Transcription Factor AP-2γ Stimulates Proliferation and Apoptosis and Impairs Differentiation in a Transgenic Model

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Abstract

AP-2 transcription factors play pivotal roles in orchestrating embryonic development by influencing the differentiation, proliferation, and survival of cells. Furthermore, AP-2 transcription factors have been implicated in carcinogenesis, a process where the normal growth and differentiation program of cells is disturbed. To experimentally address the potential involvement of AP-2 in mammary gland tumorigenesis, we generated mice overexpressing AP-2γ by transgenesis using the mouse mammary tumor virus-long terminal repeat as the transgene-driving promoter unit. In the mammary gland, transgene expression elicited a hyperproliferation that, however, was counterbalanced by the enhanced apoptosis of epithelial cells leading to a hypoplasia of the alveolar epithelium during late pregnancy. In addition, secretory differentiation was impaired, resulting in a lactation failure. In male transgenic mice, the seminal vesicles were sites of strong transgene expression. There the effects of AP-2γ on proliferation and apoptosis were even more pronounced, and differentiation was impaired, too, as revealed by the absence of androgen receptor immunoreactivity. In both tissues, the mammary gland and the seminal vesicles, enhanced steady-state transcript levels of the AP-2 target gene *IGFBP-5* were detected, revealing a potential mechanism of AP-2-induced apoptosis. Our results suggest a role of AP-2 transcription factors in the maintenance of a proliferative and undifferentiated state of cells, characteristics not only important during embryonic development but also in tumorigenesis.

Introduction

The AP-2 family of transcription factors consists of four homologous proteins of M_r 50,000, AP-2α, AP-2β, AP-2γ, and AP-2δ (1), encoded by different genes (reviewed in Ref. 2). These transcription factors homo- or heterodimerize via helix-span-helix motifs and transactivate their target genes by binding to GC-rich consensus sequences in the respective promoter regions (3, 4). AP-2 proteins have been implicated in the regulation of cell type-specific processes based on their expression in neural, neural crest, kidney, and epithelial cells during development. The importance of *AP-2* genes is highlighted by the embryonic lethal phenotypes of mice lacking AP-2α, β, or γ (5–8). Results from a screen for genes regulated by AP-2α indicate that AP-2 might play a central role at the checkpoint proliferation/differentiation by repressing differentiation-specific markers and inducing proliferation-specific genes during development (9). While support of proliferation during development is desirable for gestational processes, activation of proliferation and unwanted suppression of differentiation-specific genes in the adult organism may lead to hyperplastic growth and cancer. The expression of AP-2 proteins has been associated with neoplasia of the breast, a process where the normal growth and differentiation program of epithelial cells is disturbed (10). It has therefore been suggested that *AP-2* genes might be causally involved in the tumorigenesis of the breast. This notion is supported by the fact that the genomic locus 20q13.2, where the *AP-2γ* gene maps, is frequently amplified in breast cancer cell lines and breast carcinoma (11). In addition, several studies have linked AP-2γ expression with the expression of the receptor tyrosine kinase ErbB-2 (encoded by the HER-2/c-*neu* proto-oncogene), representing a clinical marker for a poor prognosis in breast carcinoma (12). In mammary tumor cell lines, a high expression of AP-2α and AP-2γ correlated with the expression of the *erbB-2* gene; moreover, the *erbB-2* promoter was shown to contain an AP-2 binding site and could be transactivated by AP-2 proteins in cell transfection studies (13). Furthermore, AP-2 can abrogate the estrogen-mediated repression of the *erbB-2* gene by binding to the critical estrogen response element in the promoter region (14). Immunochemical analyses revealed a correlation between AP-2α and AP-2γ expression and ErbB-2 on breast tumor specimens, whereas AP-2α alone was associated with the estrogen receptor (ER; 15), the gene of which had previously been shown to be controlled by AP-2 (16). In a different study, however, AP-2α or β correlated inversely with ErbB-2 in invasive breast cancer, and it was suggested that disease progression might involve the loss of AP-2 (17).

In this study, we established a gain-of-function model, addressing the consequences of forced AP-2γ expression in the

mammary gland. In female mice, transgene expression interfered with the proper secretory differentiation of the mammary gland epithelium during late pregnancy resulting in a lactation failure. Epithelial cells displayed an increase in proliferation rate, which was overcompensated by the concomitantly enhanced apoptosis. In addition, milk fat was not being produced and the expression of milk protein genes was delayed. In male mice, transgene expression was detected in the seminal vesicle epithelium. Here, too, enhanced proliferation and apoptosis were detected and differentiation of epithelial cells was impaired. The results presented show that AP-2γ influences proliferation, apoptosis, and differentiation of epithelial cells. Thus, AP-2 genes might contribute to tumor development by the stimulation of proliferation and dedifferentiation once apoptotic mechanisms are overcome.

## Results

Construction of Transgenic Mice

To experimentally address the potential involvement of AP-2γ in the genesis of mammary cancer, we chose a transgenic approach. Mammary gland-specific overexpression of AP-2γ was achieved by placing the murine AP-2γ cDNA under the control of the long terminal repeat (LTR) of the mouse mammary tumor virus (MMTV; Fig. 1A). We established two independent transgenic mouse lines denominated LTRγA and LTRγB. The lines were registered with mouse genome nomenclature database and the strain name Tg(Tcfap2c)Hsc was assigned. The lines were backcrossed to C57Bl/6 mice.

Expression of AP-2γ Leads to Lactation Deficiency

Neither pregnancy rates nor litter sizes were affected in female transgenic mice. However, during postpartum, it became evident that the transgenic females suffered from a lactation problem. The pups failed to thrive and had no milk in their stomachs compared to offspring from wild-type dams. Because all offspring irrespective of whether they were control or transgenic failed to thrive, we could rule out a developmental defect of the transgenic pups themselves. Fostering of the offspring to control dams resulted in normal postpartum development and was used as a routine method to keep the transgenic lines. Transgene expression in mammary glands at parturition was verified both at the RNA and at the protein level (Fig. 1, B and C). At this time point, endogenous AP-2γ is only weakly expressed. Expression of the milk-protein gene *β-casein* was unaltered in the transgenic mammary glands. Because these results were obtained using two independently derived lines of mice, the lactation failure is unlikely to be the consequence of an integration site effect of the transgene but rather results from transgene expression.

