Salivary Gland Tumors in Transgenic Mice with Targeted *PLAG1* Proto-Oncogene Overexpression

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Abstract

Pleomorphic adenoma gene 1 (*PLAG1*) proto-oncogene overexpression is implicated in various human neoplasias, including salivary gland pleomorphic adenomas. To further assess the oncogenic capacity of *PLAG1*, two independent *PLAG1* transgenic mouse strains were established, PTMS1 and PTMS2, in which activation of *PLAG1* overexpression is Cre mediated. Crossbreeding of PTMS1 or PTMS2 mice with MMTV-Cre transgenic mice was done to target *PLAG1* overexpression to salivary and mammary glands, in the P1-Mcre/P2-Mcre offspring. With a prevalence of 100% and 6%, respectively, P1-Mcre and P2-Mcre mice developed salivary gland tumors displaying various pleomorphic adenoma features. Moreover, histopathologic analysis of salivary glands of 1-week-old P1-Mcre mice pointed at early tumoral stages in epithelial structures. Malignant characteristics in the salivary gland tumors and frequent lung metastases were found in older tumor-bearing mice. *PLAG1* overexpression was shown in all tumors, including early tumoral stages. The tumors revealed an up-regulation of the expression of two distinct, imprinted gene clusters (i.e., *Igf2*/*H19* and *Dlk1*/*Gtl2*). With a latency period of about 1 year, 8% of the P2-Mcre mice developed mammary gland tumors displaying similar histopathologic features as the salivary gland tumors. In conclusion, our results establish the strong and apparently direct *in vivo* tumorigenic capacity of *PLAG1* and indicate that the transgenic mice constitute a valuable model for pleomorphic salivary gland tumorigenesis and potentially for other glands as well. (Cancer Res 2005; 65(11): 4544-53)

Introduction

Pleomorphic adenoma gene 1 (*PLAG1*) is a proto-oncogene on human chromosome 8q12 whose oncogenic activation is a crucial event in the formation of pleomorphic adenomas of the salivary glands (1, 2) and lipoblastomas (3–5). Recently, amplification and overexpression of *PLAG1* was reported for hepatoblastoma, indicating that the *PLAG1* gene might also be implicated in the molecular pathogenesis of this childhood neoplasia (6). Altogether, these observations seem to emphasize a more general importance of the *PLAG1* gene in human benign solid tumor development. Recent studies have shown that *PLAG1* and *PLAGL2* seem also to be implicated in leukemia (7). Indeed, *PLAG1* and *PLAGL2* expression was increased in 20% of human acute myeloid leukemia (AML) samples, that contain in most cases the *CBFB-MYH11* fusion gene (8, 9).

PLAG1 encodes for a zinc finger transcription factor and has two structurally related family members, *PLAGL1* (*PLAG-like 1*) and *PLAGL2* (10). The main mechanism of oncogenic activation of *PLAG1* involves recurrent chromosome translocations. This leads to promoter exchange between *PLAG1*, a gene differentially expressed and primarily during fetal development and one of a variety of possible translocation partner genes, that are more broadly and constitutively expressed. Chromosome breakpoints invariably occur in the 5′ noncoding region of *PLAG1*, causing an exchange of regulatory control elements without affecting the coding sequences of the gene. This process, also called promoter swapping, leads to ectopic expression of *PLAG1* in the genetically affected cells (1, 2). *PLAG1* activation can also be caused by cryptic rearrangements involving the gene region (11). It is assumed that ectopic *PLAG1* overexpression causes deregulation of expression of *PLAG1* target genes leading to particular tumors in humans (1, 3).

Structurally, the *PLAG1* transcription factor contains seven canonical C_2H_2 zinc fingers and a serine-rich COOH terminus with transactivation capacity (10). *PLAG1* specifically recognizes a bipartite DNA-binding consensus sequence consisting of a core sequence, GRGGC, and a G cluster, GGG, which are separated by 6 to 8 nucleotides (12). A nuclear localization sequence was identified (13) and *PLAG1* is post-translationally modified by SUMOylation (14). In recent microarray analyses (15), genes were identified that are consistently induced or repressed by *PLAG1*, and these were classified into various functional categories. Among the classes of up-regulated *PLAG1* target genes, the one encoding growth factors was the largest and included the genes for insulin-like growth factor II (*IGF-II*), cytokine-like factor-1 (*CLF-1*), bone-derived growth factor (*BPGF-1*), choriogonadotropin β chain (*CGB*), vascular endothelial growth factor (*VEGF*), and placental growth factor (*PIGF*). From *in silico* evaluation of their promoter regions, it seemed that a large proportion of them harbor several copies of the specific bipartite DNA binding consensus sequence, suggesting that they constitute direct *PLAG1* targets. Furthermore, the *in silico* studies indicated that *PLAG1*–down-regulated genes

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are likely to be regulated indirectly by *PLAG1*, because the typical *PLAG1* DNA binding consensus sequences are not present in their corresponding promoter regions (15).

The oncogenic potential of *PLAG1* has been established *in vitro* by transforming cultured cells via overexpression of *PLAG1* (16). The results of these *in vitro* studies are consistent with the *in vivo* observation that increased *PLAG1* expression is important in the development of particular tumors, such as those mentioned before. To explore the role of *PLAG1* in neoplastic transformation in the context of a complex organism, we have initially generated transgenic mice overexpressing *PLAG1* early and ubiquitously.[6] These studies revealed that such early and ubiquitous overexpression of *PLAG1* leads to embryonic lethality. Now, we have developed *PLAG1* transgenic mouse strains, in which activation of overexpression of the transgene as well as the tissue distribution of such overexpression can be manipulated (e.g., by Cre-mediated activation and targeted expression, respectively). Two independent *PLAG1* transgenic mouse strains were obtained and used to target overexpression of the *PLAG1* gene to a restricted number of tissues, including salivary gland and mammary gland tissue. *PLAG1* overexpression in the salivary glands leads to tumor formation. These *PLAG1*-induced salivary gland neoplasias were studied in detail histopathologically and molecularly. Finally, induction of mammary gland tumors, with similar morphologic features, was also studied.

Materials and Methods

Generation of transgenic mouse strains with Cre-mediated activation of PLAG1 expression. A *Kpn*I/*Xho*I cDNA fragment, comprising the complete open reading frame of human *PLAG1* followed by the coding sequences for an *HA*-tag, was cloned into the *Eco*RI site of the pCAGGS vector DNA (17) using blunt end ligation (Fig. 1*A*). At the multiple cloning site between the CAG promoter and the sequences encoding HA-tagged *PLAG1*, a *loxP*/*PGK-Neo*/*loxP* DNA fragment (a kind gift of P. Kastner, Institut de Chimie Biologique, Université Louis Pasteur, Strasbourg, France; ref. 18) was inserted as a Cre-dependent removable stop cassette (Fig. 1*A*). This resulted in a DNA construct conditionally allowing targeted activation of *PLAG1* expression. The resulting DNA was digested with restriction endonucleases *Sal*I and *Sfi*I, and upon size fractionation by electrophoresis, a 6.0-kb *Sal*I/*Sfi*I DNA fragment (Fig. 1*A*) was purified using the QIAquick extraction kit (Qiagen, Chatsworth, CA). This purified DNA was used for microinjection into the male pronucleus of fertilized eggs of (FVB × FVB) mice (Charles River Laboratories, Brussels, Belgium). Upon oviductal implantation of these eggs in pseudopregnant foster females, offspring was obtained and these mice were bred to select *PLAG1* transgenic founders. To target *PLAG1* expression to the salivary gland and mammary gland, these founders were crossed with B6129-Tgn(MMTV-LTR/Cre)1Mam transgenic mice (The Jackson Laboratories, Bar Harbor, ME; ref. 19).

