aP2-Cre-mediated expression activation of an oncogenic *PLAG1* transgene results in cavernous angiomatosis in mice

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Abstract. The developmentally regulated *PLAG1* proto-oncogene has been implicated in the development of various human tumor types, such as pleomorphic salivary gland adenomas, lipoblastomas, hepatoblastomas and AML. In previous studies, we generated two independent *PLAG1* transgenic founder strains, PTMS1 and PTMS2, in which *PLAG1* could be activated via Cre-mediated excision of a stop cassette. With these founders, PLAG1-induced tumor formation in salivary and mammary glands of mice was studied. To further delineate the oncogenic spectrum of PLAG1 in mice, we induced aP2-Cre-mediated overexpression of *PLAG1* in offspring from crossbreeding PTMS1 mice with *aP2-Cre* transgenic mice. More than 80% of *aP2-Cre*$^{+/-}$/*PLAG1*$^{+/-}$ (P1-ACre) mice developed a vascular tumor type within one year, which could be classified histopathologically as cavernous angiomatosis. The lesions occurred in various regions of the mouse body but almost exclusively in the immediate surrounding of fat cells. Validation of available PLAG1-induced gene expression profiling data, using targeted tissues, revealed that expression activation of *PLAG1* is functional because it leads to elevated levels of PLAG1 target gene transcripts in those tissues, such as for instance those of *H19, Dlk1*, and *Igf-2*, similarly as observed in PLAG1-induced salivary and mammary gland tumors. In conclusion, we present the first evidence that links *PLAG1* to the molecular pathogenesis of vascular tumorigenesis, known as cavernous angiomatosis, with the possible involvement of Igf signaling and, moreover, further delineate the oncogenic spectrum of *PLAG1* in mice, increasing the potential of this transgenic mouse tumor model system for research and therapeutic drug testing.

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Introduction

Pleomorphic adenoma gene 1 (PLAG1) is a developmentally regulated proto-oncogene on human chromosome 8q12, whose oncogenic activation is a crucial event in the development of several human tumors (see recent review by Van Dyck *et al* (1). These include so far pleomorphic adenomas of the salivary glands (2,3), hepatoblastomas (4), AML (5), and lipoblastomas (6,7). The oncogenic capacity of *PLAG1* has been confirmed in *in vitro* experiments, which also established IGF-II and IGF-IR as key pathway elements, similarly as in many human tumors (8). Recent comparative gene expression profiling, using *PLAG1*-transduced 293 cell lines as well as *PLAG1*-expressing human pleomorphic salivary gland adenomas, confirmed the consistent induction of IGF-II by PLAG1 (9). To define the oncogenic spectrum of PLAG1 in the context of a complex organism, conditional *PLAG1* transgenic mouse strains were developed. In these, activation of overexpression of the *PLAG1* transgene as well as the tissue distribution of such overexpression can be manipulated by Cre-mediated activation and targeted expression, respectively (2). Effective use of the Cre-*loxP* system for gene modification requires transgenic mouse strains with well-defined patterns of *Cre* expression. Two independent *PLAG1* transgenic mouse strains (PTMS1 and PTMS2) were used in crossbreeding experiments to target overexpression of the *PLAG1* gene to the salivary and mammary glands. Such targeted expression activation of *PLAG1* in the afore-mentioned organs was achieved by crossbreeding B6129-Tgn(MMTV-LTR/Cre)1Mam (10) transgenic mice (*MCre*) with PTMS1 and PTMS2 mice, which lead to the development of tumors in the targeted organs in the *PLAG1*$^{+/-}$/*MCre*$^{+/-}$ offspring (2). Such tumors expressed a variety of PLAG1 target genes at elevated levels, including Igf-2 (2,11). Complete inactivation of the *Igf-2* gene in *PLAG1*$^{+/-}$/*MCre*$^{+/-}$ offspring led to a noticeable delay in tumor appearance (Declercq, unpublished data). This, together with the *in vitro* data discussed above, suggest that Igf-signaling indeed seems critical in PLAG1-induced tumors.

