Int. J. Cancer: **118,** 643–648 (2006)

Wnt pathway is involved in pleomorphic adenomas induced by overexpression of *PLAG1* in transgenic mice

Xudong Zhao[1,2], Weihua Ren[1], Wenjun Yang[3], Yi Wang[2], Hui Kong[2], Long Wang[2,4], Lanzhen Yan[2], Guojiang Xu[2], Jian Fei[2], Jiliang Fu[2], Chenping Zhang[3] and Zhugang Wang[1,2,4]*

[1]*Laboratory of Genetic Engineering, Department of Medical Genetics, Institute of Health Science, Shanghai Institutes for Biologic Sciences of Chinese Academy of Sciences and Shanghai Second Medical University, Shanghai, China*
[2]*Shanghai Research Center for Model Organisms, Shanghai, China*
[3]*Department of Oral and Maxillofacial Surgery, Ninth People's Hospital Affiliated to Shanghai Second Medical University, Shanghai, China*
[4]*State Key Laboratory of Medical Genomics, Rui-Jin Hospital Affiliated to Shanghai Second Medical University, Shanghai, China*

Pleomorphic adenoma gene 1 (*PLAG1*) was found frequently rearranged and activated in human salivary gland pleomorphic adenomas. It encodes a developmentally regulated transcription factor. Ectopic overexpression of PLAG1 has been proposed to play a crucial role in tumorigenesis of salivary gland pleomorphic adenomas. It was reported that PLAG1 can activate the transcription of insulin-like growth factor 2 (*IGF2*), functioning as a protooncogene. In this report, we show that the salivary gland tumors developed in *PLAG1* transgenic mice share major histopathologic features with human pleomorphic adenomas. It was found that β-catenin, the key component of Wnt signaling pathway, was upregulated at transcriptional level in tumors developed in 3 independent transgenic mouse lines. Immunohistochemical staining revealed that expression of β-catenin as well as c-myc, downstream of β-catenin in Wnt signaling pathway, was highly upregulated with overexpression of *PLAG1* transgene in tumor and normal transgenic salivary gland tissues. Moreover, we found that PLAG1 can activate the transcription of mouse but not human β-catenin in the 3T3 cells cotransfected with reporter constructs. Sequence analysis shows there are 4 PLAG1 consensus binding sites in mouse β-catenin promoter region but not in human. Our findings provide the first *in vivo* evidence for the oncogenic activity of PLAG1 in pleomorphic adenoma tumorigenesis, reveal a valued animal model for human salivary gland tumors and suggest that Wnt signaling pathway may also contribute to the development of pleomorphic adenomas in transgenic mice.

Key words: pleomorphic adenoma gene 1; transgenic mice; β-catenin; pleomorphic adenomas

The pleomorphic adenoma is the most common type of salivary gland tumor, which accounts for more than 50% of all salivary gland neoplasms. It usually behaves as the benign slow-growing tumor morphologically characterized by a biphasic pattern containing both epithelial and mesenchymal areas.[1] In recent years, a series of studies on tumorigenesis of salivary gland tumors has revealed that oncogenic activation of pleomorphic adenoma gene 1 (*PLAG1*) on 8q12 plays a crucial role in the development of pleomorphic adenomas originating from salivary glands. The major form of *PLAG1* activation is reciprocal chromosomal translocations that lead to promoter swapping between *PLAG1* gene, which is not expressed or weakly expressed in adult salivary glands, and the genes ubiquitously expressed in adult tissues, such as the β-catenin gene on 3p21,[2] the leukemia-inhibitory factor receptor (*LIFR*) gene on 5p13 and the transcription elongation factor SII gene on 3p21.3-22.[3,4] The breakpoints of both fusion partner genes invariably occur in the 5′ noncoding regions and consequently lead to ectopic expression of *PLAG1* gene in salivary glands. In fact, the chromosomal translocations involving 8q12 only account for 39% of tumors. Other chromosomal abnormalities were also found to be associated with the formation of pleomorphic adenomas, such as chromosomal rearrangements involving architectural transcription factor high-mobility group protein isoform I-C (*HMGIC*) on 12q14-15 (8%), and sporadic clonal changes involving neither 8q12 nor 12q14-15 (23%).[1] It should be noted that no apparent chromosomal abnormalities were detected in about 30% of all pleomorphic adenomas.[4] Irrespective of existence of chromosomal abnormalities or types of chromosomal rearrangements, overexpression of *PLAG1* was identified in about 70% of all pleomorphic adenomas,[1–4] implicating the crucial role of PLAG1 in tumorigenesis.

In addition to pleomorphic adenomas, ectopic expression of *PLAG1* is also at least in part responsible for the development of lipoblastomas,[5,6] hepatoblastomas,[7] leiomyomas and leiomyosarcomas.[4] Although the various molecular events, including chromosomal translocations, amplification in specific chromosomal regions and even tumors with normal karyotype were also reported, overexpression of *PLAG1* was frequently detected in these tumors. Recently, PLAG1 and PLAG-like 2 (PLAGL2) were found to be overexpressed in 20% of human acute myeloid leukemia samples. PLAG1 can promote the development of acute myeloid leukemia in cooperation with CBFβ-SMMHC fusion protein *in vivo*.[8] These findings suggest that *PLAG1* overexpression may have general importance in tumorigenesis.