Genotyping by PCR analysis. Genotyping of candidate *PLAG1* founders was done by PCR analysis of tail DNA using oligonucleotide primers POS-1599 (5′-TTCTCAAGCATCGTCATCAT-3′) and β-globin (5′-AAAATTCCAACACACTATTGC-3′) at an annealing temperature of 58°C. PCR analysis was also used to evaluate Cre-mediated excision of the floxed stop cassette from the *PLAG1* DNA construct in the transgenic mouse strains. For that purpose, genomic DNA was isolated from various tissues of transgenic mice using the DNeasy tissue kit (Qiagen) according to the manufacturer's instructions. PCR analysis to detect Cre-mediated excision of the floxed stop cassette was done using the following primers: P1 forward 5′-CTACAGCTCCTGGGCAA-CGTGCTGG-3′ or P2 forward 5′-GCCTGAAGAACGAGATCAGCAGCC-3′ in combination with P2 reverse 5′-CACCACTTGTTGCGGCATGCAAGGCC-3′. The P1 forward/P2 reverse and P2 forward/P2 reverse set of primers amplified, respectively, a DNA fragment of 500 or 600 bp. The selected annealing temperature was 65°C.

DNA transfection, SDS-PAGE, and Western blot analysis. Selected DNAs (1 μg) were (co)transfected into appropriate mammalian cells using FuGENE 6 transfection reagent (Boehringer Mannheim, Mannheim, Germany) according to the protocol of the manufacturer. About 24 hours after transfection, cells were lysed in SDS-PAGE sample buffer [50 mmol/L Tris-HCl (pH 6.8), 10% glycerol, 2% SDS, 4% β-mercaptoethanol], boiled, and loaded on a 10% SDS-polyacrylamide gel. After size separation, proteins were transferred electrophoretically to PROTEAN nitrocellulose membranes (Schleicher and Schuell, Keene, NH). Expression of HA-tagged *PLAG1* was detected using mouse anti-HA monoclonal antibody (1:5,000, Roche, Nutley, NJ). Enhanced chemiluminescence Western blotting was done using Renaissance Western blotting detection reagents (Perkin-Elmer Life Sciences, Norwalk, CT; ref. 20).

Histopathology, immunohistochemistry, and immunofluorescence. For histopathology, organs and tumors of mice were removed carefully, fixed overnight in 4% paraformaldehyde, and embedded in paraffin using routine procedures. For each tumor, two to three different areas were selected to analyse histologically. Paraffin sections (5 μm) were stained with H&E, Alcian Blue (pH 0.2, 1, or 2.5), or periodic acid-Schiff (PAS).

Immunostainings were done on 5 μm paraffin sections according to standard procedures. Briefly, after antigen retrieval in citrate buffer (10 mmol/L citric acid, pH 6), the mouse anti-human smooth muscle actin (SMA; 1:500 DAKO, Carpinteria, CA), and the guinea pig anti-human cytokeratin (8–18) antibody (1:50, Progen, Heidelberg, Germany) were used as primary antibodies. Such antibodies are frequently used to stain myoepithelial and epithelial cells, respectively (21). As a secondary reagent to detect smooth muscle actin, the avidin-biotin complex system was used. For detection of cytokeratin 8/18, a secondary rabbit anti-guinea pig antibody (DAKO) was used, followed by the application of the anti-rabbit Envision+ kit (DAKO) and development with 3,3′-diaminobenzidine (Sigma FastTM DAB Tablet Set, Sigma, St. Louis, MO). Proliferation was assessed in salivary gland tumors by bromodeoxyuridine (BrdUrd) incorporation. Briefly, mice received a single i.p. injection of BrdUrd (cell proliferation labeling reagent, 1 ml/100 g, GE Healthcare, Roosendaal, the Netherlands) 1 hour before sacrification. Paraffin-embedded salivary gland samples were subjected to antigen retrieval in citrate buffer (10 mmol/L citric acid, pH 6) and immunohistochemistry using anti-BrdUrd-POD Fab (1/100, Roche). Tyramide signal amplification was obtained with the TSATM Biotin System (Perkin-Elmer Life Sciences) and STP-POD (1/100, DAKO).

Immunofluorescence analysis was used to assay *PLAG1* protein expression and nuclear staining [4′,6-diamidino-2-phenylindole (DAPI), blue] in mouse salivary gland tumor tissue of P1-Mcre and P2-Mcre mice. Five-micrometer cryostat sections were fixed in buffered 4.0% paraformaldehyde for 10 minutes, blocked in swine serum for 7 minutes (dilution 1:20), and incubated first with the primary rabbit polyclonal anti-*PLAG1* antibody, PEM 190 or PEM195 (ref. 22; dilution 1:60) for 60 minutes at room temperature and, secondarily, with fluorescein-labeled swine anti-rabbit antibody (dilution 1:20; TRITC, red, DAKO) for 45 minutes. Sections were mounted in Citifluor containing 0.5 μg/μL of DAPI. The slides were finally analyzed with a Zeiss Axiophot microscope equipped with UV optics (Carl Zeiss, Inc., Thornwood, NY). Images were recorded with a CE200A CCD camera (Photometrics, Tucson, AZ).

Northern blot analysis and DNA probes. Total RNA from mouse tissues or tumors was isolated according to the method described by Chomczynski et al. (23). Northern blot analysis was done as described previously (24). The following probes were radiolabeled with [α-^{32}P]dCTP (MP Biomedicals, Asse-Relegem, Belgium) using the Megaprime DNA Labeling kit (GE Healthcare): for *PLAG1*, a 1.5-kb cDNA fragment encompassing the complete *PLAG1* open reading frame; for *Igf2*, a 674-bp DNA fragment containing the sequence between nucleotides 1253 and 1927 in NM_010514; for *H19*, a 1.5-kb *Eco*RI/*Xba*I DNA fragment of the AA408602 cDNA clone; for *Dlk1*, a 629-bp PCR fragment containing the sequence

[6] Unpublished results.