In light of the above, exploration to further define the oncogenic spectrum of PLAG1 in mice is of interest, since it could lead to a broader relevance of this transgenic mouse model in tumor research. In this context, the *aP2-Cre* transgenic FVB mouse strain (12) was selected for further crossbreeding studies with PTMS1 mice. In these *aP2-Cre* transgenic FVB mice, a 5.4-kb DNA fragment encompassing promoter/enhancer sequences of the *aP2* gene drives expression of the *Cre* gene. The promoter/enhancer sequences of the *aP2* gene, which is also known as *A-FABP* (adipocyte fatty acid binding protein gene) or *FABP4* (fatty acid binding protein 4 gene), was selected because of its interesting expression pattern, both postnatally and prenatally. Studies have indicated that, postnatally, transgene expression driven by these *aP2* promoter/enhancer sequences is predominantly adipose tissue-specific, including brown adipose tissue (BAT) and white adipose tissue (WAT) (13,14). Although confirming such an increasingly more adipose-tissue-specific expression spectrum after birth, recent studies by Urs *et al*, however, pointed also towards a broader activity of these promoter/enhancer sequences, especially prenatally (15). During embryonic development, selective expression of an *aP2/FABP4-Cre* transgene was observed in non-adipogenic tissues, especially in cells sharing a common lineage with adipocytes such as chondrocytes, myocytes, neurons, and osteocytes; all cells arising from a common mesenchymal stem cell progenitor population. Furthermore, expression of the *aP2/FABP4-Cre* transgene was also observed in developing bone marrow (15). Interestingly, consulting the Reference Database for Gene Expression Analysis (http://157.82.78.238/refexa/main_search.jsp), we found suggestive evidence for *aP2/FABP4* expression in dermal microvasculature endothelial cells. Furthermore, *aP2/FABP4* was found to be expressed in a subpopulation of cells, associated with renal microvasculature, possibly including endothelial cells (16). It is well established that, for optimum functioning, adipose tissue requires extensive vascularization and the microvasculature in this tissue is known to be well developed. In light of the above, the selected *aP2* promoter/enhancer sequences are likely to drive *Cre* expression, and consequently *PLAG1* proto-oncogene activation, in adipose tissue, in particular in its pre-adipocytes, mature adipocytes and endothelial cells, cell types that are known to mutually and very intimately interact with each other not only during development but also after birth.

In the present studies, we first performed experiments to obtain stronger evidence that the *aP2* gene can indeed be expressed in endothelial cells. To achieve this objective, we compared *aP2* gene expression levels in several endothelial cell lines to those in pre-adipocytes that were induced to differentiate to adipocytes. The occurrence of increasingly higher *aP2* gene expression levels in these differentiating adipose cells is well established. After establishing that endothelial cells can express the *aP2* gene, we subsequently used *aP2-Cre* transgenic mice (12,13) in crossbreeding experiments with our *PLAG1* transgenic mice (PTMS1 strain) to activate expression in selected tissues of the *PLAG1*$^{+/-}$/*aP2-Cre*$^{+/-}$ (P1-ACre) offspring. The phenotypic lesions that became apparent were characterized histopathologically. Furthermore, molecular studies were performed to test expression of PLAG1 target genes in various targeted tissue specimens to test the functionality of the aP2-Cre-mediated activation of the *PLAG1* proto-oncogene.

Materials and methods

aP2-Cre-mediated activation of PLAG1 expression in transgenic mice. The generation of the afore-mentioned *PLAG1* transgenic founder lines, PTMS1 and PTMS2, has been reported recently (2). PTMS1 mice (*PLAG1*$^{+/-}$) were crossed with *aP2-Cre*$^{+/-}$ transgenic FVB mice (12,13) to generate *aP2-Cre*$^{+/-}$/*PLAG1*$^{+/-}$ (P1-ACre) offspring. In P1-ACre offspring, Cre-mediated excision of a stop-cassette was achieved, which was inserted between the promoter region and the PLAG1 encoding sequences of the original *PLAG1* transgenic construct to block expression of *PLAG1*. Removal of the stop cassette allows expression of the *PLAG1* transgene in all cell types in which the selected *aP2* promotor/enhancer sequences drive transcription of the Cre-encoding sequences.

Isolation of genomic DNA from mouse tail tips. Tail tips of ~2 mm were incubated overnight at 56˚C in 200 μl PCR tissue homogenization buffer [50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2 mM $MgCl_2$, 0.1 mg/ml gelatine, 0.45% NP-40, 0.45% Tween-20, 120 μg/ml proteinase K], followed by a 10-min incubation at 95˚C to inactivate proteinase K. One μl of the homogenates was used as template in the PCR reactions.