Pleomorphic adenoma gene 1 encodes a developmentally regulated transcription factor, which contains 7 canonical C2H2 zinc finger domains, 2 putative nuclear localization signals and a serine-rich C terminus.[2] Structurally, it constitutes a new subfamily of zinc finger proteins with PLAG-like 1 (PLAGL1) and PLAGL2.[9] It has been shown that PLAG1 can bind DNA and activate the transcription of insulin-like growth factor 2 (*IGF2*) gene through the consensus PLAG1 binding site, which is a bipartite element containing a core sequence, GRGGC, and a G-cluster, RGGK, separated by 7 random nucleotides.[10] Upregulation of *IGF2* directly induced by PLAG1 was proposed to be a crucial event in the development of pleomorphic adenomas.[9,10] *In vitro* study shows that NIH3T3 cells overexpressing PLAG1 are able to form foci, to grow in soft agar and to form tumors in nude mice.[9] All these findings indicate that PLAG1 is an oncogenic protein and *IGF2* is one of its direct target genes.

To evaluate the oncogenic activity of PLAG1 *in vivo*, we previously generated human *PLAG1* transgenic mice in which transgene was driven by long terminal repeats (LTRs) of mouse mammary tumor virus (MMTV). It was shown that *PLAG1* transgenic

Grant sponsor: the Major State Basic Research Development Program of China; Grant number: 2001CB509901; Grant sponsor: the National High Technology Research and Development Program of China; Grant number: 2001AA216081; Grant sponsor: the National Natural Science Foundation of China for Outstanding Young Scientists; Grant number: 39925023; Grant sponsor: E-Institutes of Shanghai Municipal Education Commission; Grant number: E03003; Grant sponsor: The National Natural Science Foundation of China; Grant number: 30271420.

***Correspondence to:** Department of Medical Genetics, Shanghai Second Medical University, 280 South Chong-Qing Road, Shanghai 200025, China. Fax: +86-21-58951005. E-mail: zhugangw@shsmu.edu.cn

Received 12 January 2005; Accepted after revision 8 June 2005
DOI 10.1002/ijc.21400
Published online 17 August 2005 in Wiley InterScience (www.interscience.wiley.com).

mice developed salivary gland tumors.[11] Here we present the results showing that the salivary gland tumors induced by *PLAG1* overexpression possess the main pathologic features of human pleomorphic adenomas. β-catenin, the key component of Wnt signaling pathway, and c-myc, downstream of β-catenin in Wnt signaling pathway, were found to be highly upregulated with overexpression of *PLAG1* transgene in the normal transgenic salivary gland and tumor tissues, suggesting that Wnt signaling pathway might be involved in the tumorigenesis of pleomorphic adenomas in mice.

Material and methods

MMTV-PLAG1 *transgenic mice*

MMTV-*PLAG1* transgenic mice were generated in Shanghai Research Center for Model Organisms and maintained in C57BL/6 background. The transgenic mice were genotyped with PCR by using specific primers as described previously.[11] All transgenic mice used in this study are heterozygous for *PLAG1* transgene.

Preparation and analysis of RNA

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. For Northern blot analysis, 20 μg of total RNA was electrophoresed through a 1.5% agarose formaldehyde gel and transferred to charged nylon membrane (S&S, Keene, NH). Probes were labeled and hybridized in ExpressHyb hybridization solution (Clontech, Palo Alto, CA). The membrane was washed and autoradiographed at −80°C.

Histopathologic examination and immunohistochemistry

Tissues for histopathologic analysis were rapidly dissected and fixed in 10% buffered formalin. The tissues were embedded in paraffin and 5 μm sections were cut. Sections were stained with hematoxylin and eosin. For immunohistochemistry analysis, sections were deparaffinized through xylene and dehydrated with graded alcohol. Endogenous peroxidase was then blocked with 3% H_20_2 for 30 min at room temperature. Antigen retrieval was performed by treating slides with citrate buffer in a microwave for 20 min. The slides were then incubated in moist chamber with first antibody at 4°C overnight. After a brief wash in PBS, the slides were treated with a second antibody for 45 min at 37° C. The slides were developed in 0.05% freshly prepared diaminobenzedine solution (DAB; Sigma, St. Louis, MO) for 5–10 min, then counterstained and mounted.

The antibodies used in this study include antibodies against cytokeratin and α-smooth muscle actin isoform (Research Diagnostics, Concord, MA), β-catenin (Santa Cruz Biotechnology, Santa Cruz, CA) and c-myc (kind gift from Professor Xintai Zhao, Shanghai Institute of Cancer, Shanghai, China).

In situ *hybridization*

For *in situ* hybridization, tissues were rapidly dissected and fixed in freshly prepared 4% paraformaldehyde for 3–4 hr and transferred to PBS (pH 7.4) until embedding in paraffin. The sense and antisense riboprobes were transcribed from *PLAG1* cDNA cloned into pGEM-Teasy vector (Promega, Madison, WI). Sections were hybridized with riboprobes labeled with digoxin and incubated with antidigoxin antibody conjugated with alkaline phosphatase, stained with NBT/BCIP solution (Roche, Mannheim, Germany).