Secretory Differentiation Is Impaired in Transgenic Mice

Using histological sections, we analyzed the pathological changes underlying the observed lactation failure (Fig. 2, A–D). Histological examination of mammary glands at day 18.5 of pregnancy revealed that the area covered by alveolar epithelium was smaller in transgenic mice. Accordingly, adipose tissue was still much more prominent than in wild-type mammary glands. The individual alveoli appeared condensed and smaller in
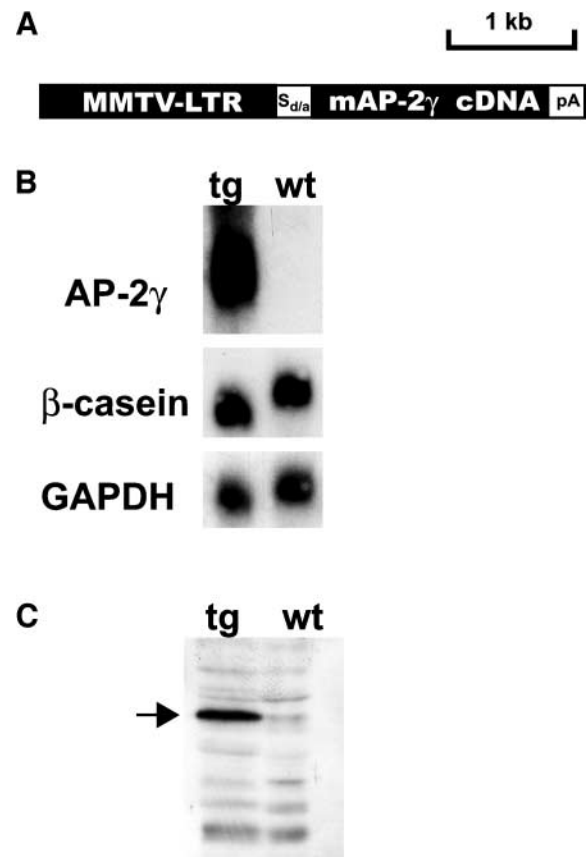
FIGURE 1.   **A.** Transgenic construct: MMTV promoter driving murine AP-2γ cDNA, which was placed between splice donor/acceptor ($S_{d/a}$) sequence and a polyadenylation signal (*pA*) from SV40 virus. **B.** Northern blot of 15 μg total RNA of wild-type (*wt*) or transgenic (*tg*) mammary glands at parturition. Membrane was hybridized sequentially to probes specific for AP-2γ and β-casein. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) served as a control for equal RNA amounts. **C.** Western blot of 50 μg of protein derived from wild-type (*wt*) or transgenic (*tg*) mammary glands at parturition. *Arrow* shows AP-2 protein (M_r 50,000).

volume compared to the wild-type situation (Fig. 2, A and B), suggesting that the overall amount of milk produced by the transgenic mice was reduced. In addition, droplets indicative for milk fat production were absent in the transgenic mammary epithelial cells and also in the alveolar lumina. This was analyzed in more detail using electron micrographs. As shown in Fig. 2 (C and D), alveolar cells and lumina were devoid of milk fat globules in the transgenic mammary glands. By contrast, casein-micelles represented by the electron-dense granules inside the alveolar lumina were present both in wild-type and in transgenic mammary glands, which is consistent with the only slightly altered expression levels of the *β-casein* gene seen on Northern blots (Fig. 1C and 3A).

Taken together, these findings show that in addition to being hypoplastic, the transgenic mammary epithelium suffers from a very specific secretory defect involving the synthesis of milk fat.

Lactation Deficiency Is a Cell Autonomous Defect Which Cannot Be Overcome by Lactogenic Hormones

To verify that the observed phenotype was intrinsic to the mammary epithelium and not due to hormonal deregulation in

the transgenic mice, mammary gland organ cultures from day 13.5 of pregnancy were prepared and exposed to lactogenic hormones *in vitro*. At this time point, transgenic mammary glands are histologically indistinguishable from wild types, and transgenic AP-2γ protein is not detectable on Western blots (see below and data not shown). Under these conditions, wild-type cultures differentiated nicely, whereas the transgenic cultures reflected the hypoplastic phenotype observed *in vivo* (Fig. 2, E and F, compare to C and D). This result indicates that overexpression of AP-2γ leads to a compromised responsiveness of the mammary epithelium to lactogenic stimuli.

