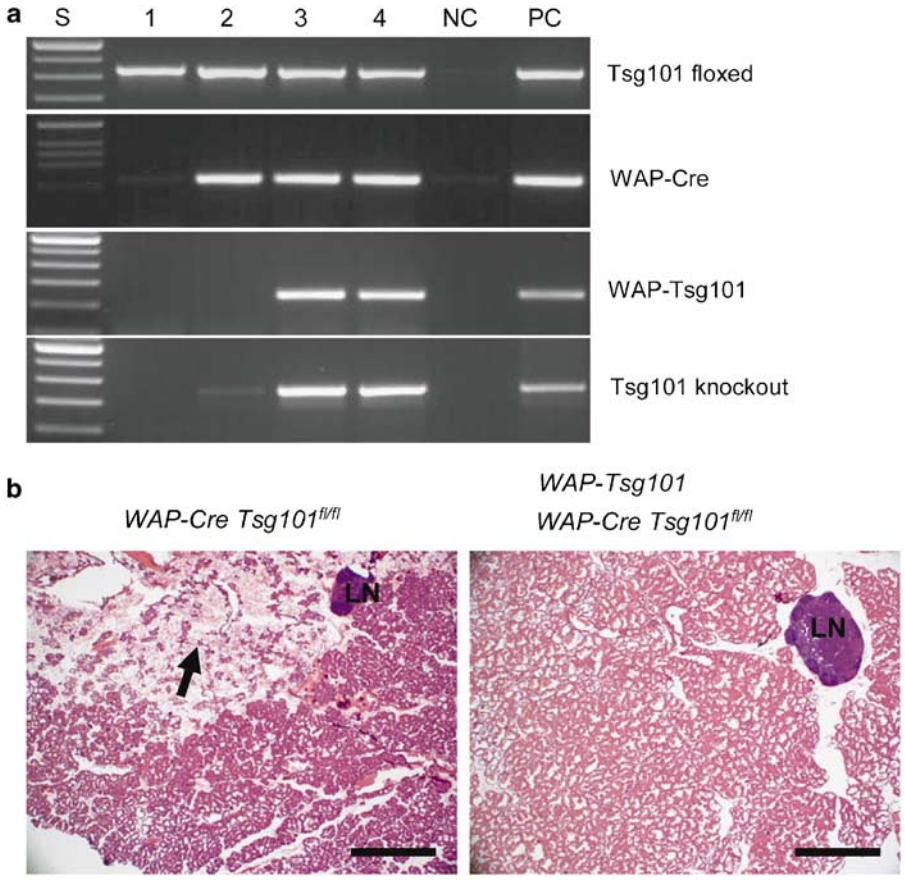

Figure 4 Expression of exogenous Tsg101 abolishes the negative selection pressure of mammary epithelial cells (MECs) conditionally deficient in endogenous Tsg101. (**a**) PCR assay to monitor the survival of MECs carrying *Tsg101* knockout alleles in mammary glands of Tsg101 conditional knockout mice (*WAP-Cre Tsg101$^{fl/fl}$*) in the presence (lanes 3 and 4) or absence (lane 2) of an additional WAP-Tsg101 transgene. Genomic DNA was isolated at day 10 of lactation when the negative selection of Tsg101-deficient cells was almost complete in the conditional knockouts (lane 2). In contrast, the presence of the WAP-Tsg101 transgene in the conditional knockout females rescues the survival of MECs lacking endogenous *Tsg101* (lanes 3 and 4). A lactating *Tsg101$^{fl/fl}$* female without the WAP-Cre transgene served as a negative control (lane 1). NC, no DNA control; PC positive controls for the specific PCR reactions of the various alleles. (**b**) H&E-stained sections of mammary glands from a Tsg101 conditional knockout mouse (left panel) and the genetic rescue of Tsg101 deficiency (right panel) at day 10 of lactation (bar = 1 mm). Note the heterogeneous phenotype in the conditional knockout mouse, which is caused by a mosaic expression pattern of the WAP-Cre transgene and the negative selection of Tsg101-deficient MECs (left panel, arrow). Such severely underdeveloped regions of the gland were not observed in genetically rescued animals (right panel). LN, lymph node.