Transfection and reporter gene assays

Mouse (−5126 to 892) and human (−3373 to 1249) β-catenin promoter fragments were cloned into pGL3-basic (Promega) and named MCTNNB1-luc and HCTNNB1-luc, respectively. PLAG1 expression vector pCI-neo-PLAG1 and empty pCI-neo vector were cotransfected into NIH3T3 cells in triplicate on 24-well plates with MCTNNB1-luc and HCTNNB1-luc, respectively. Luciferase activity was measured 48 hr after transfection with luciferase assay system (Promega) according to the manufacturer's instruction. Relative luciferase activity was normalized with β-galactosidase activity derived from pSV-β-galactosidase control vector (Promega).

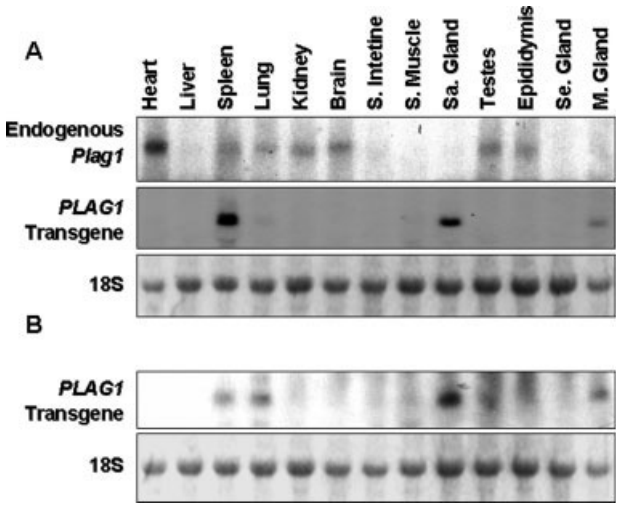

Figure 1 – Expression of endogenous *Plag1* and *PLAG1* transgene in mice. Total RNA was extracted from the indicated tissues of normal transgenic mice from lines 9 (*a*) and 42 (*b*), respectively. The specific probes for human *PLAG1* transgene and endogenous *Plag1* at equal amount were used for Northern blot. 18S RNA was shown as loading control. Note that expression of endogenous *Plag1* was not detected in salivary glands and expression pattern of transgene was found similar between 2 lines. s. intestine, small intestine; s. muscle, skeletal muscle; sa. gland, salivary gland; se. gland, seminal gland; m. gland, mammary gland.

Results

Expression of PLAG1 *transgene in mice*

Expression of *PLAG1* transgene in salivary glands of mice was detected in 5 of 6 transgenic mouse lines. Among them, line 42 shows the highest expression level of transgene.[11] To study the expression pattern of *PLAG1* transgene and possible effect of endogenous *Plag1* on phenotype analysis, we tested the expression of transgene and endogenous *Plag1* in various tissues of mice from lines 9 (Fig. 1*a*) and 42 (Fig. 1*b*) by Northern blot, respectively. The results shown in Figure 1 indicate that *PLAG1* transgene mainly expresses in salivary gland, spleen, mammary gland and lung tissues. The expression pattern seems comparable between 2 mouse lines. The transgene expression profile is consistent with the previous studies on MMTV LTR-based transgenic mice.[12,13] Endogenous *Plag1* expression was detected in several tissues as indicated in Figure 1(*a*), but no expression was found in wild-type salivary glands.

Incidence of salivary gland tumors in 3 transgenic mouse lines

All transgenic mice were inspected on a regular basis. They developed normally and looked healthy till tumors in salivary glands were identified. As we expected, the transgenic mice from lines 42 and 9 spontaneously developed salivary gland tumors at the age of 1–5 months. The tumor incidence was found 100% in line 42 and 68% in line 9 (Fig. 2). In addition, the transgenic mice from line 104 developed salivary gland tumors with lower incidence (2/16; 12.5%) and longer latency (usually more than 10 months) in comparison with lines 42 and 9 (data not shown). It should be mentioned that no tumors were found in the transgenic mice from lines 53 and 59 during their life span, although the transgene expression level in salivary glands was comparable to

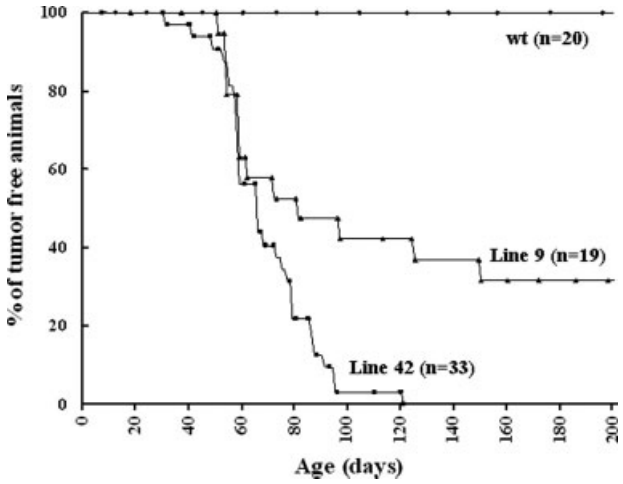

FIGURE 2 – Salivary gland tumor-free curves in MMTV-*PLAG1* transgenic mice. All transgenic mice heterozygous for *PLAG1* transgene were inspected on a regular basis. The time points when tumors became visible in the mice from lines 42 ($n = 33$) and 9 ($n = 19$) were plotted against the percentages of tumor-free animals.