Mammary Gland Development Is Altered in Transgenic Mice

Impairment of lactation is most likely the consequence of an impaired mammary gland development during pregnancy, which in the mouse takes 3 weeks. In virgin mice, the mammary gland consists of a system of branching ducts residing in the mammary fat pad. During pregnancy, alveolar buds begin emanating from the ductal epithelium, which, during the third week of pregnancy, give rise to lobuloalveolar structures capable of milk production. To determine the approximate developmental stage when aberrant development began within the transgenic mammary gland, whole mount preparations of mammary tissue taken from transgenic *versus* wild-type mice at different time points of pregnancy were compared (Fig. 3, A–F). During the first 2 weeks of pregnancy, development of the mammary gland appeared to be unaltered. At day 14.5, both transgenic and wild-type mammary glands displayed a similar degree of ductal branching and formation of alveolar buds (Fig. 3, A and B). Variations up to this stage reflect individual differences between the mice rather than the presence of the transgene. From day 16.5 onwards, however, a consistent finding was an apparent delay in alveolar development in the transgenic mammary glands. In the wild-type mammary glands, lobuloalveoli expanded by day 16.5 and had begun to obscure the ducts, which were completely covered by day 18.5 (Fig. 3, C and E). By contrast, the transgenic alveoli remained small and condensed, and even by day 18.5, ducts
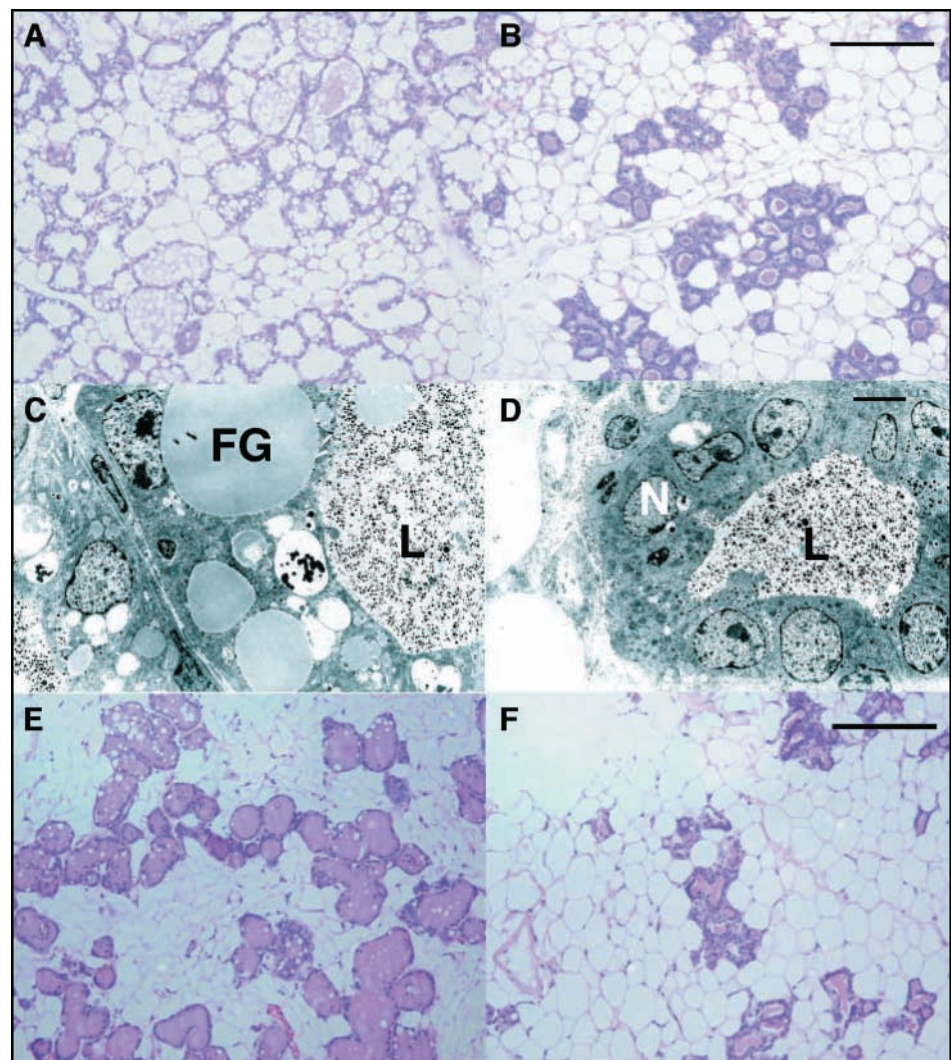
FIGURE 2. Mammary glands of wild-type (**A** and **C**) and transgenic (**B** and **D**) mice at day 18.5 of pregnancy (plug date defines day 0.5). **A** and **B.** H&E-stained paraffin sections. *Scale bar,* 100 μm. **C** and **D.** Ultrastructure of mammary epithelium revealed by transmission electron microscopy. *L,* lumen of the alveolus; *FG,* milk fat globule; *N,* nucleus. *Scale bar,* 5 μm. **E** and **F.** H&E-stained sections through organ cultures of transgenic (**E**) and wild-type (**F**) mammary glands after 8 days of culture. *Scale bar,* 100 μm.
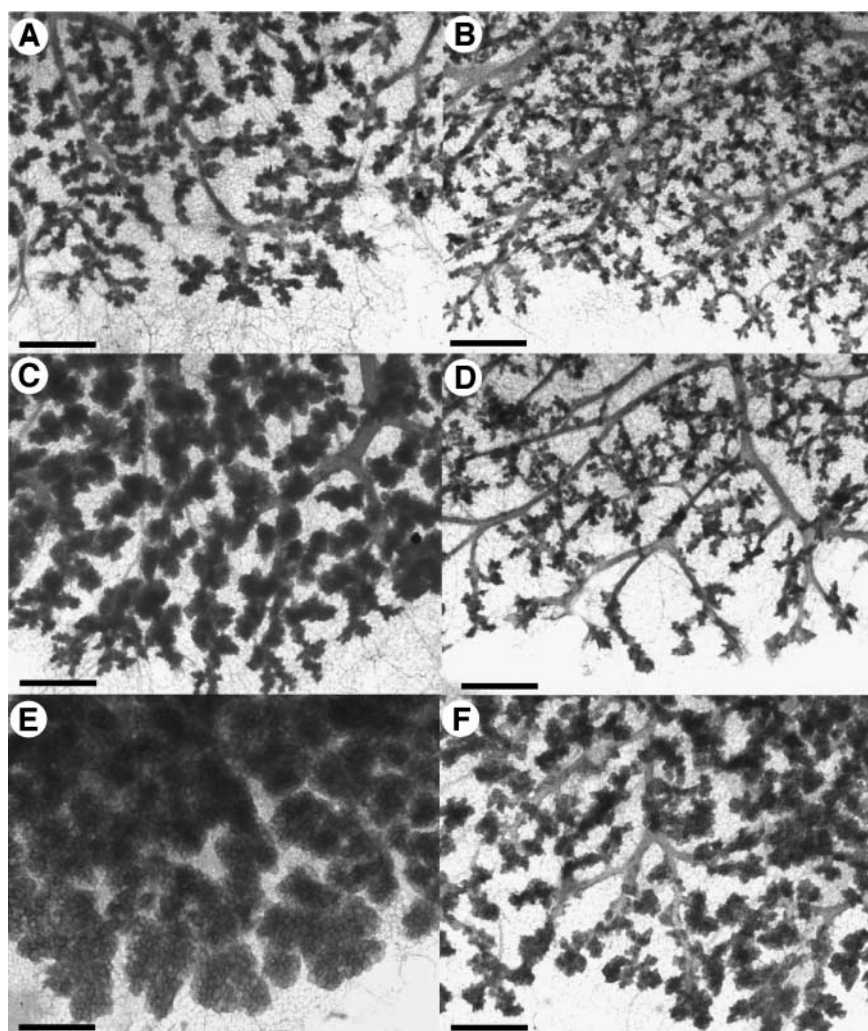
FIGURE 3. Whole mount preparations of mammary glands of wild-type (**A**, **C**, **E**) and transgenic (**B**, **D**, **F**) mice at day 14.5 (**A** and **B**), 16.5 (**C** and **D**), and 18.5 (**E** and **F**) of pregnancy (plug date defines day 0.5), stained with carmine red. *Scale bar,* 500 μm.

were easily visible (Fig. 3, D and F). Based on these findings, the major differences in development of the mammary epithelium take place by day 16.5 of pregnancy. This stage was therefore analyzed in more detail with respect to proliferation and apoptosis.