degradation (Katzmann *et al*., 2001). To determine whether the overexpression of Tsg101 perturbs signaling through major hormonal signaling pathways, we analysed the activation Stat5, Stat3 and MAP kinases during key stages of mammary gland development (Figure 5). These selected signal transducers are all important for mammary gland development and they are activated by multiple hormone and cytokine receptors at defined stages of mammogenesis (Hennighausen and Robinson, 2001). The initial analysis of these three signal transducers revealed that overexpression of Tsg101 resulted in an increase in the phosphorylation of Erk1/2 and Stat3, whereas Stat5 did not exhibit consistent differences in its activation between transgenics and wild-type controls throughout the gestation cycle. Surprisingly, Stat3 was activated in the WAP-Tsg101 transgenics during late pregnancy and early lactation (i.e. developmental stages that normally exhibit very little to moderate expression of phospho-Stat3) (Figure 5a). This increase in Stat3 activation correlates with the upregulated expression of Tsg101 during late pregnancy (Figure 3). The level of phospho-Stat3 remained higher in the WAP-Tsg101 transgenics during early stages of the postlactational involution period (Figure 5b and Supplementary Figure S2), in which Stat3 becomes highly activated and functions as an inducer of programmed cell death of secretory epithelial cells (Chapman *et al*., 1999). The elevated activity of Stat3, however, appeared not to result in accelerated remodeling of the involuting gland within 2 days after weaning the offspring (data not shown).

Functional differentiation of mammary epithelial cells is normally associated with reduced activation of downstream signals, such as MAP kinases, that mediate a proliferative response (Cerrito *et al*., 2004). Concurrent with this notion, phosphorylated Erk was expressed during pregnancy but barely detectable at day 4 and day 10 of lactation in the mammary gland of wild-type mice

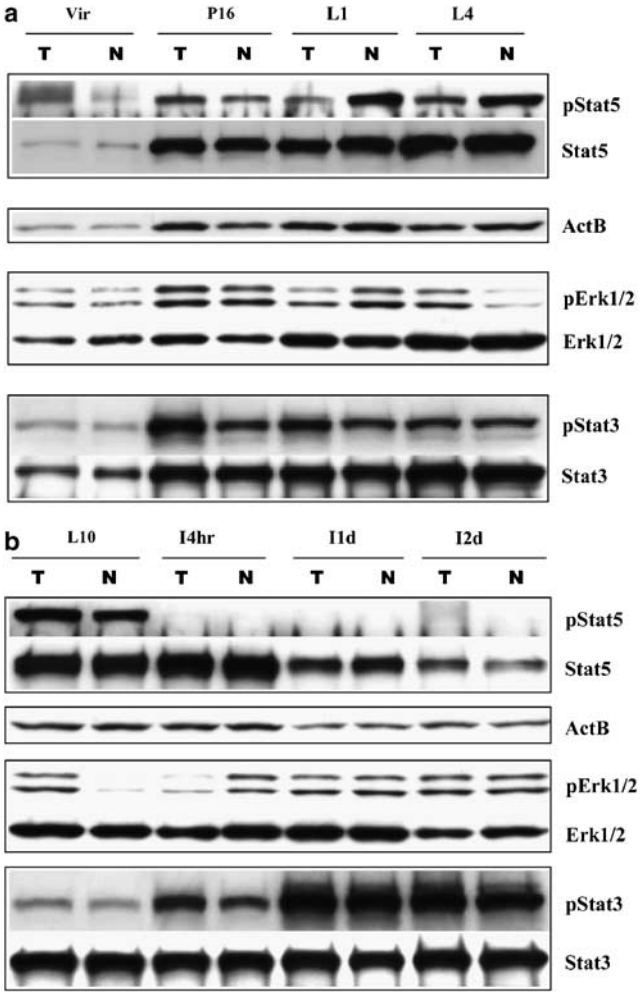

Figure 5 Expression analysis of major signal transducers of growth factor signaling pathways in the developing mammary gland of WAP-Tsg101 transgenic (T) females and their non-transgenic (N), wild-type controls. (**a**) Western blot analysis of activated Stat5, Stat3 and Erk1/2 in virgin (Vir) females and during mid-gestation (P16, pregnancy day16) as well as the 1st and 4th day of lactation (L1 and L4). (**b**) Analysis of phosphorylated Stats and MAP kinases at day 10 of lactation (L10) and during postlactational involution (I, 4 h, days 1 and 2).