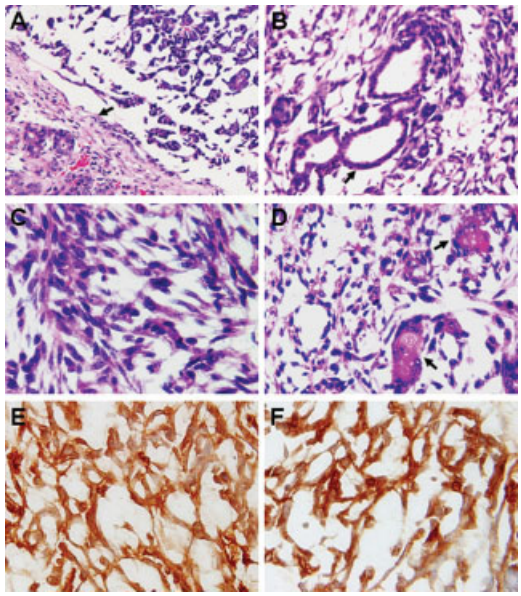

FIGURE 3 – Histopathologic features of salivary gland tumors in transgenic mice. (*a*) The tumor tissue is completely encapsulated. There is a clear border to normal tissue as indicated by an arrow (H&E, 200×). (*b*) The tumor cells form into luminal or cyst-like structure (H&E, 100×). (*c*) Myoepithelial cells can be seen and arranged in a disordered pattern in tumor tissue (H&E, 400×). (*d*) Epithelial keratinization (pointed by arrows) can be observed in some cases (H&E, 400×). Positive immunostaining for cytokeratin (*e*; 400×) and α-smooth muscle actin isoform (*f*; 400×) can be seen in tumor tissues.

other lines. It might be associated with different chromosomal integration sites or other mutations occurred in these 2 lines.

The majority of tumors in 3 transgenic mouse lines were identified in submandibular glands and a few tumors were found to be derived from parotids. Since the transgene expression was detected in spleen, mammary gland and lung, we also checked these organs grossly and failed to find tumors. We also failed to find any evidence of tumor metastasis before the mice with tumors were sacrificed to prevent them from suffering. The time for follow-up was between 3 and 10 months after the tumors were identified.

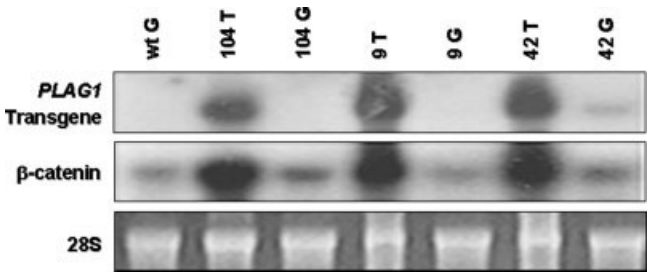

FIGURE 4 – Northern blot analysis for *PLAG1* and β-catenin expression. Northern blot was performed with total RNA extracted from tumor (T) and the adjacent normal salivary gland tissues (G) collected from lines 104 (4 month), 9 (5 month) and 42 (2 month) transgenic mice. β-catenin expression is upregulated with overexpression of *PLAG1* in the tumors developed in 3 independent transgenic mouse lines. It should be noted that *PLAG1* transgene expression can be detected in adjacent normal salivary gland tissues in line 42 and in the other 2 lines with longer exposure time (Northern blot film for *PLAG1* expression was exposed to a shorter time to get clear image since *PLAG1* is highly upregulated in tumor tissues).

Salivary gland tumors in transgenic mice share major pathologic features with human pleomorphic adenomas

To assess the type of tumors developed in transgenic mice, we performed pathologic analysis of 20 cases at age of 1–5 months from different lines. Pathologically, all salivary gland tumors in transgenic mice revealed the major features of human pleomorphic adenomas. Shown in Figure 3 are tumors from line 42. Grossly, no signs of skin ulcers and masses from other sites were found in the mice with salivary gland tumors. All tumors are completely encapsulated (Fig. 3*a*). They are solid and muculant, but no apparent cysts were found. Microscopically, the luminal and cyst-like structures containing mucus were found (Fig. 3*b*). Myoepithelial cells and keratinized epithelium could be found in the tumors as spindle-shaped cells arranging in diffused sheets or short fascicles (Fig. 3*c* and *d*). Immunohistochemical analysis showed that a majority of tumor cells were strongly immunoreactive to cytokeratin (Fig. 3*e*) and α-smooth muscle isoform (Fig. 3*f*). All these pathologic findings demonstrate that salivary gland tumors in the transgenic mice share the major features with human pleomorphic adenomas.

Expression and localization of PLAG1 transgene detected by in situ *hybridization*

To demonstrate the association of *PLAG1* expression and tumor formation and identify the origination of tumors, we performed *in situ* hybridization using *PLAG1*-specific riboprobes on the sections of wild-type and normal transgenic salivary gland and tumor tissues. The results indicate that transgene is specifically expressed in ductal epithelium in normal transgenic tissues (Fig. 5*b*), and more extensive expression was found in tumor tissues (Fig. 5*c*). No apparent signal was detected in wild-type mice (Fig. 5*a*).