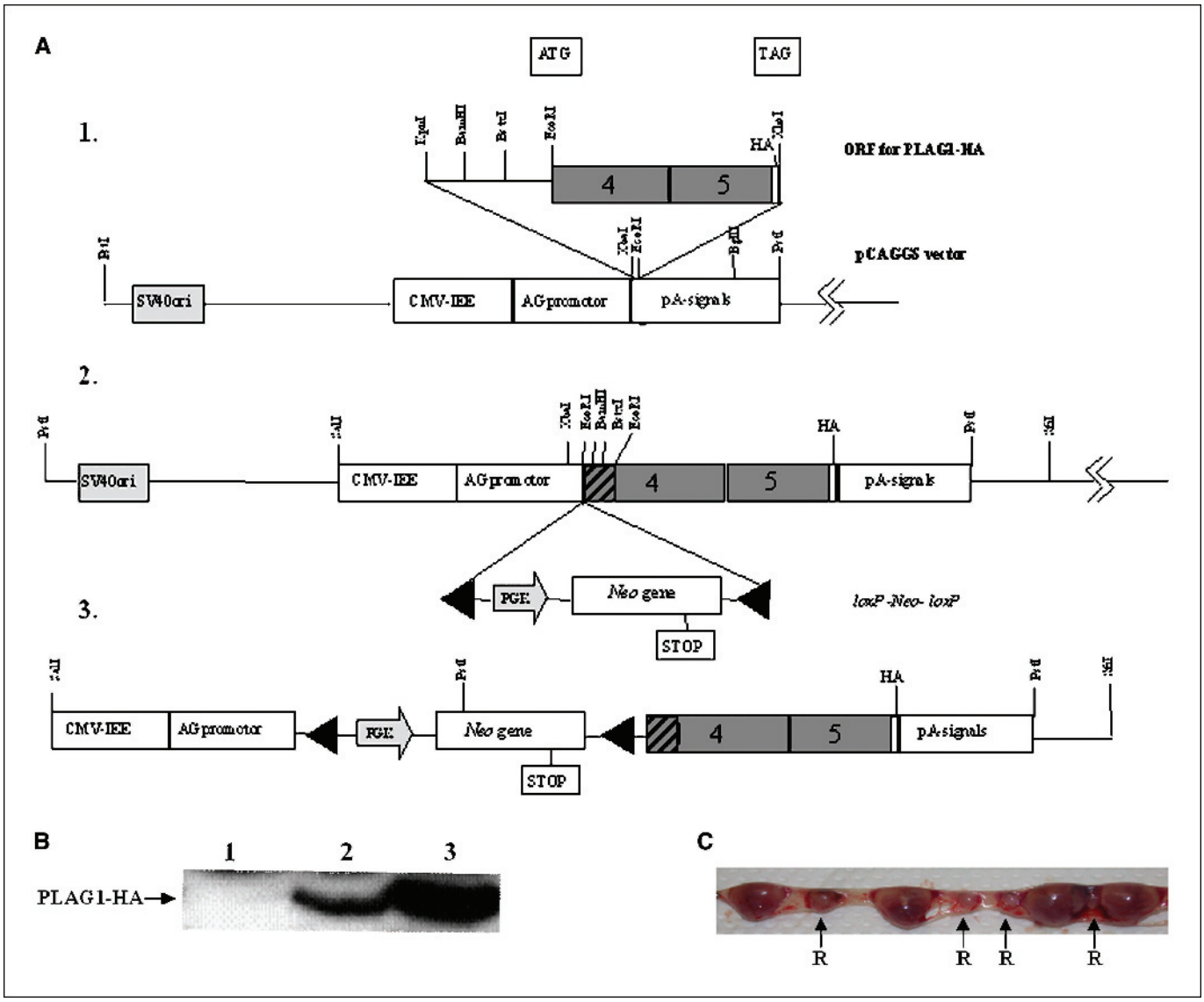

Figure 1. *A,* schematic representation of the cloning strategy to obtain the *PLAG1* transgene DNA construct used in the generation of the two *PLAG1* transgenic mouse strains, PMTS1 and PMTS2. Human *PLAG1* cDNA, containing the complete open reading frame (*ORF*) for *PLAG1* and sequences encoding a hemaglutinin tag at the 5′ end of the *PLAG1* cDNA, was cloned in the pCAGGS vector, bringing its expression under control of the CMV-IEE-AG promoter. Subsequently, a stop cassette was inserted between the CAG promoter and the PLAG1-HA coding sequences. The stop cassette consists of the *Neo* gene, which is flanked on both sides by loxP sites and which is under expression control of the PGK promoter. Expression of *PLAG1-HA* is therefore dependent on Cre-mediated removal of the stop cassette. The *Sal*I/*Sfi*I DNA fragment, shown under "3" was used for zygote injection. *CMV-IEE,* CMV immediate early enhancer; *AG,* chicken β-actin/rabbit β-globin hybrid promoter; *pA,* polyadenylation. *B,* functional validation *in vitro* cell culture of the *PLAG1* transgene DNA construct used in the generation of the two *PLAG1* transgenic mouse strains. Cre-mediated excision of the stop cassette was tested by Western blot analysis of lysates of 293T cells transfected with the *PLAG1* transgene DNA construct shown under "2" in the absence (*lane 1*) or presence (*lane 2*) of *pCAGGS-Cre* plasmid DNA. As a postive control, a lysate of cells transfected with *CMV-PLAG1-HA* plasmid DNA expressing constitutively the PLAG1-HA protein was used. The PLAG1-HA protein was detected using a monoclonal antibody with specificity for the HA-tag. *C,* developing *PLAG1*$^{-/-}$ embryos and resorbed (*R*) embryos, which presumably possess a *PLAG1*$^{+/-}$ genotype.

between the nucleotides 64 and 1270 of NM_003836.3; for *Gtl2*, a PCR fragment containing the sequence between the nucleotides 284 and 778 of AJ320506; and for the *β-actin* gene, a PCR fragment containing the sequence between nucleotides 825 and 1112 of mouse *β-actin* (X03672).

Results

Generation of two independent *PLAG1*$^{+/-}$ transgenic mouse strains with *PLAG1* expression dependent on Cre-mediated removal of a stop cassette. Results of initial experiments to generate PLAG1 transgenic mice indicated that early and ubiquitous expression of the *PLAG1* gene leads to embryonic lethality. Therefore, a *PLAG1* DNA construct was designed from which a floxed "stop cassette" had to be removed via Cre-mediated excision to allow *PLAG1* expression. The cloning strategy is described in Materials and Methods and the genetic composition of the generated DNA construct is shown in Fig. 1*A*. The integrity and functionality of the *PLAG1* DNA construct was validated *in vitro* by Western blot analysis of HEK293T cells, transfected with DNA of the *PLAG1* construct and this in the presence or absence of DNA coding for the Cre enzyme. Upon transfection of only DNA representing the *PLAG1* construct, the *PLAG1* protein could not be detected (Fig. 1*B*, *lane 1*). Upon

cotransfection of the *PLAG1* DNA and DNA encoding the Cre enzyme, readily detectable levels of the *PLAG1* protein were present (Fig. 1*B*, *lane 2*), indicating efficient Cre-mediated removal of the stop cassette.

Upon independent zygote injection experiments using the *Sal*I/*Sfi*I linearized and purified DNA fragment of about 6 kb (Fig. 1*A*) and subsequent breeding of the offspring, two independent hemizygous *PLAG1*$^{+/-}$ transgenic mouse strains (PTMS), PTMS1 and PTMS2, were obtained. To test the integrity and functionality of the *PLAG1* DNA construct *in vivo*, PTMS1 and PTMS2 mice were crossed with *PGK-Cre*$^{+/+}$ transgenic mice. It is known that the *PGK*-promoter is already expressed in the two-cell stage during embryogenesis (25). Therefore, early and widespread *PLAG1* expression could be expected in the offspring. Intercrossing of PTMS1 mice with *PGK-Cre*$^{+/+}$ transgenic mice did not result in any *PGK-Cre*$^{+/-}$*/PLAG1*$^{+/-}$ offspring. This was most likely due to early embryonic lethality, as can be deduced from the fact that about 50% (39 of 70) of the embryos appeared embryonically resorbed (Fig. 1*C*). The genotype of the resorbed embryos could not be determined, however, that of the normally developed embryos (31 of 70) was invariably *PGK-Cre*$^{+/-}$*/PLAG1*$^{-/-}$. In contrast, intercrossing of PTMS2 mice with *PGK-Cre*$^{+/+}$ transgenic mice resulted in litters in which 50% of the mice were *PGK-Cre*$^{+/-}$*/PLAG1*$^{+/-}$. Although theoretically ubiquitous expression was predicted for these *PGK-Cre*$^{+/-}$*/PLAG1*$^{+/-}$ mice, *PLAG1* expression seems restricted mainly to the bladder on Northern blot analysis (data not shown). However, discriminating PCR analysis of DNA isolated from various tissues of these *PGK-Cre*$^{+/-}$*/PLAG1*$^{+/-}$ mice, including stomach, small intestine, caecum, colon, heart, seminal vesicle, uterus, kidney, and bladder, clearly established that excision of the stop cassette had taken place, at least partially, in all these tissues (data not shown).