Transgenic Mammary Glands Display Hyperproliferation and Enhanced Apoptosis

Reasoning that a reduced cell proliferation might account for the impaired lobuloalveolar development, we first examined proliferation of epithelial cells. Much to our surprise, Ki-67 staining of tissue sections of mammary glands taken at day 16.5 of pregnancy revealed an increased percentage of proliferating cells in the transgenic mice (see Table 1). This unexpected finding was therefore verified with a different set of mice using BrdUrd labeling. Unlike Ki-67 staining, BrdUrd incorporation is strictly limited to cells having undergone DNA synthesis. Here, too, transgenic mammary glands displayed a significantly higher percentage of proliferating cells as compared to wild-type mammary glands (see Table 1 and Fig. 4A). Therefore, we next determined the percentage of cells undergoing cell death using TUNEL analysis. Tissue sections of mammary glands

taken at day 16.5 of pregnancy showed a more than 2-fold increase in the percentage of cells undergoing apoptosis in transgenic mice as compared to wild types (Fig. 4B and Table 1). This cell loss by the enhanced apoptosis in the transgenic mammary epithelium is apparently overcompensating the gain in cell number due to the enhanced proliferation, the net result being the hypoplasia observed. Hence, AP-2γ seems to stimulate both proliferation and apoptosis in the mammary gland.

Table 1. Comparison of Proliferation and Apoptosis in Mammary Epithelial Cells Between Transgenic and Wild-Type Mice at Day 16.5 of Pregnancy

Staining	Ki-67	BrdUrd	TUNEL
Transgenic	25.9 ± 4.6%	11.4 ± 1.9%*	2.0 ± 0.4%**
Wild type	17.2 ± 3.6%	6.8 ± 0.87%*	0.7 ± 0.2%**

Note: Numbers represent the average percentage of positive cells ± SD. TUNEL, terminal deoxynucleotidyl transferase (Tdt)-mediated dUTP nick end labeling.
*$P < 0.02$ (Student's t test).
**$P < 0.05$ (Student's t test).
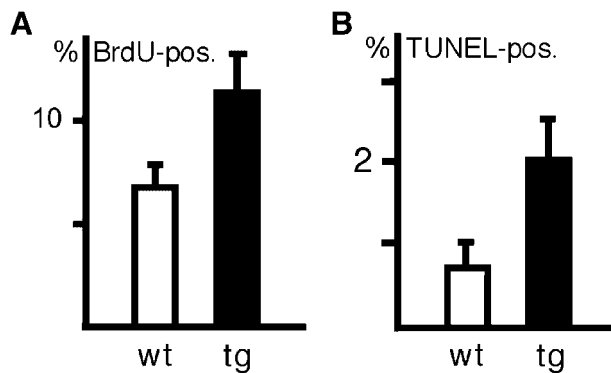
FIGURE 4. Proliferation (**A**) and apoptosis (**B**) in transgenic and wild-type mammary epithelium. **A.** Percentages of BrdUrd-positive nuclei of wild-type (□, *wt*) and transgenic (■, *tg*) mammary epithelium at day 16.5 of pregnancy. **B.** Percentages of TUNEL-stained nuclei of wild-type (□, *wt*) and transgenic (■, *tg*) mammary epithelium at day 16.5 of pregnancy. *Error bars,* SDs.

Forced Expression of AP-2γ Leads to Induction of Proliferation Markers and Repression of Differentiation Markers

It is known that transgenes driven by the MMTV-LTR are usually strongly induced from mid pregnancy to parturition (18). As seen in Fig. 5, the AP-2γ transgene is being expressed in a continuously increasing manner from day 14.5 until parturition. We also were able to detect a weak signal for AP-2 in control animals indicative for endogenous expression during day 14 to 18 with declining intensity. As a transcription factor, transgenic overexpression of AP-2γ is expected to influence cellular processes by the activation or repression of target genes at the transcriptional level. We therefore decided to analyze the expression levels of several genes possibly involved in the proliferation, apoptosis, and differentiation of the mammary epithelium during pregnancy using Northern blots (Fig. 5). As putative breast cancer-related AP-2 target genes, erbB-2 and ER expression were analyzed. ErbB-2 transcript amounts were increased in transgenic mammary glands starting from day 16.5 on, whereas in wild-type glands, the gene is down-regulated toward the end of pregnancy (Fig. 5). At parturition, erbB-2 was overexpressed in the transgenic mammary glands and undetectable in controls (not shown). Despite the transgene being also weakly expressed in the spleen, there was no detectable erbB-2 up-regulation in that organ, suggesting that cofactors might be necessary for activation by AP-2. Expression of the ER decreased to undetectable levels during pregnancy in both wild-type and control animals (Fig. 5). As a marker of alveolar differentiation (19), sulfated glycoprotein 2 (*SGP-2*) gene expression was found to be decreased in the transgenic mammary glands through the whole period analyzed. Expression of the milk-protein gene encoding the whey acidic protein (WAP) was undetectable in transgenic glands at day 14.5 and reduced at days 16.5 and 18.5, which is in contrast to the controls, where WAP transcripts could be detected at all days analyzed. Likewise, expression of β-casein was much lower at days 14.5 and 16.5 of pregnancy. However, at day 18.5 (Fig. 5) and at parturition (Fig. 1C), levels of β-casein from transgenic and wild-type mammary glands were comparable. This result suggests a deficiency in the proper timing of

differentiation. Furthermore, we analyzed the expression of IGFBP-5, a transcriptional target of AP-2 (20), being involved in the apoptosis of alveolar cells during postlactational mammary gland involution (21, 22). Whereas IFGBP-5 expression was declining in the control mammary glands with time, it was strongly induced in the transgenic mice at all days analyzed (Fig. 5). This result suggests that enforced expression of AP-2γ might induce apoptosis via transcriptionally activating the *IGFBP-5* gene.