Wnt signaling pathway is activated through upregulation of β-catenin in mice

β-catenin, the most common partner gene frequently involved in chromosomal translocations associated with human pleomorphic adenoma, was previously reported to be downregulated in tumors with t(3;8).[2] To study the possible role of β-catenin in tumorigenesis of pleomorphic adenomas in transgenic mice, we checked β-catenin expression in both tumors and adjacent salivary glands. To our surprise, β-catenin, coupling with overexpression of *PLAG1*, was also remarkably upregulated in tumors compared

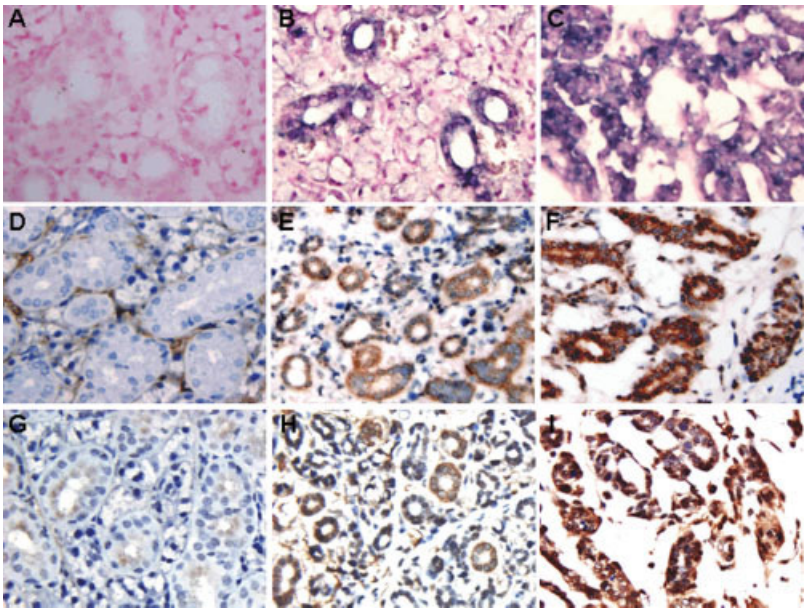

FIGURE 5 – Expression of *PLAG1*, β-catenin and c-myc in salivary gland and tumor tissues. *In situ* hybridization for *PLAG1* (*a–c*; 400×) and immunohistochemistry for β-catenin (*d–f*; 400×) and c-myc (*g–i*; 400×) were performed on wild-type (*a*, *d* and *c*) and normal transgenic salivary glands (*b*, *e* and *h*) and salivary gland tumors (*c*, *f* and *i*) collected from a littermate of line 42 at the age of 6 weeks. *PLAG1*, β-catenin and c-myc share similar expression pattern in normal transgenic salivary gland and tumor tissues.

with their adjacent normal salivary glands of the transgenic mice from lines 42, 9 and 104 by Northern blot (Fig. 4). Immunohistochemistry analysis showed increased expression of β-catenin (Fig. 5*d–f*) and c-myc (Fig. 5*g–i*) in normal transgenic salivary glands (Fig. 5*e* and *h*) and tumors (Fig. 5*f* and *i*) compared with wild-type salivary glands (Fig. 5*d* and *g*), respectively. The expression pattern of both β-catenin and c-myc was similar to that of *PLAG1* transgene detected by *in situ* hybridization (Fig. 5*a–c*).

Since upregulation of β-catenin was detected in transgenic salivary gland and tumor tissues by Northern blot and immunohistochemical staining, we checked human and mouse β-catenin promoter region for PLAG1 consensus binding sites. Indeed, 4 consensus binding sites existed in the mouse promoter region but not in human (Fig. 6*a*). We decided to determine whether PLAG1 could specifically transactivate the mouse but not human β-catenin promoter in transient transfection assay. As expected, cotransfection of PLAG1 expression vector (pCI-neo-PLAG1) with human and mouse β-catenin reporter constructs as indicated in Figure 6(*a*) resulted in a dose-dependent increase in luciferase activity only when the cotransfection was performed with mouse β-catenin reporter construct (Fig. 6*b*), indicating that PLAG1 can specifically activate the transcription of mouse but not human β-catenin.

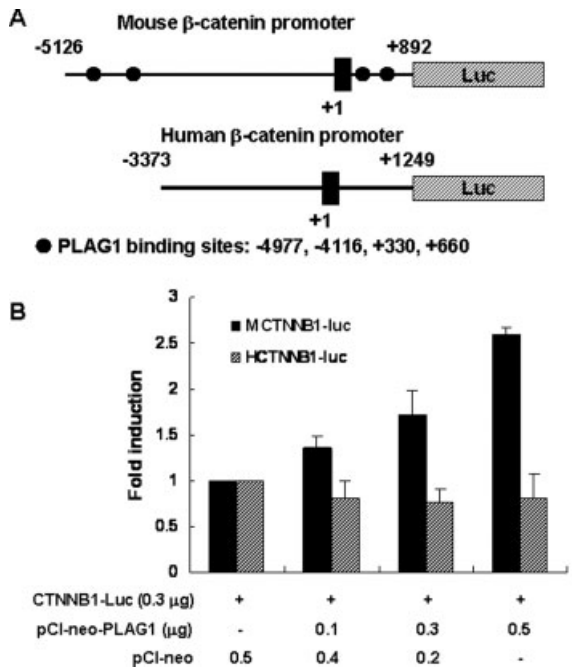

FIGURE 6 – Transactivation of β-catenin promoter by PLAG1. (*a*) Putative PLAG1 consensus binding sites in human and mouse β-catenin promoter regions. Four binding sites (−4977, −4116, 330, 660) were identified in the mouse promoter region from −5126 to 892 and no typical binding site was found in the human β-catenin promoter region from −3373 to 1249. (*b*) The expression vector pCI-neo-PLAG1 was cotransfected with mouse and human CTNNB1-luc reporter vectors as shown in (*a*) into NIH 3T3 cells. Relative luciferase activity was normalized with β-galactosidase activity derived from pSV-β-galactosidase control vector. The result shown as average of triplicate with SD is representative of 2 independent experiments with similar results.