Targeting stop cassette excision to the salivary glands results in salivary gland tumor development. In previous *in vitro* studies, the tumorigenic capacity of *PLAG1* was established (16). The aim of the present studies was to establish whether *PLAG1* is capable to induce salivary gland tumors in mice and also to investigate in what way such tumors resemble human pleomorphic adenomas with *PLAG1* overexpression. Therefore, PTMS1 and PTMS2 mice were intercrossed with B6129-Tgn(MMTV-LTR/Cre)1Mam transgenic mice (19) to target stop cassette excision and subsequent *PLAG1* expression activation to particular tissues. This mouse strain was used because the expression pattern of the Cre enzyme has been well defined and includes the salivary and the mammary glands (19). Such intercrossing between PTMS1 and B6129-Tgn(MMTV-LTR/Cre)1-Mam transgenic mice resulted in the *MMTV-LTR-Cre*$^{+/-}$*/PLAG1*$^{+/-}$ (P1-Mcre) offspring. Within 5 weeks, 100% (37 of 37) of the P1-Mcre mice developed a large tumor mass (Fig. 2*A*) in the ventral neck region, which upon necropsy always seemed to consist of bilateral, multifocal salivary gland tumors (Fig. 2*B*). These tumors seemed to preferentially originate from submandibulary salivary glands, but tumors originating from the sublingual salivary glands were also observed. In contrast, only about 6% (6 of 97) of the P2-Mcre mice developed salivary gland tumors, and all of these originated from the submandibulary salivary glands, arose unilaterally, and constituted a single tumor mass. Furthermore, the tumors in the P2-Mcre mice became apparent much later (i.e., in most cases only after a latency period of several months). Discriminating PCR analysis of DNA isolated from salivary gland tumors of these P1-Mcre and P2-Mcre mice clearly showed that excision of the stop cassette had taken place in all these tumors (data not shown).

Activation of expression of the *PLAG1* transgene in P1-Mcre and P2-Mcre mice. To study activation of expression of the *PLAG1* transgene after Cre-mediated excision of the stop cassette resulting from intercrossing PTMS1 or PTMS2 mice with B6129-Tgn(MMTV-LTR/Cre)1Mam transgenic mice, Northern blot analysis was done on RNA from tissues from the offspring using the open reading frame sequences of the human *PLAG1* cDNA as

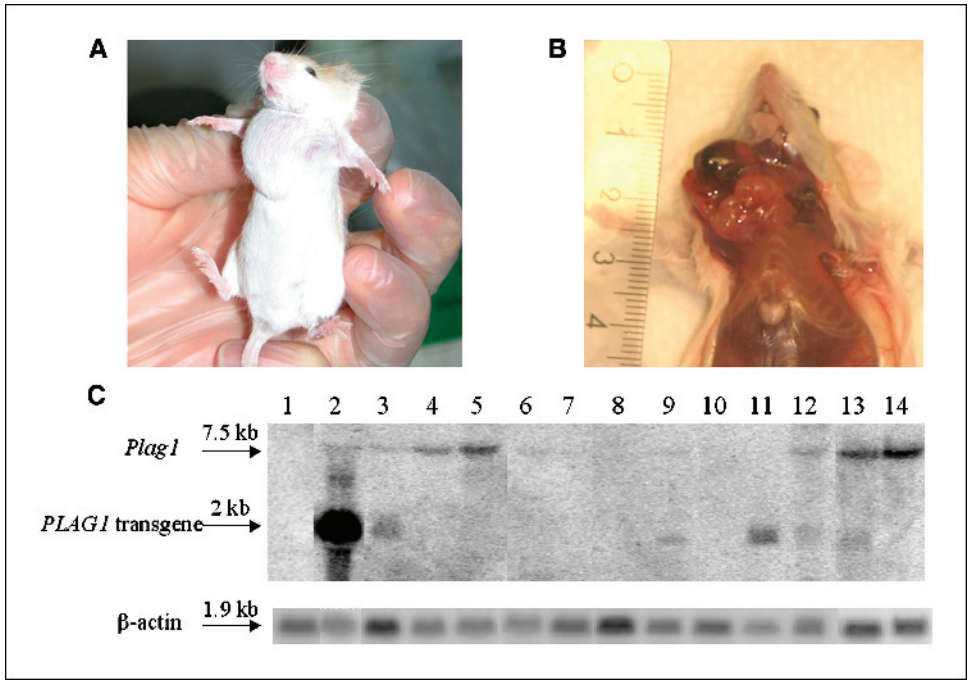

Figure 2. *A,* large salivary gland tumor mass in a 5-week-old *MMTV-LTR-Cre*$^{+/-}$/*PLAG1*$^{+/-}$ mouse (P1-Mcre). *B,* macroscopic appearance at necropsy of the tumor mass of the 5-week-old P1-Mcre mouse shown in (*A*). *C,* Northern blot analysis of RNA isolated from salivary gland tissue of PTMS1 mice (*lane 1*) or various tissues of 5-week-old P1-Mcre mice (*lanes 2-14*), including submandibular salivary gland tumor (*lane 2*), mammary gland (*lane 3*), adrenal gland (*lane 4*), kidney (*lane 5*), stomach (*lane 6*), small intestine (*lane 7*), colon (*lane 8*), ovary (*lane 9*), uterus (*lane 10*), seminal vesicle (*lane 11*), testis (*lane 12*), lung (*lane 13*), and brain (*lane 14*). As molecular probes, a ^{32}P-labeled 1.5-kb human *PLAG1* cDNA probe corresponding to the complete open reading frame of *PLAG1* or a 290-bp *β-actin* probe was used. The transcripts of *PLAG1* and *PLAG1* (*arrows*) and their sizes.

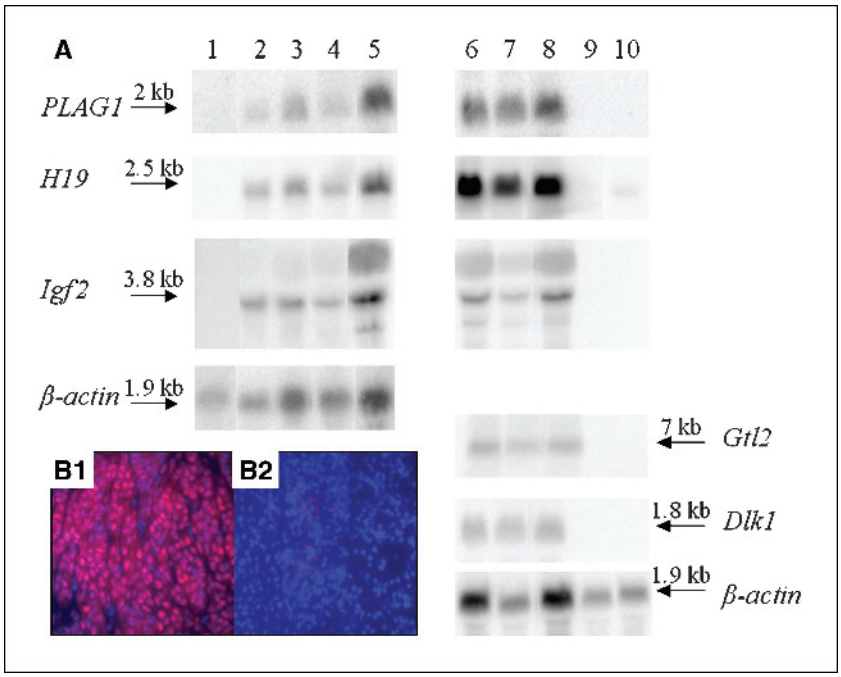

Figure 3. *A,* Northern blot analysis of expression of the *PLAG1* transgene in salivary gland tissue or tumors of P1-Mcre and P2-Mcre mice and upregulation of *PLAG1* target genes *H19*, *Igf2*, *Gtl2*, and *Dlk1*. RNA was isolated from salivary gland tissue of PTMS1 mice (*lanes 1* and *9*), P1-Mcre mice (*lanes 2-7*), a P2-Mcre mouse (*lane 8*), or a B6129-Tgn(MMTV-LTR/Cre)1Mam mouse (*lane 10*). The P1-Mcre mice varied in age and included mice ages 1 week (*lane 2*), 2 weeks (*lane 3*), 2 weeks (*lane 4*), or 5 weeks (*lanes 5-7*). Salivary gland tissue from mice ages 1 to 4 weeks possessed early tumoral stages, whereas in mice of a later age, large salivary gland tumor masses were always present. The P2-Mcre mouse was about 11 weeks old and also showed a salivary gland tumor mass. The nature and size of the transcripts that were detected with the various probes, which have been described in Materials and Methods, are indicated. Qualitative and quantitative validation of the RNA loaded in various lanes was done using *β-actin* DNA as a molecular probe. *B1,* immunofluoresence analysis of a cryostat section of a salivary gland tumor of a 5-week-old P1-Mcre mouse using PLAG1-specific antibody PEM-195. *B2,* DAPI staining of the same section as shown in (*B1*).