Genotyping. Genotyping of mice to identify *PLAG1* transgenic offspring was performed by PCR analysis of tail DNA using oligonucleotide primers POS-1599 (5'-TTCTCAAGCATCG TCATCAT-3') and ß-globin (5'-AAAATTCCAACACACTA TTGC-3') at an annealing temperature of 58˚C. Identification of mice, carrying the *Cre* transgene, was performed similarly by PCR analysis using oligonucleotide primers Cre1 (5'-CCT GTTTTGCACGTTCACCG-3') and Cre3 (5'-ATGCTTC TGTCCGTTTGCCG-3').

Quantitative RT-PCR. Total RNA was isolated from tumor lesions and from tissues of non-tumor bearing control littermates (PTMS1 as well as *aP2-Cre*$^{+/-}$ transgenic mice) using the Qiagen RNeasy lipid tissue midi kit or Rneasy lipid tissue mini kit, as described by the manufacturer. To compare expression levels of *aP2* in endothelial cells, relative to those in differentiating 3T3-F442A preadipocytes, total RNA from 3T3-F442A preadipocytes was prepared using the Macherey-Nagel Nucleospin kit, according to the manufacturer's protocol. Total RNA (5 μg) was reverse transcribed using random hexamer primers and M-MLV reverse transcriptase from Invitrogen in a volume of 20 μl. cDNA from the endothelial cell lines fEnd5 (17), PlKO [a home-made primary endothelial cell line, derived from neonatal mouse hearts as described elsewhere (18)], and E2 (19) was a kind gift from Dewerchin *et al*, VIB Center for Transgene Technology and Gene Therapy, K.U. Leuven, Belgium. qRT-PCR was performed in the MyIQ 2.0 system (Bio-Rad) using qPCR Master Mix for SYBR Green I detection and fluorescein as internal standard (Eurogentec), in accordance with the manufacturer's guidelines.

The relative amount of gene expression was calculated using the comparative C_t method ($2^{-\Delta\Delta Ct}$). qRT-PCR for *GAPDH* was used as a reference gene (forward primer: (5'-ATGGCC TTCCGTGTTCCT-3' and reverse primer: 5'-CAGGCGGCA CGTCAGAT-3'). Gene-specific primers were designed for the following genes: *PLAG1* (forward 5'-CCACGTTTCCAT CAAGCTTTTC-3' and reverse 5'-AGGCAGCCTGCACCT GAG-3'), *Igf-2* (forward 5'-TGTCTGTTCGGACCGCG-3' and reverse 5'-GTTGGCACGGCTTGAAGG-3'), *H19* (forward 5'-AAGAGCTCGGACTGGAGACTAGG-3' and reverse 5'-GGCACATCCACCTCTGCTG-3'), *Dlk1* (forward 5'-TGCGCGTCCTCTTGCTC-3' and reverse 5'-CATTCAG CCCCATAGGTGCT-3'), *Gtl2* (forward 5'-CTCCAACCCA CTGCTTCCTG-3' and reverse 5'-AGCGAGAGCCGTTCG ATG-3'), and *aP2* (forward 5'-ACACGAGATTTCCTTCAAA CTG-3' and reverse 5'-TAACACATTCCACCACCAGCTT-3').

Histopathology. Tumor lesions were dissected, fixed overnight in 4% paraformaldehyde and embedded in paraffin using routine procedures. Paraffin sections (6 μm) were stained with hematoxylin and eosin, according to standard procedures, for histopathological evaluation.

Cell lines, culture and differentiation conditions, and morphology. Murine 3T3-F442A (ECACC 70654) preadipocytes (20,21) were grown in basal medium (Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% bovine calf serum (BCS) and 5% penicillin/streptomycin). To induce differentiation, 3T3-F442A cells were seeded at a cell density of 3.6x10^4 cells/cm^2 and grown to confluence in DMEM with 10% fetal bovine serum (FBS). When cell cultures reached confluence, medium was removed and fresh medium added. After two days, cells were treated for 2 days with induction medium [DMEM supplemented with 10% FBS, 17 nM insulin, 2 nM triiodothyronine (T3), 100 nM dexamethasone (DEX) and 100 μM methylisobutylxanthine (collectively designated IBMX)]. Cultures were then switched to a differentiation medium (DMEM containing 10% FBS, 17 nM insulin and 2 nM T3) for 2 weeks. During the differentiation procedure, medium was renewed every 2-3 days.