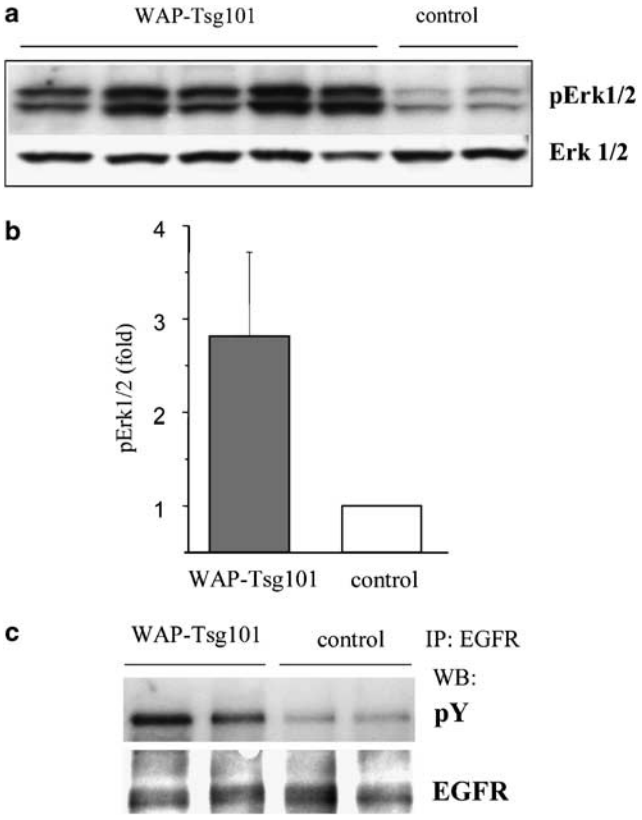

Figure 6 Tsg101 overexpression in the mammary gland of transgenic mice leads to abnormal activation of MAP kinases and increased phosphorylation of the epidermal growth factor receptor (EGFR) at day 10 of lactation. (**a**) Western blot analysis of activated Erk1/2 in the mammary gland of WAP-Tsg101 transgenic females and their non-transgenic, wild-type controls. Pan-Erk1/2 served as loading control. (**b**) Relative upregulation of active MAP kinases (fold change) in the mammary gland of Tsg101 overexpressing mice in comparison to their wild-type controls. The level of phosphorylated Erk1/2 was normalized against the total amount of Erk1/2 in individual mice shown in (**a**), and the normalized values of the controls were set as 1 (right bar). The left bar illustrates the mean fold change in the relative activation of Erk1/2 in the WAP-Tsg101 transgenics including the standard variation. (**c**) Immunoprecipitation and Western blot analysis to determine the level of tyrosine phosphorylation (pY) of the EGFR in mammary gland extracts of WAP-Tsg101 transgenic females and their wild-type controls.

(Figure 5a and b). With the exception of lactation day 1, Tsg101-overexpressing mice exhibited unusually high levels of active MAP kinases during pregnancy and lactation. The high-level expression of activated Erk1/2 in postpartum WAP-Tsg101 females was verified by analysing several mammary glands of transgenics and wild-type controls at day 10 of lactation (Figure 6a) Compared with non-transgenic females, the increase in the level of Erk phosphorylation was nearly threefold (Figure 6b). Previous studies suggested that Tsg101 is involved in the regulation of the trafficking of the activated epidermal growth factor receptor (EGFR) (Babst *et al*., 2000). Two other well-established facts support the idea that the EGFR might be a likely candidate responsible for the irregular expression of phosphorylated Erk in the transgenic mice: (a) the ligand-activated EGFR signals through MAP kinases and (b) lowering EGF-mediated signaling is a prerequisite for functional differentiation of mammary epithelial cells (Merlo *et al*., 1996). Indeed, using immunoprecipitation/Western blot analysis, we can demonstrate that the EGFR exhibits a sustained high level of tyrosine phosphorylation in the lactating mammary gland of WAP-Tsg101 transgenic females but not the wild-type controls (Figure 6c). This observation is unexpected as Tsg101 is a suggested mediator for the sorting and lysosomal degradation of the activated EGFR (Babst *et al*., 2000). When overexpressed, Tsg101 might actually function very similar to Hrs (a Tsg101-binding partner). The overexpression of Hrs inhibits the down-regulation of the activated EGFR by disturbing receptor trafficking from early to late endosomes (Chin *et al*., 2001; Raiborg *et al*., 2001). In conclusion, exogenous

Tsg101 might perturb the correct assembly and function of the endosomal sorting complex, which, in turn, might lead to a sustained activation of the EGFR and MAP kinases, which, after a long latency, might contribute to neoplastic transformation of mammary epithelial cells and tumor progression.