molecular probe (Fig. 2*C*). In salivary gland specimens of PTMS1 mice (*PLAG1*$^{+/-}$), no expression of the transgene could be detected in line with the inhibition of expression by the stop cassette (Fig. 2*C*, *lane 1*). Furthermore, no expression of endogenous *Plag1* could be detected either (Fig. 2*C*, *lane 1*). Analysis of RNAs isolated from a variety of tissue samples of salivary gland tumor-bearing male and female 5-week-old P1-Mcre mice revealed that expression of the *PLAG1* transgene was very high in the samples of salivary gland tumors (Fig. 2*C*, *lane 2*). Furthermore, weak *PLAG1* expression was found in samples of mammary glands, ovary, and seminal vesicles (Fig. 2*C, lanes 3, 9, and 11),* and very weak expression in testis and lung (Fig. 2*C*, *lanes 12* and *13*). This correlates with the expression pattern of the Cre enzyme in the B6129-Tgn(MMTV-LTR/Cre)1Mam transgenic mice as described by Wagner et al. (19). Expression of the endogenous mouse *Plag1* gene was also detected in some of the tissues tested, including mammary gland, adrenal gland, kidney, stomach, ovary, testis, lung, and brain but at low and varying levels (Fig. 2*C*, *lanes 3-6*, *9*, *12-14*). Similar studies with tissues from salivary gland tumor bearing P2-Mcre mice (data not shown) also revealed high levels of ***PLAG1*** expression in these tumors.

Time course of activation of *PLAG1* overexpression. Salivary gland specimens from P1-Mcre mice were studied by Northern blot analysis at different ages after birth. For each stage, at least two mice were analyzed, and 10 in the case of 5-week-old mice. As shown in Fig. 3*A*, *PLAG1* transcripts could be observed in salivary gland tissues at all ages analyzed, including mice ages 1 week (*lane 2*), 2 weeks (*lane 3*), 4 weeks (*lane 4*), or 5 weeks (*lanes 5-7*). This points towards activated expression of the *PLAG1* transgene already at the very first measuring point. It should be noted that tumor nodules were not macroscopically visible in salivary gland specimens of 1- or 2-week-old mice; even not at necropsy. Salivary gland tumor nodules were macroscopically visible in 4-week-old mice but only at necropsy. Between 4 and 5 weeks, large salivary gland tumors became clearly visible as large protruding tumor masses that grew rapidly (Fig. 2). Upon analysis of a salivary gland tumor specimen of a P2-Mcre mouse of about 11 weeks old, similar levels of *PLAG1* transcripts were detected (Fig. 3*A*, *lane 8*). Northern blot analysis of tissue specimens of salivary glands of PTMS1 mice, respectively, 1 and 3 weeks after birth, revealed no *PLAG1* expression (Fig. 3*A*, *lanes 1* and *9*). The transgene transcript was obviously also not detectable in salivary gland tissue of *B6129-Tgn(MMTV-LTR/Cre)1Mam* transgenic mice (*MMTV-LTR/Cre*$^{+/+}$; Fig. 3*A*, *lane 10*). Immunofluorescence experiments using the well-characterized *PLAG1*-specific antibody PEM-195 revealed a widespread nuclear staining in the salivary gland tumors of about 5-week-old P1-Mcre mice. Mostly, such staining comprised whole lobules (Fig. 3, *B1* and *B2*). Similar staining was seen in tumors of P2-Mcre mice (data not shown).

Expression up-regulation of other genes resulting from activation of *PLAG1* overexpression. Previously, we showed that the *IGF-II* gene is strongly up-regulated in human pleomorphic salivary gland adenomas with *PLAG1* overexpression, (12). Moreover, recent microarray studies revealed that the human *IGF-II* gene is a genuine target gene of *PLAG1* (15). To test whether *PLAG1* overexpression in the salivary glands of P1-Mcre and P2-Mcre mice also led to induction of *Igf2* expression, Northern blot analysis of the same salivary gland tissue specimens was done with an *Igf2*-specific probe. In all cases in which *PLAG1* transcripts were detectable, the multiple *Igf2* transcripts (4.8, 3.8, and 3.6 kb in size) were clearly expressed (Fig. 3*A*, *lanes 2-8*). Furthermore, we found that expression of another gene within the imprinted *Igf2* gene cluster on mouse chromosome 7 (i.e., the *H19* gene that encodes an untranslated RNA) was also strongly up-regulated in the salivary gland tumors of these mice but not in control salivary gland specimens (Fig. 3*A, lanes 1 and 9).* Of interest to note is that expression of another but similarly imprinted gene cluster is also affected by *PLAG1*. This cluster, located on mouse chromosome 12, contains the genes *Dlk1* and *Gtl2*, of which *Gtl2* encodes an untranslated RNA, like *H19*. As can be seen in Fig. 3*A*, a 1.8-kb *Dlk1* and a

7-kb *Gtl2* transcript are expressed in the salivary gland tumors (Fig. 3*A*, *lanes 6-8*) but not in the control glands (Fig. 3*A*, *lanes 9* and *10*). These results clearly establish the up-regulation of expression of several genes in two distinct, but structurally somewhat similar organized, imprinted gene clusters.

Early tumoral stages of the salivary glands in P1-Mcre mice. Submandibulary glands of P1-Mcre mice and those of the wild-type controls were examined microscopically at weekly intervals starting at 1 week after birth. Histologic analysis of salivary glands of P1-Mcre mice ages 1 week (one case), 2 weeks (one case), and 4 weeks (four cases) revealed multiple foci most likely representing early tumoral stages. Most of the lobules show these abnormalities. They contain epithelial cells with a large basal oval nucleus and basophilic cytoplasm that are densely packed around a small empty lumen. Dilated ducts with a flat epithelium, sometimes multilayered, are also visible. Mitotic figures were regularly observed (Fig. 4*A*) and BrdUrd labeling revealed active proliferation in early as well as in later tumoral stages (Fig. 4*B*).

Pleomorphic adenoma in P1-Mcre and P2-Mcre mice. In P1-Mcre mice, the tumors were clearly visible macroscopically in the ventral neck region after 5 weeks. Lobulated tumors were observed bilaterally at necropsy, consisting of a large white, encapsulated mass. The largest tumors possessed hemorrhagic parts. Similar observations were made at dissection of salivary gland tumors of six P2-Mcre mice (tumor 1-6, respectively, 11, 16, 23, 38, 36, and 70 weeks old at sacrifice), but it should be emphasized that those tumors arose after a much longer latency period (several months) and not bilaterally.