Results

Expression of the aP2 gene in endothelial cells. Since the initial discovery that the *PLAG1* proto-oncogene is causally implicated in pleomorphic adenomas of the salivary glands with chromosome 8q21 aberrations (3), *PLAG1* has not only been implicated in additional human tumors, as outlined above, but experiments with *PLAG1* transgenic mice have revealed its even broader tumorigenic capacity (2). The latter experiments were performed with *PLAG1* transgenic mouse strains, which were developed in such a way that the *PLAG1* proto-oncogene could be specifically activated in and targeted to selected cells via Cre-mediated excision of a stop cassette in the *PLAG1* transgene (see Materials and methods). In an attempt to further delineate the oncogenic spectrum of the *PLAG1* proto-oncogene in mice, crossbreeding experiments between PTMS1 and *aP2-Cre* transgenic mice seemed a promising possibility based on the intriguing expression profile of the *aP2* gene pre- and postnatally. An important question that we wanted to address first pertained to the issue whether the *aP2* gene can indeed be expressed in endothelial cells, as tentatively suggested in the literature. Therefore, expression of *aP2* in different immortalized endothelial cell lines, including the E2, fEnd5, and PlKO cell line, was investigated by qRT-PCR (Fig. 1). Since expression of *aP2* is known to increase during induced differentiation of pre-adipocyte 3T3-F442A cells to fully differentiated adipocytes (22), the *aP2* mRNA levels in the selected endothelial cell lines were compared to those observed in 3T3-F442A pre-adipocyte cells, at different differentiation stages. From these experiments, it became clear that the *aP2* gene was indeed expressed in the tested endothelial cell lines and noticeably at readily detectable levels, which even exceeded those in undifferentiated 3T3-F442A pre-adipocyte cells (3T3d-0). This observation indicated that our planned crossbreeding experiments between the PTMS1 and *aP2-Cre* transgenic mice could lead to activation of the *PLAG1* proto-oncogene in various cell types, including endothelial cells. Expression in the latter cell type is of importance in light of the extensive (micro)vasculature in many tissues, e.g. adipose tissue.

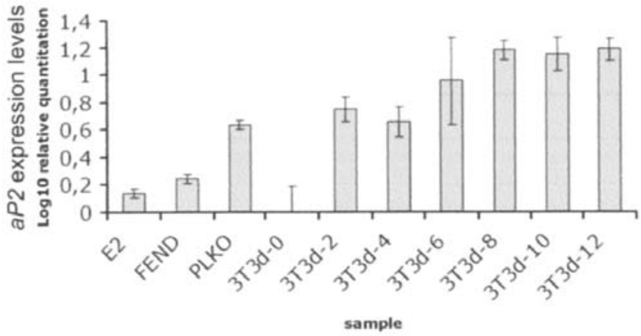

Figure 1. Expression of the *aP2* gene in endothelial cells. In qRT-PCR experiments, expression levels of *aP2* mRNA in endothelial cell lines E2, FEND and PLKO were compared, relative to *GAPDH* levels. 3T3-F442A preadipocytes, showing an increase in *aP2* mRNA levels along their differentiation into adipocytes (days 0-12), were included as positive controls. The results establish that *aP2* mRNA is expressed in various endothelial cell lines, as indicated.

Expression activation of the PLAG1 proto-oncogene upon crossbreeding PTMS1 and aP2-Cre$^{+/-}$ mice. Intercrossing of PTMS1 mice (*PLAG1*$^{+/-}$) with *aP2-Cre*$^{+/-}$ transgenic mice resulted in offspring with an *aP2-Cre*$^{+/-}$/*PLAG1*$^{+/-}$ (*P1-ACre*) genotype in a Mendelian fashion. To establish activation of expression of the *PLAG1* transgene resulting from Cre-mediated excision of the stop-cassette, quantitative RT-PCR was performed on RNA from fat-rich tissues (abdominal fat and mammary gland tissue, which is rich in adipose tissue) of the *P1-ACre* offspring. Expression of the *PLAG1* transgene could indeed be detected in such tissues (data not shown).

Functionality of PLAG1 proto-oncogene expression activation. In previous micro-array-based expression profiling studies (9), it was found that PLAG1 induces a variety of target genes to strongly elevated levels. The most extensively studied PLAG1 target gene is the Igf-2 (2,11), which is also