Discussion

To elucidate the role of PLAG1 overexpression in the development of pleomorphic adenomas *in vivo*, we previously generated the transgenic mice in which human *PLAG1* was overexpressed in salivary glands. It was found that *PLAG1* transgenic mice spontaneously developed salivary gland tumors. In this report, we show the salivary gland tumors developed in transgenic mice share the major pathologic features with human pleomorphic adenomas, thus supplying the direct *in vivo* evidence for oncogenic activity of PLAG1 in tumorigenesis of pleomorphic adenoma.

Previous studies on the transgenic mice show that the expression of transgenes, such as *Ras* or *Ras* plus *Myc*,[14] *Wnt-1*,[15] *Wnt-3*,[16] *Fgf-8*,[17] *Neu*[18,19] and SV40 T antigen,[20] driven by MMTV promoter, can result in salivary gland hyperplasia or tumors. The above transgenic mice develop tumors more frequently in mammary gland than in salivary glands and none of them pathologically displays the typical characteristics of human pleomorphic adenomas. The pathologic analysis of mammary gland tumors in MMTV driving *Ras*, *Myc* and *Neu* transgenic mice reveals that the different oncogenes activated in the same cell type result in different tumor types,[21] and SV40 T-antigen overexpression in different cells in salivary glands results in tumors with similar morphol-

ogy,[20,22] suggesting that the tumor types in transgenic mice are closely associated with the activated oncogenes in certain cell types. The fact that *PLAG1* overexpression specifically induces pleomorphic adenomas of salivary glands in mice indicates that MMTV-*PLAG1* transgenic mice would be an ideal animal model for the study of human pleomorphic adenomas in terms of tumor pathology and molecular mechanism of tumorigenesis. Human pleomorphic adenomas mainly developed from parotid. However, most of the tumors observed in the transgenic mice originated from submandibular gland. Parotid is the biggest gland among 3 pairs of salivary glands in human while in mouse the biggest one is submandibular gland. This is probably why the tumors mainly derived from submandibular gland of transgenic mice. Expression of *PLAG1* transgene was also detected at lower level in some other tissues in the transgenic mice of lines 9 and 42 (Fig. 1). We did not find any tumors grossly or even hyperplasia pathologically in these tissues. The reason that PLAG1 overexpression preferentially results in pleomorphic adenoma in salivary gland remains unknown.

PLAG1 is a developmentally regulated transcription factor, which contains 7 canonical C2H2 zinc finger domains, 2 putative nuclear localization signals and a serine-rich C terminus.[2] *PLAG1* was found frequently activated and overexpressed in human pleomorphic adenomas due to chromosomal translocations or other unknown reasons.[1–4] Identification of PLAG1 target genes revealed that *IGF2* is upregulated through its consensus PLAG1 binding site.[10] We also tested mouse *Igf2* expression level in 3 transgenic mouse lines and found remarkable upregulation of *Igf2* in tumor tissues in which *PLAG1* transgene is highly expressed in comparison with their adjacent normal salivary glands (data not shown). IGF2 is an embryonic growth promoter and cell survival factor. Excess IGF2 has detrimental systemic and local effects *in vivo*, promoting somatic overgrowth and an increased frequency of tumors.[23,24] Overexpression of IGF2 has been found in human colorectal and many other cancers.[24–28] However, studies on the transgenic mice in which *IGF2* was overexpressed in salivary glands, mammary glands, lung, uterus and spleen, also under the control of MMTV promoter, show no pleomorphic adenomas or any other salivary gland tumors.[29,30] It strongly suggests that overexpression of IGF2 alone might not be enough for initiation of tumorigenesis in the salivary glands. Other genes potentially regulated by PLAG1 may be involved in pleomorphic adenoma tumorigenesis. In fact, PLAG1, as a transcription factor, has a broad effect on transcriptional regulation of a series of target genes, including growth factors and their related genes, cell cycle-related genes, oncogenes, and so on.[31]