IGFBP-5 and Endogenous AP-2γ Are Coexpressed During Pregnancy and Involution

The potential functional link between transgenic AP-2γ and IGFBP-5 expression prompted us to analyze the expression pattern of the *IGFBP-5* gene and the endogenously expressed *AP-2γ* gene in wild-type mammary glands at different developmental stages. In agreement with recently published data (23),
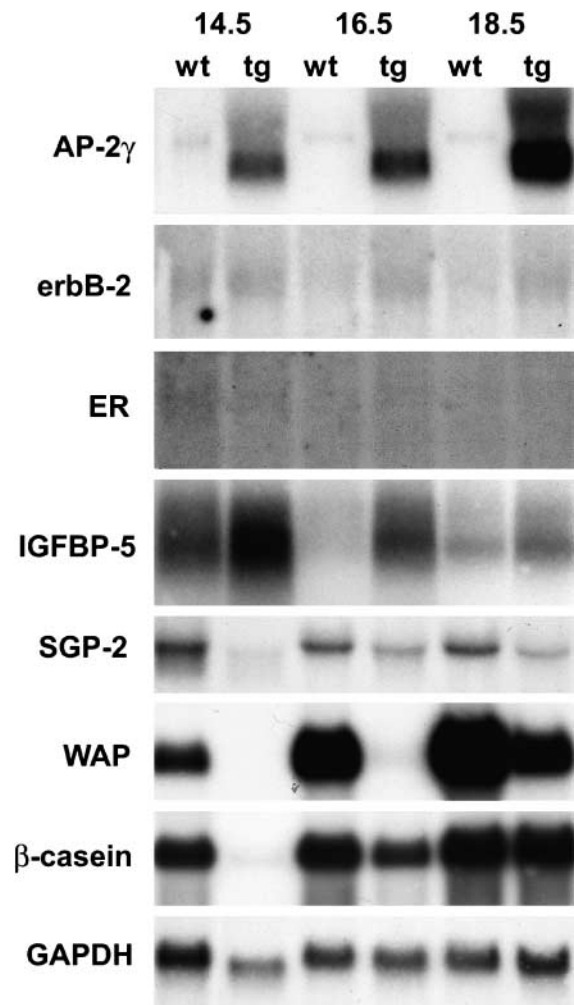
FIGURE 5. Northern analysis of gene expression at days 14.5, 16.5, and 18.5 of pregnancy. Northern analysis of 15 μg of total RNA of wild-type (*wt*) or transgenic (*tg*) mammary glands sequentially hybridized with the probes indicated. Note that the transgene-derived AP-2γ transcript differs in size from the endogenous transcript because of the replacement of part of the 3′ untranslated region of the cDNA by the SV40 polyA sequence in the transgene construct.
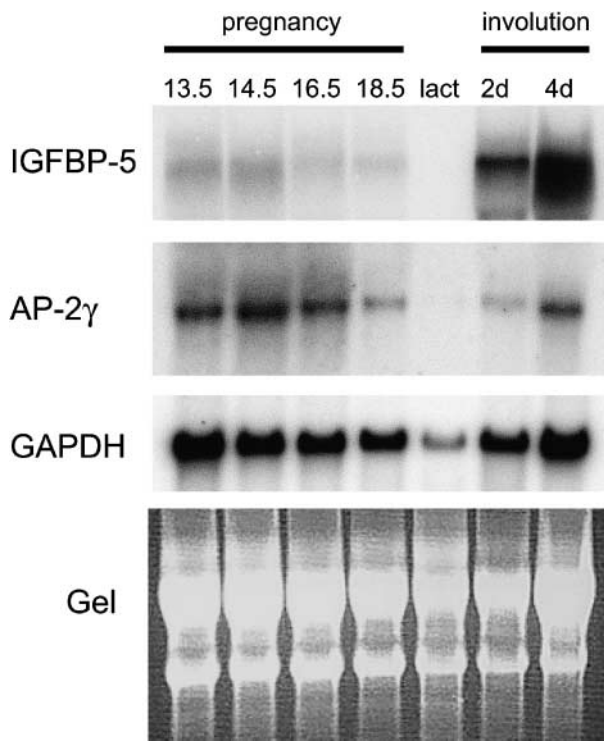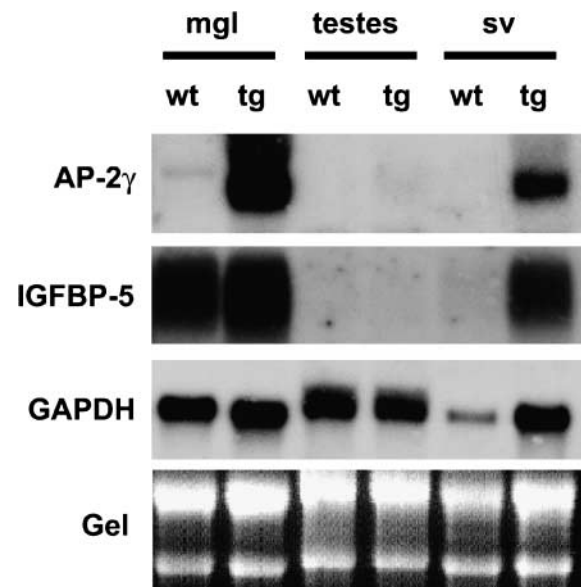
FIGURE 6. Northern analysis of expression of endogenous AP-2γ and IGFBP-5 during pregnancy, lactation, and involution at the days indicated. Fifteen micrograms of total RNA of wild-type mammary glands were sequentially hybridized with the probes indicated. Note that GAPDH expression itself is regulated during mammary gland development; therefore, the ethidium bromide-stained gel is shown to verify equal loading.