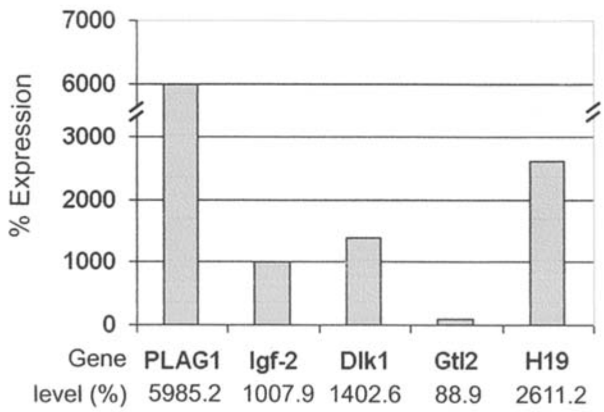

Figure 2. Functionality assessment of aP2-Cre-mediated *PLAG1* expression activation in P1-ACre mice. Expression levels of the PLAG1 target genes *Igf-2, H19, Dlk1* and *Gtl2* in *PLAG1* expressing abdominal fat tissue specimens of P1-ACre mice were obtained by qRT-PCR experiments and are compared to the levels in corresponding tissue specimens of control littermates. Expression levels in abdominal fat tissue specimens of control littermate mice is set as 100%.

known to play a critical role in the formation of many tumors. To assess the functionality of aP2-Cre-mediated *PLAG1* activation, expression of *Igf-2* and some other previously reported PLAG1 target genes was tested in PLAG1 expressing tissues of P1-ACre mice using qRT-PCR. In this context, our studies initially focused upon abdominal fat tissue of P1-ACre mice without any visible vascular lesions and compared those to levels in abdominal fat tissue of control littermates. As already reported above, *PLAG1* expression is strongly elevated in abdominal fat tissue of P1-ACre mice as compared to those in similar tissue of control littermates (see also Fig. 2). Furthermore, expression of *Igf-2, H19* and *Dlk1* appeared also clearly upregulated in *PLAG1* expressing abdominal fat tissue of P1-ACre mice as compared to the corresponding tissues of control littermate mice (Fig. 2). Expression studies, using PLAG1-overexpressing adipose-rich tissue specimens from mammary glands, gonads, the scapulae region, or other adipose-rich regions in which lesions, corresponding to cavernous angiomatosis, frequently originated, also yielded similar results (data not shown). Altogether, these results indicate that aP2-Cre-mediated *PLAG1* activation results in