Tsg101-overexpressing mice develop mammary gland abnormalities and mammary cancer after a long latency

To investigate the possible involvement of Tsg101 in mammary tumorigenesis, we maintained a cohort of 40 WAP-Tsg101 transgenic females and 21 wild-type controls over a period of 12–24 months that were monitored weekly for mammary tumor formation. Animals were maintained as parous mice to (a) repeatedly induce transgene expression and (b) increase the overall proliferation rate of mammary epithelial cells in response to higher estrogen and progesterone levels during pregnancy. Three WAP-Tsg101 transgenic females developed palpable mammary cancers before 18 months of age, and four females developed solid lesions between 18 and 21 months of age. Of the latter four, one female had three mammary tumors in her thoracic mammary glands. The tumor latency did not seem to depend on the number of pregnancies. The necropsy and analysis of mammary gland whole mounts of 19 tumor-free animals at the study endpoint (20–24 months of age) revealed that more than half of all WAP-Tsg101 transgenic females displayed mammary gland abnormalities (Figure 7). Inflammatory nodules with squamous metaplasia and ectatic ducts were observed in almost all abnormal glands (Figure 7a and b). Atypical hyperplasia (Figure 7c) and squamous metaplasia (Figure 7d) were common premalignant lesions present in a number of glands. The palpable mammary cancers were identified as typical adenocarcinomas (Figure 7d) and one scirrhous carcinoma (Figure 7f). The transplantation of fragments from both