Histologically, all salivary gland tumors from the P1-Mcre (12 tumors studied) as well as from the P2-M-Cre mice (six tumors) emerged from the submandibulary gland. The tumors show histologic features that are reminiscent of those of human pleomorphic adenomas. A thin capsule was usually visible, sometimes with capsular ingrowth of tumoral lobes. The tumors were composed of epithelial structures and myoepithelial cells (Fig. 4*C*). Epithelial components of the tumors showed a high

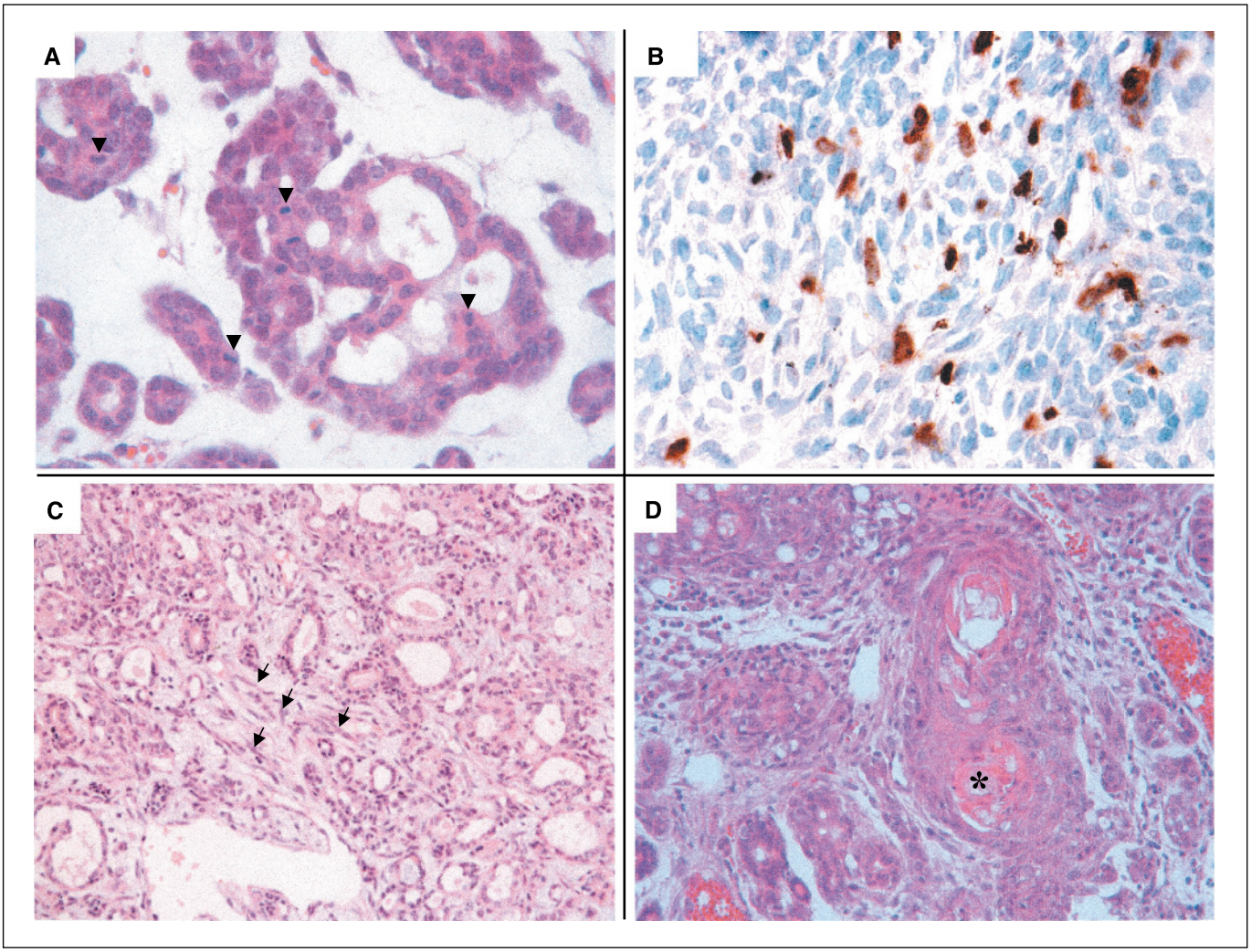

Figure 4. *A,* early tumoral stage in a 4-week-old P1-Mcre mouse, submandibulary gland, H&E. Original magnification, ×400. Irregular, tubular structures, with sometimes a multilayered epithelium are observed in loose stroma. Epithelial cells lining these structures are irregular, with nuclei of variable size, and loss their normal polarity. Mitotic figures are numerous (*arrowheads*). *B,* BrdUrd incorporation into salivary gland tumor cells of a 5-week-old P1-Mcre mouse to visualize proliferation. Original magnification, ×400. *C,* pleomorphic adenoma features in a salivary gland tumor of a 23-week-old P2-Mcre mouse, H&E. Original magnification, ×200. The tumor is composed of epithelial structures (tubes, *ribbons*) in a myxoid stroma in which a few spindle or stellate cells are observed (*arrows*). *D,* pleomorphic adenoma features in a salivary gland tumor of an 8-week-old P1-Mcre mouse, H&E. Original magnification, ×200. Squamous differentiation with keratin pearls (*asterisk*).