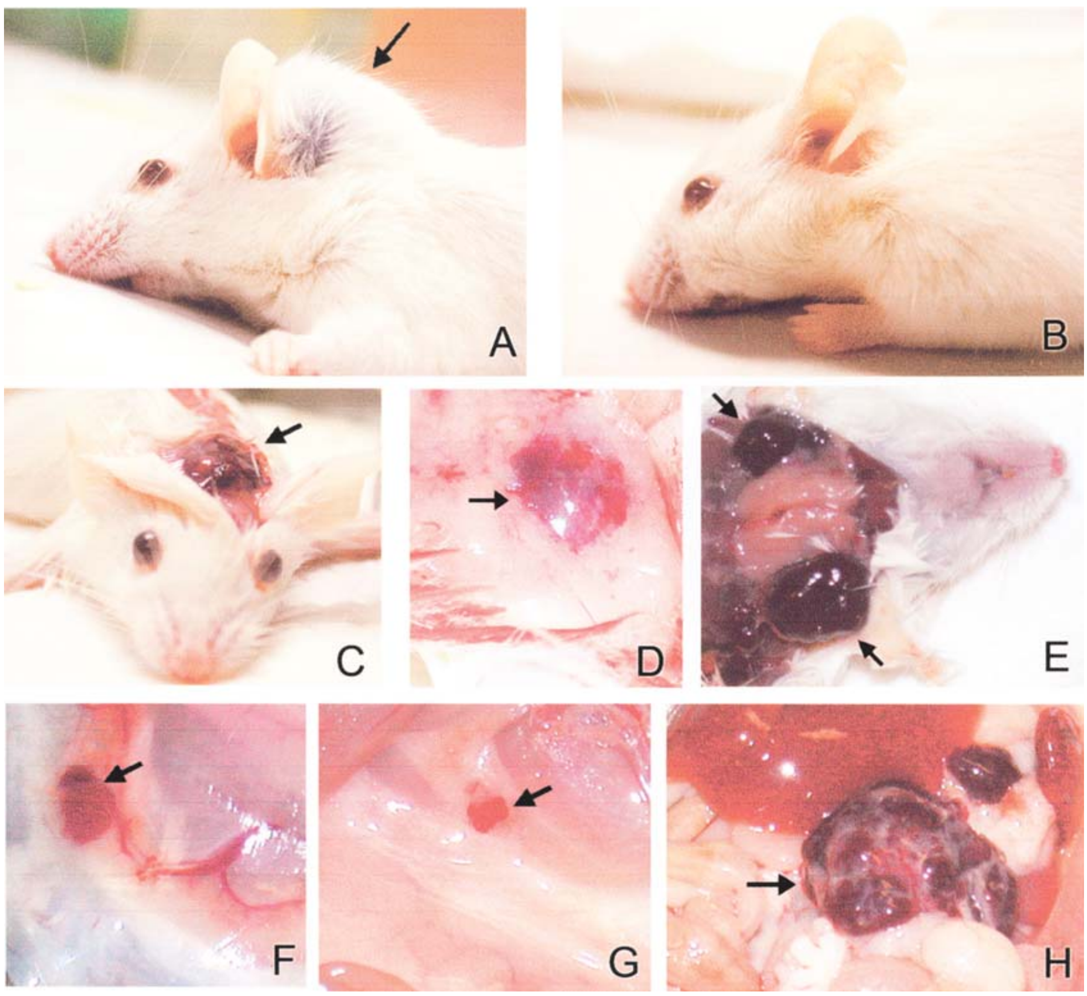

Figure 3. Vascular lesion or malformation development at different sites in P1-ACre transgenic mice. Vascular lesions or malformations, arising in P1-ACre mice (A), are often visible before autopsy (arrow); (B) Control littermate mouse. Upon autopsy, lesions can be found at different sites within the body, such as (C) head region (on top of skull), (D) lower abdominal region (near tail base), (E and F) mammary gland region, (G) abdominal wall region (near peritoneum), (H) region harboring abdominal fat tissue.