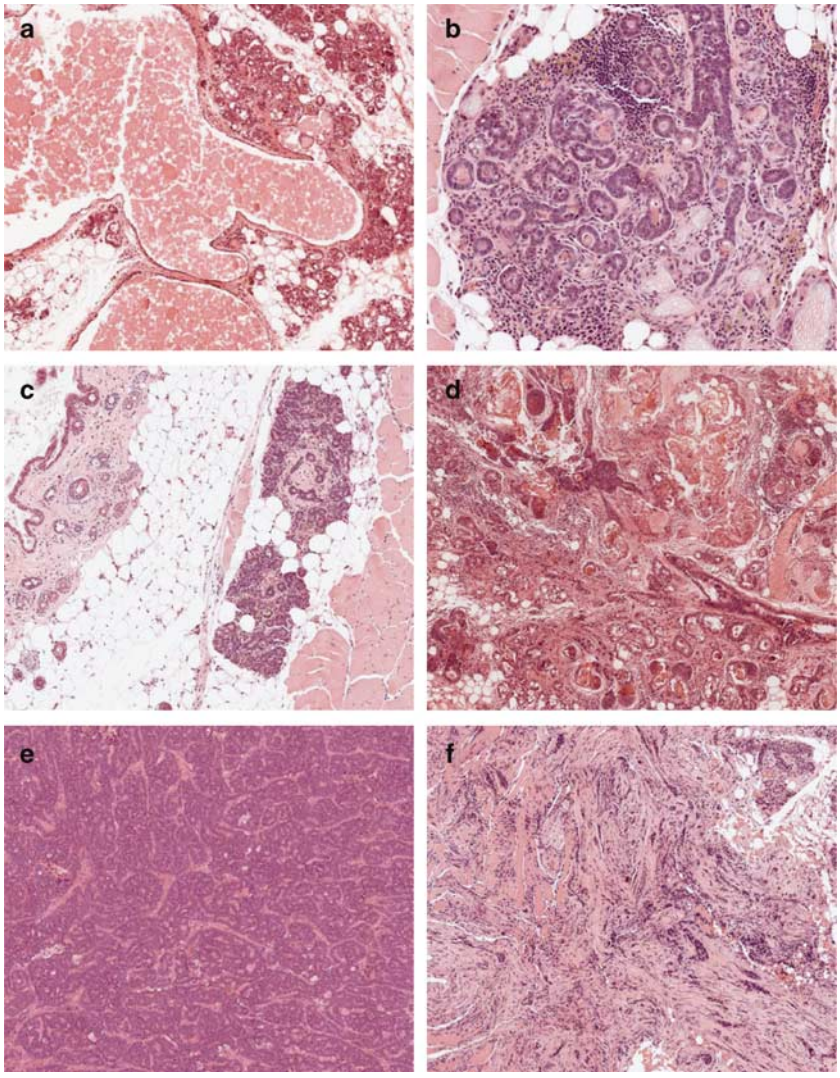

Figure 7 Types of mammary lesions from WAP-Tsg101 females. (**a**) ectatic ducts; (**b**) inflammatory nodule, squamous metaplasia; (**c**) atypical hyperplasia; (**d**) adenocarcinoma with squamous metaplasia; (**e**) adenocarcinoma and (**f**) scirrhous carcinoma (magnification $\times 50$).

types of solid tumors into athymic nude recipient females resulted in the formation of secondary cancers, suggesting that these were true cancerous lesions in the transgenic females. Among the wild-type controls, one female developed lymphoma after 10 months, and only one female developed a palpable mammary tumor at 20 months of age. Ectadic ducts and small hyperplastic nodules were also present in the mammary glands of a few wild-type females that were analysed at the study end point. It has been reported that approximately 15% of FvB mice develop pituitary abnormalities by the age of 2 years (Mahler *et al*., 1996), which can induce secondary effects on mammary gland differentiation in selected colonies of FvB substrains (Wakefield *et al*., 2003) owing to an increase in the secretion of lactogenic hormones. Therefore, it was not surprising to notice that mammary glands of a few wild-type control mice exhibited a morphology, which resembled that of a mid-pregnant female (data not shown).

In summary, the overexpression of Tsg101 seemed to increase the susceptibility of mammary epithelia toward malignant transformation. However, owing to the long latency of tumor formation and the rare occurrence of bona fide mammary cancers, we conclude that the wild-type Tsg101 protein has only weak oncogenic properties for cancer initiation. In this regard, the phenotype of the transgenic model corresponds to our findings in human breast cancers. As Tsg101 was mostly upregulated in a number of advanced stages of human breast cancer, it might be more likely that Tsg101 plays a more predominant role in the progression of spontaneously arising neoplasia in the mammary gland.

Materials and methods

Generation of WAP-Tsg101 transgenic mice and genotyping protocol

The cloning of a Tsg101 expression vector carrying an N-terminal HA-tag was described previously (Krempler *et al*., 2002). The HA-tagged Tsg101 cDNA was excised from the pJ3H vector using *Sal*I and *Eco*RI, blunted and cloned into the blunted *Asp*718 and *Sal*I sites of the WAP-based mammary-specific expression vector (Burdon *et al*., 1991), thereby replacing the coding region of exons 1–3 of *WAP*. The entire transgene (approximately 6.6 kb in size) was excised from the vector backbone and microinjected into pronuclei of zygotes from FvB/N mice using standard protocols. Transgenic founders were identified by PCR using primers 1826 and 1827 (5′-GGA GTC ATA GAC CTG GAT GTG TT-3′ and 5′-TTG ATC TGA TGC AGG GTA AGG TG-3′) that amplify a 240 bp fragment of the junction between the 3′ end of the *Tsg101* cDNA and the third intron of the *WAP* gene. Established lines of WAP-Tsg101 founders were maintained in an FvB/N background. All animals used in the described studies were treated humanely and in accordance with institutional guidelines and federal regulations

RT–PCR methodology and primer sequences

Total RNA was isolated from cell pellets using standard guanidinium thiocyanate-phenol-chloroform extraction. A SuperScript II kit from Invitrogen (Carlsbad, CA, USA) with oligo dT primers was used to perform the first strand synthesis. PCR amplification of the *WAP-Tsg101* and the endogenous *WAP* transcript was conducted using the following primer pairs: *WAP-Tsg101* transgene (5′-ATG CAG AGG AAA ATG CTA TTG AA-3′ and 5′-CTT GCT GTA TAG ACT TGG GTG GT-3′, these primers amplify about 250 bp fragment from *Tsg101* cDNA and 3′ untranslated region of exon IV of *WAP*), second endogenous *WAP* gene (5′-TGG AAT CTA CTC CAA ACG ATC AG-3′ and 5′-CTT GCT GTA TAG ACT TGG GTG GT-3′; both primers amplify the *WAP* cDNA of exons 3 and 4).