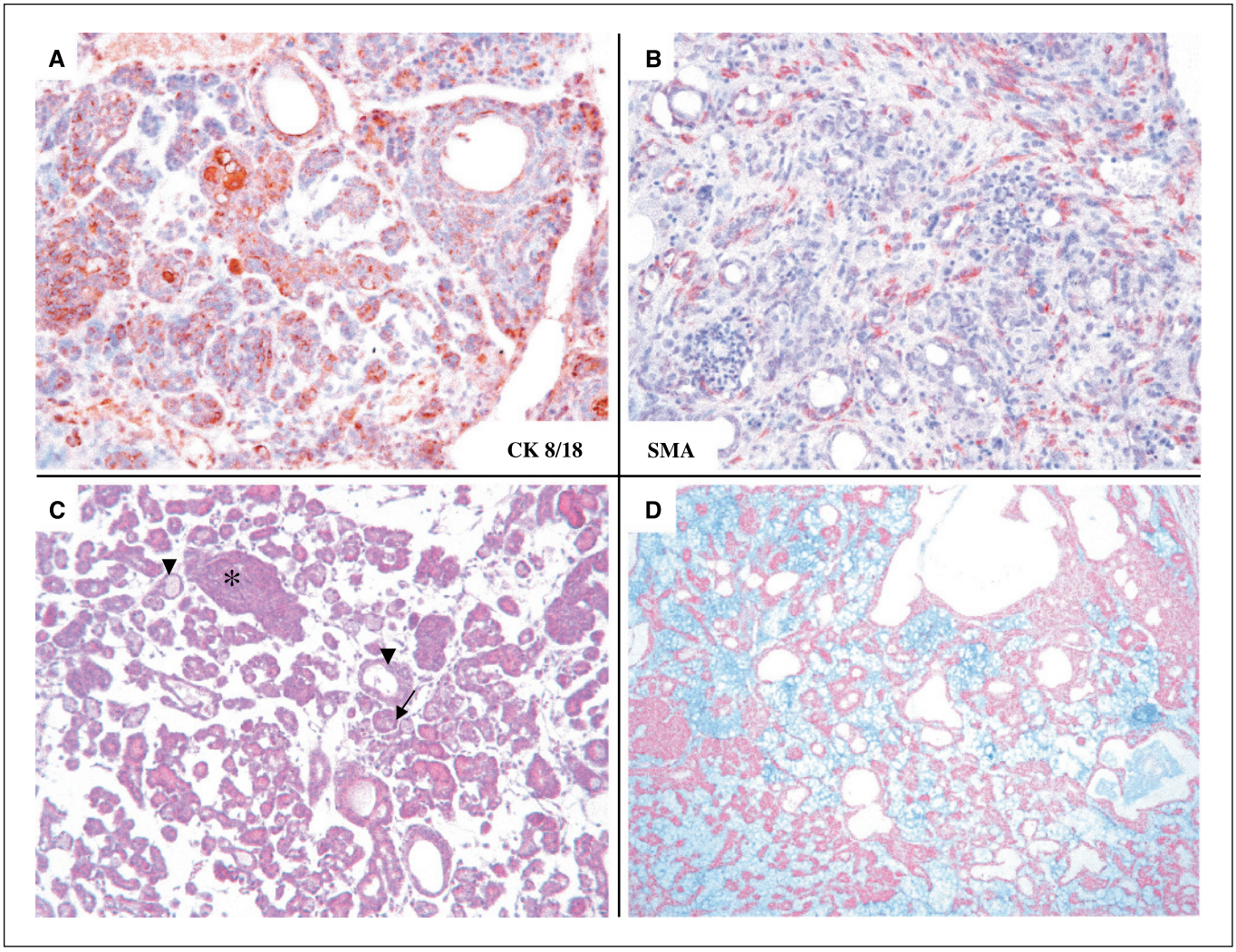

Figure 5. *A,* immunohistochemical staining, using an anti-CK8/18 antibody, of a salivary gland tumor of a 5-week-old P1-Mcre mouse. Original magnification, ×200. Most of the tumoral cells in the ductal structures, solid sheets or areas of squamous differentiation are positive for CK8/18. *B,* immunohistochemical staining, using an anti-SMA antibody, of a salivary gland tumor in a 34-week-old P2-Mcre mouse. Original magnification, ×200. Spindle and stellate cells are positive, as well as some cells underlying the epithelial tumoral acini. *C,* pleomorphic adenoma features in a salivary gland tumor of a 5-week-old P1-Mcre mouse, H&E. Original magnification, ×200. The tumor is composed of epithelial structures in a loose stroma. Components show a high morphological diversity, with acinar (*arrow*) and ductal structures, which are empty or filled with fluid (*triangles*), lined by one or more cellular rows, or solid sheets of cells (*asterisk*). *D,* Alcian Blue staining (pH 2.5) of a salivary gland tumor of a 2-month-old P1-Mcre mouse. Original magnification, ×100. Myxoid stroma surrounding epithelial ducts and cysts is stained pale blue.

morphologic diversity, because within the same tumor, ductal structures lined by one or more cellular rows, cysts filled with fluid, papillary patterns, solid sheets of cells, sebaceous differentiation, and frequently squamous areas with keratinization (Fig. 4*D*) could be observed. Spindle and stellate cells, more or less numerous, that have the morphologic characteristics of myoepitelial cells were closely associated with epithelial islands. Immunohistochemical analysis showed that the epithelial parts of the tumors were strongly positive for anti-cytokeratin 8/18 staining, as illustrated in Fig. 5*A*. A high proportion of the spindle or stellate cells were positive after anti-SMA staining, as illustrated in Fig. 5*B*. The myxoid stroma (Fig. 5*C*) seemed alcianophilic, after Alcian Blue staining at pH 0.2, 1, and 2.5 (Fig. 5*D*) and mucus secreting, PAS-positive cells, were numerous. In older tumors of the P1-Mcre mice (8 weeks to 4 months, eight tumors studied) and of the P2-Mcre mice (six tumors), malignant features were observed. These included large areas of necrosis (Fig. 6*A*), hemorrhages and cellular polymorphism. Besides some regions with typical characteristics of pleomorphic adenomas, some of the older tumors of the P1-Mcre and P2-Mcre mice contained regions resembling carcinomas. In 3 tumors of the P1-Mcre mice (9, 14, and 15 weeks old) and one tumor of the P2-Mcre mice (23 weeks old), lung metastases were observed. These showed the same histologic characteristics of the primary salivary gland tumor as illustrated in Fig. 6*B* and *C*.

Mammary gland tumors in P2-Mcre mice. Expression of the *PLAG1* transgene in the P1-Mcre mice was not restricted to the salivary glands, which is consistent with the Cre expression pattern reported for the B6129-Tgn(MMTV-LTR/Cre)1Mam transgenic mice (19). Normal mammary glands of the P2-Mcre mice also expressed *PLAG1* transcript levels detectable by Northern blot analysis (Fig. 2*C*, *lane 3*). About 8% of the P2-Mcre mice developed tumors of the mammary gland with a latency period of about 1 year. These tumors shared some histologic features with the salivary gland tumors (Fig. 6*D*). They were also composed of

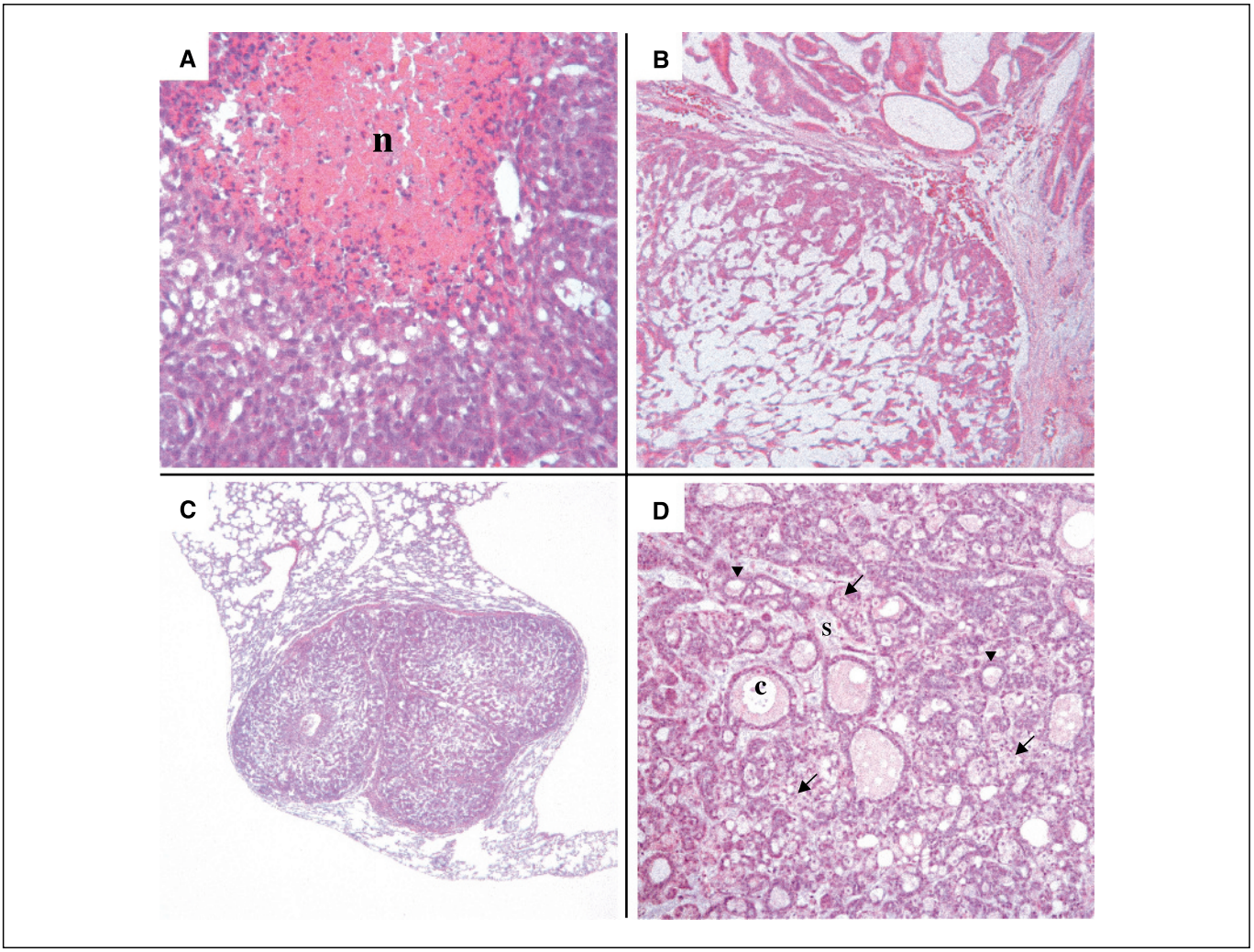

Figure 6. *A,* malignant features in an 8-week-old P1-Mcre mouse salivary gland tumor, H&E. Original magnification, ×200. Necrotic area (*n*). Salivary gland tumor in a 2-month-old P1-Mcre mouse, with fusiform cells. Original magnification, ×200. *C,* lung metastasis of the salivary gland tumor shown in (*B*), with similar morphologic features. *D,* mammary gland tumor in a 34-week-old P2-Mcre mouse, H&E. Original magnification, ×100. The tumor shows variable patterns: tubes (*arrowheads*) or cysts (*c*) that can be filled with an eosinophilic material, ribbons, among a myxoid pale blue stroma (*s*). Cells undergoing sebaceous differentiation are frequent (*arrows*).