β-catenin gene on 3p21 is the most common partner gene frequently involved in human pleomorphic adenomas with 8q12 rearrangements. It has been showed that β-catenin was downregulated in the tumors with t(3;8), probably due to loss of one allele or formation of *PLAG1*-β-catenin fusion transcript.[2] β-catenin is a pivotal part of the Wingless/Wnt signaling pathway as a component of complex that also includes adenomatous polyposis coli (APC) gene, GSK-3β serine/threonine protein kinase and axin/conductin protein.[32,33] Formation of the complex results in the subsequent proteolysis of β-catenin by ubiquitin-proteasome pathway. Wnts activate Wnt signaling pathway through inhibiting the degradation and leading to the translocation of free β-catenin from cytoplasm to nucleus, where β-catenin regulates expression of target genes together with transcription factors of T-cell factor/lymphoid enhancer-binding factor 1 (TCF/LEF) family.[33] High level of free β-catenin resulting from stabilization due to mutation of APC or β-catenin itself or more release from β-catenin/E-cadherin complex, sometimes also from increased β-catenin mRNA, was found to be engaged in several types of tumors (strongly established in colorectal cancer and melanomas).[32–34] In order to study the possible role of β-catenin in tumorigenesis of pleomorphic adenomas, we tested β-catenin expression and found that the expression of β-catenin was remarkably upregulated with overexpression of *PLAG1*. Increased β-catenin and c-myc expression was also found in transgenic salivary gland and tumor tissues with similar pattern to PLAG1 overexpression, implying that it is the result of PLAG1 overexpression. Recent study has shown that IGF2 can activate Wnt signaling pathway through stimulating β-catenin accumulation in nucleus but not affect the transcription of β-catenin.[35] The sequence analysis reveals that 4 PLAG1 consensus binding sites could be identified in the promoter region of mouse but not human β-catenin gene, combined with the facts that upregulation of β-catenin in transgenic salivary gland and tumor tissues at mRNA and protein levels, strongly suggesting that PLAG1 could activate the transcription of mouse β-catenin. As expected, cotransfection of PLAG1 expression vector (pCI-neo-PLAG1) with human and mouse β-catenin reporter constructs resulted in a dose-dependent increase in luciferase activity when cotransfection was performed with mouse β-catenin reporter construct. No transactivation activity of PLAG1 on human β-catenin reporter construct was found (Fig. 6*b*), indicating that PLAG1 can specifically activate the transcription of mouse, but not human β-catenin. Thus, we may conclude that Wnt pathway is activated by overexpression of PLAG1 through functional activation of β-catenin in transgenic mice.

Acknowledgements

The authors gratefully acknowledge Professor Xingtai Zhao for the technical assistance of *in situ* hybridization. They also thank Professor Yin Jin and Dr. Peter Chung for critical review. Supported by E-Institutes of Shanghai Municipal Education Commission (E03003; to Z.G.W.) and the National Natural Science Foundation of China (30271420; to C.P.Z.).

References

1. Enlund F, Nordkvist A, Sahlin P, Mark J, Stenman G. Expression of PLAG1 and HMGIC proteins and fusion transcripts in radiation-associated pleomorphic adenomas. Int J Oncol 2002;20:713–6.
2. Kas K, Voz ML, Roijer E, Astrom AK, Meyen E, Stenman G, Van de Ven WJ. Promoter swapping between the genes for a novel zinc finger protein and beta-catenin in pleiomorphic adenomas with t(3;8)(p21;q12) translocations. Nat Genet 1997;15:170–4.
3. Voz ML, Astrom AK, Kas K, Mark J, Stenman G, Van de Ven WJ. The recurrent translocation t(5;8)(p13;q12) in pleomorphic adenomas results in upregulation of PLAG1 gene expression under control of the LIFR promoter. Oncogene 1998;16:1409–16.
4. Astrom AK, Voz ML, Kas K, Roijer E, Wedell B, Mandahl N, Van de Ven W, Mark J, Stenman G. Conserved mechanism of PLAG1 activation in salivary gland tumors with and without chromosome 8q12 abnormalities: identification of SII as a new fusion partner gene. Cancer Res 1999;59:918–23.
5. Hibbard MK, Kozakewich HP, Dal Cin P, Sciot R, Tan X, Xiao S, Fletcher JA. PLAG1 fusion oncogenes in lipoblastoma. Cancer Res 2000;60:486972.
6. Astrom A, D'Amore ES, Sainati L, Panarello C, Morerio C, Mark J, Stenman G. Evidence of involvement of the PLAG1 gene in lipoblastomas. Int J Oncol 2000;16:1107–10.
7. Zatkova A, Rouillard JM, Hartmann W, Lamb BJ, Kuick R, Eckart M, von Schweinitz D, Koch A, Fonatsch C, Pietsch T, Hanash SM, Wimmer K. Amplification and overexpression of the IGF2 regulator PLAG1 in hepatoblastoma. Genes Chromosomes Cancer 2004;39:126–37.
8. Landrette SF, Kuo YH, Hensen K, Khosrovani B, Perrat PN, Van de Ven WJ, Delwel R, Castilla LH. Plag1 and Plagl2 are oncogenes that induce acute myeloid leukemia in cooperation with Cbfb-MYH11. Blood 2005;105:2900–7.
9. Hensen K, Van Valckenborgh IC, Kas K, Van de Ven WJ, Voz ML. The tumorigenic diversity of the three PLAG family members is associated with different DNA binding capacities. Cancer Res 2002;62:1510–7.
10. Voz ML, Agten NS, Van de Ven WJ, Kas K. PLAG1, the main translocation target in pleomorphic adenoma of the salivary glands, is a positive regulator of IGF-II. Cancer Res 2000;60:106–13.