Northern blot analyses revealed tight regulation of AP-2γ expression during pregnancy, lactation, and involution (Fig. 6). Expression levels of IGFBP-5 followed the same kinetics. Both AP-2γ and IGFBP-5 are expressed during pregnancy in a constantly declining manner from day 13.5 up to day 18.5. During lactation, neither *AP-2γ* nor *IGFBP-5* transcripts were detectable. However, both genes were found strongly up-regulated in parallel during involution (Fig. 6). These data demonstrate coexpression of both genes during the growth and involution phases of the mammary gland cycle, suggesting that *AP-2γ* might control the expression of *IGFPB-5* in this tissue.

Phenotypic Consequences of AP-2γ Expression in Seminal Vesicles

Data from the breeding program of the transgenic lines revealed that the male transgenic mice from both lines displayed infertility, thus further complicating the propagation of the transgenic lines. Because it had been reported that MMTV-LTR driven transgenes are also expressed in the male genital system (24, 25), we decided to analyze the reproductive tracts of transgenic animals. Northern blot analyses revealed that the transgene is only weakly expressed in testes and at high levels in the seminal vesicles (Fig. 7). Similar to the transgenic mammary gland, IGFBP-5 mRNA levels were strongly increased in the transgenic seminal vesicle (Fig. 7), suggesting that cells might be undergoing apoptosis.

Gross examination showed that the testes and epydidimides appeared normal both morphologically and histologically (data not shown). *In vitro* fertilization experiments using sperm isolated from the epydidimides of transgenic males and oocytes from wild-type females revealed that the transgenic sperm was not compromised in fertilization capacity (data not shown). The seminal vesicles of 3 months old transgenic males, however, were much smaller than their wild-type counterparts. Surprisingly, despite this reduction in size, the epithelium of the seminal vesicles displayed a clear hyperplasia with an irregular and multilayered organization instead of the single cell layer composing the wild-type epithelium (Fig. 8, A and B). Furthermore, the seminal fluid produced from the secretory cells in the transgenic mice was of a different appearance because it displayed a more granular and flaky structure on H&E-stained sections (L in Fig. 8, A and B). Because this phenotype was reminiscent of the mammary phenotype of transgenic females, we decided to analyze proliferation (Ki-67) and apoptosis (TUNEL) in sections of transgenic and wild-type material. Ki-67 staining revealed a high number of proliferating cells in transgenic seminal vesicles ($3.2 \pm 0.2\%$) that were barely detectable in wild types (less than 0.2%; Fig. 8, C and D). Furthermore, the transgenic seminal vesicle epithelium displayed a high proportion of cells undergoing apoptosis ($1.6 \pm 0.5\%$), whereas in most of the sections through the wild-type epithelium, no TUNEL-positive cells could be detected (less than 0.15%; Fig. 8, E and F). These differences in proliferation and apoptosis between the groups are statistically highly significant ($P < 0.01$; Student's *t* test). Because the transgenic mammary glands had displayed an impaired differentiation, we analyzed the expression of a marker for terminally differentiated seminal vesicle epithelial cells, the androgen receptor,

FIGURE 7. Northern analysis of gene expression in the testes and the seminal vesicles (*sv*) of wild-type (*wt*) and transgenic (*tg*) males at 3 months of age and mammary glands (*mgl*) of female mice at day 18.5 of pregnancy. Fifteen micrograms of total RNA of wild-type (*wt*) or transgenic (*tg*) organs were sequentially hybridized with the probes indicated. The ethidium bromide-stained gel is shown as a loading control.
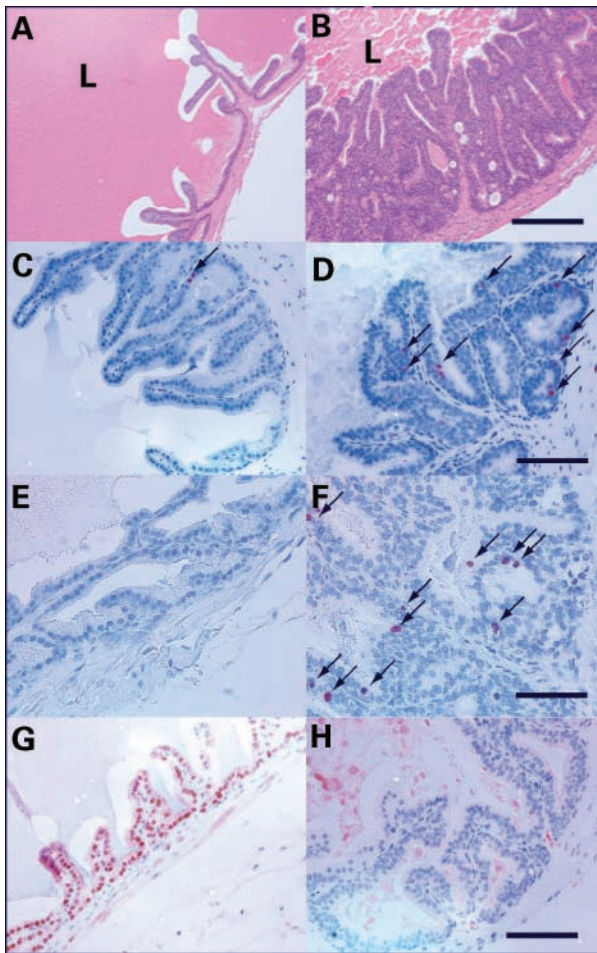
FIGURE 8. Seminal vesicle epithelium of wild-type (**A**, **C**, **E**, **G**) and transgenic mice (**B**, **D**, **F**, **H**) at 3 months of age. **A** and **B.** H&E staining. *Scale bar,* 100 μm. **C** and **D.** Immunohistochemical staining for the proliferation marker Ki-67 (*red-labeled* nuclei, *arrows*). *Scale bar,* 50 μm. **E** and **F.** TUNEL assay, nuclei of cells undergoing apoptosis are labeled in *red* (*arrows*). *Scale bar,* 25 μm. **G** and **H.** Immunohistochemical staining for androgen receptor expression (*brown-stained* nuclei). *Scale bar,* 50 μm.

using immunohistochemistry. Whereas the nuclei of the wild-type epithelium uniformly stained positive, androgen receptor was completely absent in the nuclei of transgenic epithelial cells (Fig. 8, G and H), indicating that overexpression of AP-2γ leads to impaired differentiation. These results are in agreement with those obtained from the mammary glands of female transgenic mice. In both tissues affected, we observed an increase in proliferation and apoptosis and disturbed differentiation processes.