epithelial and myoepithelial cells in a myxoid stroma. They also showed solid areas of poorly differentiated basophilic epithelial cells, with a high mitotic index. A sebaceous differentiation was often seen. Mammary gland tumors were not observed in P1-Mcre mice, because these mice had to be sacrificed for ethical reasons before the age of 4 months because of their enormous salivary gland tumors. Nevertheless, some histologic abnormalities were found in mammary glands of P1-Mcre mice, showing abnormal differentiation with sebaceous differentiation, multilayered cells around the ducts and cystic structures (data not shown).

Discussion

It is well established that overexpression of the developmentally regulated *PLAG1* gene occurs frequently in various benign human solid tumors, such as pleomorphic salivary gland adenomas (1, 2), lipoblastomas (3, 4), and also in the childhood neoplasia hepatoblastoma (6). In the first two tumor types, it has been suggested that overexpression of *PLAG1* is directly responsible for the formation of this type of tumors. This is mainly based on the causal link between the cytogenetically and molecularly well-defined recurrent chromosome 8q12 aberrations and the invariably strong up-regulation of *PLAG1* expression in such tumors. Direct solid proof, however, is still lacking (1). Recently, *PLAG1* and *PLAGL2* have also been found implicated in AML (7, 9). Furthermore, experiments have clearly established the *in vitro* oncogenic potential of *PLAG1* (16). Altogether, these findings have demarcated the *PLAG1* gene as a versatile proto-oncogene and attracted considerably attention to the study of its function. As an approach to assess *in vivo* the consequences of overexpression of *PLAG1* in the complex genetic background of a mammalian model system, two independent *PLAG1* transgenic mouse strains were developed and various features are reported here.

Our *PLAG1* transgenic mouse strains constitute a valuable model to study pleomorphic salivary gland tumorigenesis. With the P1-Mcre model, tumor progression can easily be followed in the time. Indeed, already after 1 week, 100% of the salivary glands of these mice contain early tumoral stages in the ductal region. It should be

noticed that the expression pattern of the Cre recombinase in the salivary glands of B6129-Tgn(MMTV-LTR/Cre)1Mam transgenic mice is also limited to these regions (19). Already after 5 weeks, P1-Mcre mice develop pleomorphic adenomas, which share many histopathologic features with human pleomorphic adenomas. Like the human tumors (26), they are mixed tumors containing epithelial and myoepithelial cells. Epithelial elements are highly polymorphic within the tumor, because a tubular, acinar, cystic, papillary, or solid pattern with frequent squamous differentiation can occur. Tumors are embedded in a pale myxoid stroma in which fusiform to stellate myoepithelial SMA-positive cells are seen. Usually, the tumor is encapsulated and epithelial structures are well differentiated. In some of the older P1-Mcre and P2-Mcre mice, besides typical features of pleomorphic adenoma also malignant characteristics were observed, including areas of necrosis, hemorrhages, and metastasis to the lungs. Some regions even resembled carcinomas. Consistently, it has been well described that also human pleomorphic adenomas can progress to malignancy. In humans, 2% to 17% of the pleomorphic adenomas can progress to carcinomas ex pleomorphic adenomas (27–29). Sometimes, the pleomorphic part of the tumor is only very small. The malignant characteristics observed in our mouse model suggest a similar malignant progression.

Although the *PLAG1*-induced salivary gland tumors in the transgenic mouse strains possess the basic hallmarks of human pleomorphic adenoma, a major difference with the human tumors pertains to the aggressive growth of the tumors, at least for those of the P1-Mcre strain. The engineered genetic intervention and/or the different genetic background of the mouse can most likely explain this difference. Human pleomorphic adenomas of the salivary glands are assumed to arise following a genetic accident, presumably in a single progenitor cell, and leading to a clonal activation of *PLAG1* expression long after birth. In contrast, in the *PLAG1* transgenic mouse model system, *PLAG1* activation starts already during embryonic development and occurs multiclonally and at an early phase in the epithelial duct cells of the developing salivary glands. We cannot exclude that some of the alterations observed in the early stages are developmental changes. In any case, a major phenotypic effect of overexpression of the *PLAG1* transgene is the onset of fast growing salivary gland tumors.

There are some differences between our two *PLAG1* transgenic mouse strains. First, in P1-Mcre mice lesions occur bilaterally and in most of the lobules. Moreover, 100% of the mice develop tumors. In contrast, however, in the P2-Mcre strain, the tumor frequency is low and the tumorigenic process has a long latency period (several months). Moreover, such tumors arose invariably locally and unilaterally. Zhao et al. reported a similar variation in tumor incidence in *PLAG1* transgenic mouse strains (30). Second, crossing of the PTMS1 with PGK-cre mice to generate PGK-Cre$^{+/-}$/Plag1$^{+/-}$ mice resulted in embryonic lethality, due to early and ubiquitous expression of the transgene. Embryonic lethality was not observed in similar experiments with PTMS2, probably because there is almost no expression of the *PLAG1* transgene in the offspring. We assume that the differences in expression of the *PLAG1* transgene in the two founder strains are due to the effect of the integration site of the transgene (31).

Of interest to note, finally, is that after a latency period of about 1 year, 8% of the P2-Mcre mice developed mammary gland tumors, which possessed histologic features similar to those observed in the salivary gland tumors. Similar observations were not made with the P1-Mcre mice, because they developed fast growing salivary gland tumors and had to be sacrificed before mammary gland tumors could arise. For this strain, more direct and specific targeting of expression of the *PLAG1* transgene to the mammary gland, excluding the salivary gland, seems the strategy to follow. It should be mentioned that *PLAG1* expression has never been described in primary human mammary gland tumors.

In conclusion, our studies clearly establish first that ubiquitous overexpression of the *PLAG1* proto-oncogene leads to lethality during embryonic life. Second, a direct link was shown between overexpression of the *PLAG1* proto-oncogene and tumorigenesis. Third, activation of genes from independent imprinted gene clusters (*Igf2*, *H19*, *Dlk1*, and *Gtl2*; refs. 32–34) may play a role in this tumorigenic process, although the precise nature of it remains to be established. In human salivary gland pleomorphic adenomas, the *IGF-II* gene, which is an established important element in tumor cell growth (35), has been identified as a genuine target gene of *PLAG1* (12, 15). On the other hand, it is possible that the mechanism of imprinting is involved. Finally, the PTMS1 and PTMS2 strains described here have been designed in such a way that activation of expression of the *PLAG1* transgene is Cre mediated. With the availability of mouse strains with different tissue-specific Cre expression (36), valuable models can now be obtained to study the contribution of *PLAG1* to the development of other types of tumors.

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