11. Zhao XD, Yang WJ, Wang L, Kong H, Ren WH, Zhang M, Fei J, Zhang CP, Wang ZG. Development of salivary gland tumors in pleomorphic adenoma gene 1 transgenic mice. Zhonghua Yi Xue Yi Chuan Xue Za Zhi 2003;20:390–5.
12. Xie W, Paterson AJ, Chin E, Nabell LM, Kudlow JE. Targeted expression of a dominant negative epidermal growth factor receptor in the mammary gland of transgenic mice inhibits pubertal mammary duct development. Mol Endocrinol 1997;11:1766–81.
13. Pierce DF Jr, Johnson MD, Matsui Y, Robinson SD, Gold LI, Purchio AF, Daniel CW, Hogan BL, Moses HL. Inhibition of mammary duct development but not alveolar outgrowth during pregnancy in transgenic mice expressing active TGF-beta 1. Genes Dev 1993;7:2308–17.
14. Sinn E, Muller W, Pattengale P, Tepler I, Wallace R, Leder P. Coexpression of MMTV/v-Ha-ras and MMTV/c-myc genes in transgenic mice: synergistic action of oncogenes *in vivo*. Cell 1987;49:465–75.
15. Tsukamoto AS, Grosschedl R, Guzman RC, Parslow T, Varmus HE. Expression of the int-1 gene in transgenic mice is associated with mammary gland hyperplasia and adenocarcinomas in male and female mice. Cell 1988;55:619–25.
16. Jhappan C, Gallahan D, Stahle C, Chu E, Smith GH, Merlino G, Callahan R. Expression of an activated Notch-related int-3 transgene interferes with cell differentiation and induces neoplastic transformation in mammary and salivary glands. Genes Dev 1992;6:345–55.
17. Daphna-Iken D, Shankar DB, Lawshe A, Ornitz DM, Shackleford GM, MacArthur CA. MMTV-Fgf8 transgenic mice develop mammary and salivary gland neoplasia and ovarian stromal hyperplasia. Oncogene 1998;17:2711–7.
18. Bouchard L, Lamarre L, Tremblay PJ, Jolicoeur P. Stochastic appearance of mammary tumors in transgenic mice carrying the MMTV/c-neu oncogene. Cell 1989;57:931–6.
19. Lucchini F, Sacco MG, Hu N, Villa A, Brown J, Cesano L, Mangiarini L, Rindi G, Kindl S, Sessa F. Early and multifocal tumors in breast, salivary, harderian and epididymal tissues developed in MMTV-Neu transgenic mice. Cancer Lett 1992;64:203–9.
20. Furth PA, Li M, Hennighausen L. Studying development of disease through temporally controlled gene expression in the salivary gland. Ann NY Acad Sci 1998;842:181–7.
21. Cardiff RD, Sinn E, Muller W, Leder P. Transgenic oncogene mice: tumor phenotype predicts genotype. Am J Pathol 1991;139:495–501.
22. Dardick I, Ho J, Paulus M, Mellon PL, Mirels L. Submandibular gland adenocarcinoma of intercalated duct origin in Smgb-Tag mice. Lab Invest 2000;80:1657–70.
23. Rooman R, Koster G, Bloemen R, Gresnigt R, van Buul-Offers SC. The effect of dexamethasone on body and organ growth of normal and IGF-II-transgenic mice. J Endocrinol 1999;163:543–52.
24. Toretsky JA, Helman LJ. Involvement of IGF-II in human cancer. J Endocrinol 1996;149:367–72.
25. Cui H, Cruz-Correa M, Giardiello FM, Hutcheon DF, Kafonek DR, Brandenburg S, Wu Y, He X, Powe NR, Feinberg AP. Loss of IGF2 imprinting: a potential marker of colorectal cancer risk. Science 2003; 299:1753–5.
26. Sachdev D, Yee D. The IGF system and breast cancer. Endocr Relat Cancer 2001;8:197–209.
27. Butterwith SC, Goddard C. Regulation of DNA synthesis in chicken adipocyte precursor cells by insulin-like growth factors, platelet-derived growth factor and transforming growth factor-beta. J Endocrinol 1991;131:203–9.
28. Scharf JG, Dombrowski F, Ramadori G. The IGF axis and hepatocarcinogenesis. Mol Pathol 2001;54:138–44.
29. Moorehead RA, Fata JE, Johnson MB, Khokha R. Inhibition of mammary epithelial apoptosis and sustained phosphorylation of Akt/PKB in MMTV-IGF-II transgenic mice. Cell Death Differ 2001; 8:16–29.
30. Moorehead RA, Sanchez OH, Baldwin RM, Khokha R. Transgenic overexpression of IGF-II induces spontaneous lung tumors: a model for human lung adenocarcinoma. Oncogene 2003;22:853–7.
31. Voz ML, Mathys J, Hensen K, Pendeville H, Van Valckenborgh I, Van Huffel C, Chavez M, Van Damme B, De Moor B, Moreau Y, Van de Ven WJ. Microarray screening for target genes of the proto-oncogene PLAG1. Oncogene 2004;23:179–91.
32. Peifer M. Beta-catenin as oncogene: the smoking gun. Science 1997;275:1752–3.
33. Moon RT, Bowerman B, Boutros M, Perrimon N. The promise and perils of Wnt signaling through beta-catenin. Science 2002;296: 1644–6.
34. Saito T, Oda Y, Kawaguchi K, Tanaka K, Matsuda S, Tamiya S, Iwamoto Y, Tsuneyoshi M. Possible association between higher beta-catenin mRNA expression and mutated beta-catenin in sporadic desmoid tumors: real-time semiquantitative assay by TaqMan polymerase chain reaction. Lab Invest 2002;82:97–103.
35. Morali OG, Delmas V, Moore R, Jeanney C, Thiery JP, Larue L. IGF-II induces rapid beta-catenin relocation to the nucleus during epithelium to mesenchyme transition. Oncogene 2001;20:4942–50.