Discussion

In this study, we have produced transgenic mouse lines expressing AP-2γ under the control of a mammary gland specific promoter element, the LTR of the MMTV, to test the hypothesis that AP-2γ acts as an oncogene. Female transgenic mice displayed a lactation failure resulting from an impaired mammary gland development. The number of alveolar cells was reduced and milk secretion was impaired, resulting in

smaller lobuloalveolar structures and an apparently decreased invasion of the fat pad by the secretory epithelium. Furthermore, male mice expressing the transgene in the seminal vesicles were found to have dysmorphic seminal vesicles and, as a consequence, to be infertile. In both epithelial tissues where *AP-2γ* overexpression was achieved in the late-pregnant mammary gland of female transgenic mice and the seminal vesicles of male transgenic mice, an increase in cell proliferation, enhancement of apoptosis, and the impairment of proper tissue-specific differentiation was observed. During pregnancy, apoptotic and proliferative processes as well as expression of *AP-2γ* occur endogenously in the mammary gland, which conceivably mask the effect of the transgene expression. In contrast, in the adult seminal vesicle of wild-type mice, neither proliferation and apoptosis are pronounced, nor is AP-2γ detectable on Northern blots. Therefore, the consequences of forced expression of AP-2γ are more concise.

AP-2 genes have been shown to regulate proliferation. Loss of AP-2α resulted in decreased proliferation of embryonic fibroblasts (9), and mice deficient for AP-2γ displayed an embryonic lethal phenotype due to the decreased proliferation of placental cells (8). From these data, a correlation between AP-2α and γ levels and cell proliferation can be inferred, suggesting that deregulated expression of AP-2 transcription factors might play a role in tumorigenesis. Indeed, in the transgenic mammary gland, erbB-2 has been found up-regulated, a molecule long known for its oncogenic potential. Furthermore, the *erbB-2* gene has been shown to be a direct target of AP-2 (13). Hence, a direct transcriptional activation of *erbB-2* by AP-2γ might result in the enhanced proliferation observed.

The epithelial cells of the mammary gland and the seminal vesicle display a delayed and largely impaired differentiation as shown by different markers indicative for terminal differentiation such as SGP-2, WAP, and androgen receptor. Results from a study of target genes for AP-2α suggest that one functional role of this transcription factor is the suppression of differentiation by repressing differentiation-inducing genes, as genes inducing differentiation and senescence were found up-regulated in a gene knock-out model (9). The data obtained from the transgenic model presented are in agreement with this hypothesis, overexpression of AP-2γ leads to the suppression of differentiation. The question remains, whether *WAP*, *SGP-2*, and androgen receptor are direct target genes of AP-2γ. An *in silico* search revealed AP-2 binding sites in the promoter regions of all three genes, making a direct regulation possible (data not shown). On the other hand, the expression of these marker genes could be indirectly influenced by the otherwise impaired differentiation.

Besides enhanced proliferation and suppressed differentiation, our analysis revealed that AP-2γ elicits an apoptotic signal. We found a dramatic up-regulation of the *IGFBP-5* gene in both tissues overexpressing AP-2γ. This gene had been shown to harbor active AP-2 binding sites in its promoter (20) and the encoded protein recently turned out to be one of the key regulators of apoptosis during mammary gland involution (21, 22). A model has been put forward where *IGFBP-5* is sequestering and counteracting insulin-like growth factors (IGFs), which act as survival factors for mammary epithelial

cells, thus inducing apoptosis (21). In fact, transgenic mice overexpressing IGFBP-5 in the late pregnant mammary gland display a phenotype very similar to our AP-2γ transgenic mice (22). Consistent with a stimulation of *IGFBP-5* transcription by the transgenic AP-2γ, we found a similar expression pattern of both genes during mammary development in wild-type mice. It is interesting to note that in the transgenic mammary glands, *IGFBP-5* levels decline at the end of pregnancy despite the fact that the transgene continues to be expressed at high levels. Possibly, further transcription factors or cofactors are required for *IGFBP-5* gene transcription which become down-regulated at the end of pregnancy, or *IGFBP-5* expression is actively repressed at this time, either at the level of transcription and/or message stability. Alternatively, it is possible that IGFBP-5-secreting cells are lost with time because of apoptosis and a cell type that persists lacks cofactors that are necessary for *IGFBP-5* transcription. In summary, the results suggest that AP-2γ might interact with the IGF signaling pathway by controlling the levels of IGFBP-5 and thus might be causally involved in the post-lactational involution process.

Zhang *et al.* (23) recently described the pathophysiological consequences of overexpressing AP-2α in the mammary gland using a similar experimental setup like ours. Therefore, the phenotypic consequences can be compared precisely. Both strains of mice display a lack of secretory differentiation, manifesting in hypoplastic mammary gland tissue and in both strains of mice AP-2 overexpression stimulated apoptosis. The major discrepancy compared to our study, however, consists of the different effects of the particular AP-2 isoforms on cell proliferation, with AP-2α being inhibiting and AP-2γ stimulating. These differences suggest very specific cellular functions of the particular AP-2 isoforms despite their high degree of homology. Consistent with this notion is the finding of very distinct phenotypic consequences of inactivating the particular *AP-2* genes in transgenic mice despite their overlapping expression patterns during embryogenesis (5, 7). Furthermore, the different impact of transgenic overexpression of AP-2α and AP-2γ on cellular proliferation in the mammary gland is experimentally supporting the different roles these isoforms are supposed to play in mammary carcinoma, with AP-2α correlating with a better prognosis and AP-2γ (together with AP-2α) with a poor prognosis (15).