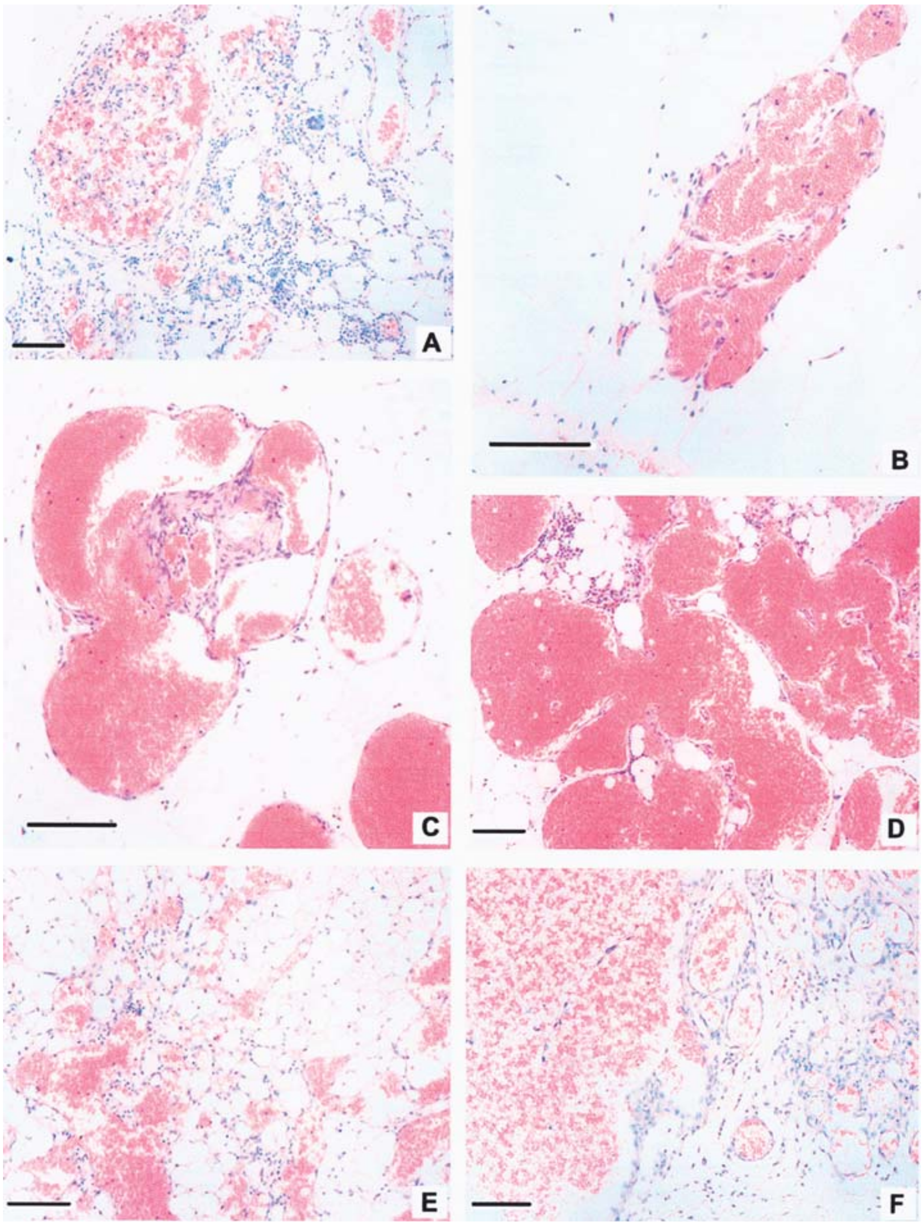

Figure 4. Histopathological evaluation reveals that aP2-Cre-mediated PLAG1-induced expression activation leads to cavernous angiomatosis. P1-ACre mice develop blood-filled lesions in fat-rich tissues, such as (A and B) in fat tissue in the lower abdominal region (near tail base); (C and D) in gonadal fat tissue; (E) in mammary gland tissue; (F) in abdominal fat tissue. Such lesions were never observed in littermate control mice (*aP2-Cre*$^{-/-}$ and/or *PLAG1*$^{-/-}$). Note the nature of large vascular caverns filled with blood cells.

functional PLAG1 expression and tentatively suggest that PLAG1-induced Igf signaling might play a role in the development of the observed and most likely PLAG1-induced cavernous angiomatosis lesions.

Development of anomalies in P1-ACre mice. P1-ACre offspring mice were initially screened weekly for externally observable aberrations or malformations. After about 20 weeks, the first externally visible abnormality was observed in a P1-ACre mouse. This anomaly consisted of an abnormal extrusion on the top of the skull of the animal (Fig. 3A), which has so far not been seen in any of the control littermates (Fig. 3B). Subsequent autopsy clearly revealed a macroscopically visible area, which apparently contained an extensive build up of blood vessels, suggesting locally aberrant development of the vascular system or malformations in it (Fig. 3C). Further autopsy of the animal revealed similar lesions in different parts of the body. Within a period of 1 year, at least 80% (n >30; range 8-60 weeks of age) of the P1-ACre mice developed such abnormalities. Lesions in the head-neck region could often be detected macroscopically around 20 weeks after birth, as illustrated in Fig. 3A. Upon autopsy, multiple lesions, possibly 'angiomatosis', could be observed at the same time at different locations other than the head region in the bodies of the P1-ACre mice studied. Other regions where such lesions were frequently found included the lower abdominal region (often near the tail base) (Fig. 3D), the mammary gland region (Fig. 3E and F), the abdominal wall near the peritoneum (Fig. 3G), and in abdominal fat tissue (Fig. 3H). Of special interest to note here is the fact that these lesions were found preferentially in fat-rich tissues or organs, such as the mammary glands, fat tissue in between the scapulae, or abdominal fat tissue.

Histopathological characterization of the anomalies, induced by aP2-Cre-mediated functional PLAG1 expression activation, as cavernous angiomatosis embedded in adipose tissue. To characterize the lesions histopathologically, paraffin sections of resected lesions were stained with hematoxylin and eosin. Extensive evaluation established that the lesions could be classified as angiomatosis, a vascular tumor lesion (Fig. 4). The small vascular lesions, as illustrated for instance in Fig. 4A, were seen to evolve into irregularly distributed, large dilated vascular spaces or large caverns (Fig. 4B-D) filled with blood and lined by a thin layer of normal looking flattened endothelial cells (data not shown). Based on all the histopathological data obtained, the vascular tumor lesions were ultimately classified as cavernous angiomatosis. Histopathological examination further confirmed the above tentatively claimed presence from autopsy (Fig. 3) of a prominent fatty component around the lesions, since the extensive build up of blood vessels mostly if not always appeared to originate in the immediate surrounding of fat cells. The presence of fat tissue around vascular tumor lesions is illustrated for such lesions in the lower abdominal region near the tail base (Fig. 4A and B), lesions in the gonadal region (Fig. 4C and D), in the mammary gland (Fig. 4E), and in the abdominal region (Fig. 4F).