Immunoprecipitation and Western blot analysis

The preparation of whole cell extracts of clarified tissue lysates and the experimental procedures for immunoprecipitation and Western blot analysis were described previously (Wagner *et al*., 2003). The following antibodies were used: α-ActB (I-19) (1:2000 dilution), α-HA (Y-11, 1:2000 dilution) from Santa Cruz Biotechnology (Santa Cruz, CA, USA); α-phosphotyrosine antibody (4G10; 1 μg/ml) from Upstate Inc. (Millipore, Billerica, MA, USA); α-phospho-Erk1/2 (9101, 1:1000 dilution), α-Stat3 (124H6; 1:1000 dilution) and α-phospho-Stat3 (58E12; 1:1000 dilution) from Cell Signaling Technology (Boston, MA, USA); α-EGFR (ab2430, 1:1000 dilution) from Abcam (Cambridge, MA, USA); α-panStat5 (89, 1:2000 dilution) and α-panErk (16, 1:2000 dilution) antibody from BD Biosciences (Billerica, MA, USA). The α-phospho-Stat5a/b (Y694/9) antibody (AX1; 0.5 μg/ml; Advantex Bioreagents, EL Paso, TX, USA) was kindly provided by Dr Hallgeir Rui (Thomas Jefferson University). The quantification of activated Erk1/2 and panErk1/2 was performed using the Luminescence Image Analyzer LAS-1000plus (FudjiFilm Life Science, Stamford, CT, USA).

Whole mount staining of mammary glands and histological analysis of mammary tumors

Protocols for the preparation of mammary gland whole mounts and hematoxylin and eosin (H&E)-stained sections of formalin-fixed tissues were described previously (Wagner *et al*., 2003). Entire H&E-stained sections of representative mammary gland lesions were digitized at high resolution using a whole slide image scanner. Composite images were analysed and annotated by Dr Bob Cardiff (UC Davis) using internet-based virtual microscopy software by MicroBrightField Inc (Williston, VT, USA).

Immunohistochemical staining of human breast cancer specimen

Sections of human breast specimens were obtained from archival samples of paraffin-embedded tissues (UNMC, Department of Pathology) from 16 normal and benign cases as well as 16 invasive human breast cancers. We used Vectastain Elite ABC kits (Vector, Burlingame, CA, USA) with 3,3′ diaminobenzidine (DAB) as a chromogen for the immunohistochemical detection of Tsg101. All slides were counterstained with Mayer's hematoxylin. A minimum of two slides per specimen was stained with Tsg101 antibodies, and serial sections present on the same slides were used as a negative controls without primary antibody. Initially, we tested four different antibodies against the human TSG101 protein, that is two monoclonal antibodies from Santa Cruz Biotechnology (clone C-2) and Novus Biologicals Inc. (clone 4A10), and two rabbit polyclonal antisera from Abgent Inc. (AP2155a and AP2155b). Among these four antisera, the monoclonal antibody from Novus Biologicals Inc. gave the best results at a concentration of 1:100. Individual samples

were scored for Tsg101 immunoreactivity levels on a scale from 0 to 3, where 0 indicates no significant Tsg101 staining within cancer cells throughout the section and 1–3 represented detectable staining at three steps of increasing staining intensity and proportion of stained tumor cells (low, intermediate and high). The intensity of Tsg101 was scored '1' when cancer cells exhibited Tsg101 immunoreactivity equivalent to that of normal breast tissue. A score of '2' was given when less than half of all cancer cells within a specimen exhibited elevated Tsg101 staining. An intensity value of '3' represents high immunostaining against Tsg101 in the vast majority of cancer cells within the entire section. Digital bright-field images of histological slides were taken on a Zeiss Axio Imager microscope equipped with a SPOT FLEX camera.

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