Of note, throughout the experiments, we never observed mammary carcinomas in transgenic mice, even in multiparous females of age up to 2 years, which had been kept in forced breeding to ensure continuous transgene expression. Our results can be interpreted in a way that AP-2γ on its own stimulates proliferation and apoptosis at the same time, in a manner reminiscent of the c-*myc* proto-oncogene (26). Therefore, further alterations leading to the circumvention of the AP-2-induced cell death might be necessary to accomplish tumorigenesis. Hence, *AP-2γ* might be a gene involved in tumor progression rather than tumor initiation by stimulating proliferation and dedifferentiation once apoptotic mechanisms are overcome. The AP-2γ transgenic mice described in this study therefore represent a useful tool to further define the roles of AP-2 transcription factors in mammary cancer, that is, by intercrossing the LTRγ transgenic mice with oncogene-bearing transgenic mice.

Materials and Methods

Generation of Transgenic Mice

The murine AP-2γ cDNA (1.8 kb) was ligated into the Not 1-site of LTRΔ containing a 1.6-kb fragment of the MMTV-LTR followed by a splice donor/acceptor sequence and the polyadenylation signal from SV40 virus (18). Transgenic mice were produced according to published procedures (28). Transgenic mice were identified by PCR analysis using the primers γ25s (5′-agactgtcattcgcaaaggacccat-3′) and γ25as (5′-gagagacgtgaggagagtgacgtgg-3′). PCR conditions were 35 × (95°C 45 s, 60°C 30 s, 72°C 30 s).

RNA Preparation and Analysis

Tissue RNA was prepared using the guanidinium isothiocyanate method (28). Fifteen micrograms of total RNA were loaded onto a 1.2% agarose gel supplemented with 3.3% (w/v) formaldehyde and run in MOPS buffer [20 mM morpholino-propyl-sulfonate, 5 mM sodium acetate, 0.5 mM EDTA (pH 7.0)]. The RNA was transferred to a nylon membrane (Hybond N$^+$, Amersham, Braunschweig, Germany) and hybridized to ^{32}P-labeled DNA probes. Filters were washed twice in 2× SSC, 0.1% (w/v) SDS and twice in 1×SSC, 0.1% (w/v) SDS at 65°C, 10 min each.

Protein Preparation and Analysis

Tissues were snap frozen, pulverized in liquid nitrogen, and suspended in protein buffer [50 mM Tris-HC1 (pH 6.8), 2.5% (w/v) SDS]. After heating to 100°C for 10 min, the samples were sonified and centrifuged. Supernatants were taken and protein concentrations determined spectrophotometrically. After adding 1/10 volume of sample buffer [600 mM Tris (pH 6.8), 8% (w/v) SDS, 20% (v/v) β-mercapto-ethanol] and heating to 100°C for 10 min, 50 μg of proteins were electrophoresed on an 8% SDS-polyacrylamide gel (29) and transferred to a polyvinylidene difluoride (PVDF) membrane by electroblotting. After blocking in 5% (w/v) dry milk in PBS, 0.3% (v/v) Tween 20, the membrane was incubated with rabbit anti-AP-2 antibody (C-18, Santa Cruz Biotechnology, Santa Cruz, CA) and a peroxidase-conjugated anti-rabbit antibody and detected using the ECL system (Amersham).

Histology

For whole-mount analysis, the third thoracic mammary glands were spread on slides, fixed in formalin, defatted in acetone, stained with carmine alum [0.2% (w/v) carmine red, 0.5% (w/v) KAl(SO$_4$)$_2$], and cleared in xylene before mounting.

For light microscopy, the fourth inguinal mammary glands were fixed in formalin, dehydrated, and embedded in paraffin. Four-micrometer sections were stained with H&E or subjected to immunohistochemical or TUNEL analysis. Ki-67 antigen was detected with rat anti-mouse monoclonal antibody (TEC-3, DAKO, Hamburg, Germany); for the staining of the androgen receptor antibody, AR441 (DAKO) was used. For BrdUrd labeling, mice were injected 2 h before sacrifice i.p. with 10 mg BrdUrd dissolved in 0.9% (w/v) NaCl. BrdUrd incorporation was detected in tissue sections with a biotinylated mouse anti-BrdUrd antibody using the BrdUrd Immunohistochemistry System (Oncogene Research Products, San Diego, CA). TUNEL

analysis was performed using the Apopdetect kit (Qbiogene, Heidelberg, Germany) according to the manufacturer's instructions. For quantitation of Ki-67 or TUNEL-positive nuclei, two sections of both inguinal mammary glands of two transgenic and three control female mice were analyzed and the percentage of positive nuclei was determined (2000 nuclei/section for Ki-67; 3000 nuclei/section for TUNEL analysis). For quantitation of BrdUrd-incorporating nuclei, a different set of mice was analyzed (three transgenic and three wild-type mice).

For electron microscopy, the left inguinal mammary glands were diced, fixed in 3% (w/v) glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4 at 4°C for 16 h, postfixed in 2% (w/v) osmium tetroxide (2 h), washed in cacodylate buffer, and dehydrated through a series of graded ethanol solutions. The tissue was stained with 0.5% uranyl acetate in 70% ethanol (1 h) cleared in propylene oxide and embedded in Epon. Fifty-nanometer sections were cut (LKB Ultratome V, Bromma, Sweden), stained with 3% (w/v) uranyl acetate in saturated lead citrate, and examined in a Philipps CM10 electron microscope.

Mammary Gland Organ Culture

Inguinal mammary glands were removed from transgenic or wild-type females at day 13.5 of pregnancy. The tissue was diced and placed on metal grids at the air/liquid interphase in RPMI containing insulin, prolactin, and hydrocortisone (5 μg/ml each) and cultivated for 8 days in a humidified incubator (3% CO_2). The tissue was fixed for 16 h in 10% (w/v) phosphate-buffered formalin, dehydrated, and embedded in paraffin. Five-micrometer sections were stained with H&E.

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Transcription Factor AP-2γ Stimulates Proliferation and Apoptosis and Impairs Differentiation in a Transgenic Model [1]1Grants from Deutsche Forschungsgemeinschaft (Scho 503) and HGF (SGF01SF9808) to H.S. supported this work. Note: Data deposition: The transgenic mice are registered with MGD as Tg(Tcfap2c)Hsc.

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