Discussion

The results of the present studies indicate that aP2-Cre-mediated activation of *PLAG1* expression in mice leads to the development of cavernous angiomatosis, a particular form of hemangiomas. Hemangiomas constitute 7% of all benign tumors, and, as such, are one of the most common types of soft-tissue tumors. Pathologically, a hemangioma is classified by the predominant type of vascular channel found at histological examination (capillary, cavernous, arterio-venous, or venous). Cavernous hemangiomas are composed of dilated, blood-filled spaces lined by flattened endothelium. They frequently involve deeper soft tissues and mostly manifest clinically as masses without other diagnostic features. Non-vascular components can also be seen in angiomatous lesions, particularly fat, which is often present in intramuscular hemangiomas (23). Although hemangiomas are well-known vascular lesions, little is known about mechanisms that might cause endothelial proliferation. The word 'birthmark' itself directly refers to earliest theories, stating that maternal behavior or intake of red fruits during pregnancy were the cause of hemangiomas (24). Until now, there is no universally accepted theory that explains the cause and further development of hemangiomas. The source of endothelial cells as well as the possible mechanisms by which the hemangioma endothelium interacts with surrounding cells are still unclear (24). Our studies thus present the first possible link between PLAG1 and hemangioma development. The P1-ACre mouse strain, developed in this study, represents an authentic animal model that could provide more insight into the pathogenesis of hemangiomas in future research.

As reported previously (1), the *PLAG1* proto-oncogene seems to exert its oncogenic potential in human tumors via cellular signaling triggered by IGF-II and its cognate receptor IGF-IR, at least partially (2,8,9,25). Generally, binding of the IGF-II ligand to its cognate receptor initiates this pathway, leading to receptor dimerization, autophosphorylation, and subsequent activation of downstream substrates. This results in activation of the MAPK signaling pathway, primarily responsible for mitogenesis, and the PI-3 kinase/Akt pathway with its anti-apoptotic and proliferative mode of action (26). Since IGF-IR signaling affects these two major pathways in tumorigenesis, mouse PLAG1-induced tumor model systems could provide a window of opportunity, i.e. for therapeutic intervention studies such as *in vivo* testing of agents that block IGF-IR signaling (27,28). As reported previously, the *PLAG1* proto-oncogene seems to exert its oncogenic potential also in mouse tumors (pleomorphic salivary gland adenomas and adenomyoepitheliomas of the breast) via Igf-2 signaling, at least partially (2). In our studies, expression of members of the similarly organized imprinted gene clusters *Igf2-H19* and *Dlk1-Gtl2* were also induced by PLAG1. Interestingly, IGF-II was previously found to be highly expressed in human proliferating hemangiomas (29) and IGFs are potent stimulators of VEGF production (30-33). IGF-II directly induces angiogenesis by stimulating cell migration, invasion, and tube formation (34). Correspondingly, not only the *IGF-II* gene, but also VEGF and PlGF, both potent mitogens for endothelial cells (35-37), as well as Ephrin B1, involved in the sprouting of new vessels (38), were found to be upregulated by PLAG1 in a microarray screening for PLAG1 targets (9). It is thus tempting to speculate that PLAG1 increases the levels of Vegf through enhanced levels of Igf-2 and, as such, provides an increasingly favorable local environment for endothelial cell proliferation.

As mentioned above, *aP2-Cre* transgenic mice were chosen to further define the oncogenic spectrum of PLAG1. Our studies confirm the broader activity spectrum of the aP2 promotor and, importantly, establish expression of aP2 in endothelial cells. Considering the fact that substantial endothelial cell proliferation could be observed in our P1-ACre model system, the effect of endothelial PLAG1 expression under the control of the *aP2* promoter on endothelial cell proliferation can not be ruled out. On the other hand, cavernous angiomatosis in our studies always tends to occur in the immediate surrounding of PLAG1 expressing fat cells, while it is known that PLAG1 increases the levels of Igf-2, which in its turn has been shown to be able to impact Vegf expression levels (30-33). The interplay of these factors possibly leads to a favorable local environment for endothelial cell proliferation. Therefore, not only the intracellular environment, i.e. PLAG1 expression and induction/repression of downstream target genes within the same endothelial cell, but also the PLAG1 expressing microenvironment, i.e. in this case the immediate surrounding fat cells in which the *PLAG1* gene is also aberrantly activated, seems important in the development of cavernous angiomatosis.

In conclusion, we further broadened the oncogenic spectrum of PLAG1 in mice by presenting an authentic *in vivo* biological model system that seems to mimic development of cavernous angiomatosis. Moreover, we report for the first time a link between PLAG1 and a form of hemangioma development. Further studies, in particular extensive (immuno)histopathological evaluation to obtain cell-type-specific validation of the various genetic players that are possibly causally implicated, are required to further substantiate this novel finding.

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