Upregulation of Igf and Wnt signalling associated genes in pleomorphic adenomas of the salivary glands in *PLAG1* transgenic mice

JEROEN DECLERCQ[1], FREDERIK VAN DYCK[1], BOUDEWIJN VAN DAMME[2] and WIM J.M. VAN DE VEN[1]

[1]Laboratory for Molecular Oncology, Department of Human Genetics; [2]Department of Morphology and Molecular Pathology, University of Leuven, Leuven, Belgium

Received October 11, 2007; Accepted December 5, 2007

Abstract. The Pleomorphic adenoma gene 1 *(PLAG1)* is involved in various human neoplasias, including pleomorphic adenomas of the salivary glands. Moreover, the oncogenic role of *PLAG1* was clearly demonstrated in two independent *PLAG1* transgenic mouse founders, in which *PLAG1* expression could be targeted to different tissues using the Cre/loxP system. MMTV-Cre-mediated targeted over-expression of *PLAG1* in the salivary glands of double transgenic offspring mice, referred to as P1-MCre and P2-MCre mice, induced pleomorphic adenomas in this organ. *Igf2*, a genuine PLAG1 target gene, was highly upregulated in those tumours as well as in human pleomorphic adenomas of the salivary glands. These and previous observations in other *PLAG1*-induced tumours e.g. breast adenomyoepitheliomas emphasize the importance of Igf upregulation in such tumours. In this study, further evidence for the role of *Igf2* in PLAG1-induced tumourigenesis, is reported. Inactivation of *Igf2* in P1-MCre mice leads to a significant delay in tumour development. Since tumour development is not fully abrogated by inactivation of *Igf2*, other signalling pathways are likely to contribute to PLAG1-induced tumourigenesis as well. Further studies revealed that several genes such as *H19, Dlk1, Gtl2, Igfbp2, Igfbp3* and genes involved in Wnt signalling, such as *Wnt6, Cyclin D1* and *ß-catenin* are upregulated in P1-MCre mice in which *Igf2* is inactivated. In conclusion, we clearly demonstrate upregulation of several genes associated with Igf and Wnt signalling in PLAG1-induced pleomorphic adenomas. Furthermore, inactivation of *Igf2* does not affect upregulation of genes associated with Wnt signalling, which might suggest that both signalling pathways are involved.

Correspondence to: Professor W.J.M. Van de Ven, Laboratory for Molecular Oncology, Department of Human Genetics, K.U. leuven, Herestraat 49/602, B-3000 Leuven, Belgium
E-mail: wim.vandeven@med.kuleuven.be

Key words: PLAG1 proto-oncogene, transgenic mouse strains, pleomorphic adenomas of the salivary glands, Wnt signalling associated genes, insulin-like growth factor 2

Introduction

Pleomorphic adenoma is the most common tumour in human salivary glands, accounting for about half of all neoplasms in this organ (1). About 84% of the pleomorphic adenomas occur in the parotid glands, 8% in the submandibular glands, 6.5% in the minor glands, and 0.5% in the sublingual glands (2).

Pleomorphic adenomas are slow growing, painless, encapsulated tumours that may become large if untreated. Larger lesions can have sites of necrosis, haemorrhage, focal calcification or occasionally ossification (3). Although these tumours are usually benign, they have the tendency to recur when inadequately excised. Moreover, 2-17% of the tumours can progress to malignancy and give rise to carcinoma ex pleomorphic adenoma (4-6).

Cytogenetically, pleomorphic adenomas of the salivary glands are well characterized, with several hundreds already karyotyped (7,8). Four major cytogenetic subgroups can be distinguished (9). The first consists of tumours with 8q12 rearrangements (39%). The second is composed of tumours with translocations involving chromosomal region 12q14-15 (8%). The third is composed of tumours with various non-recurrent translocations (23%) not involving chromosomal region 8q12 or 12q14-15. The last subgroup consists of tumours with an apparently normal karyotype (30%). It is clear that the translocations involving 8q12 and 12q14-15 are frequently encountered in pleomorphic adenomas of the salivary glands. By positional cloning, we identified the genes involved in those translocations. The high mobility group protein C *(HMGI-C*, now known as *HMGA2)* gene was found to be affected in tumours with 12q14-15 translocations (10) whereas the Pleomorphic adenoma gene 1 *(PLAG1)* was found to be affected in tumours with 8q12 translocations (11). The most common abnormality in the latter cases is a t(3;8)(p21;q12) translocation, found in almost half of these. The translocation results in promoter swapping between *PLAG1* and *CTNNB1* (the gene for ß-catenin). As a result of the t(3;8)(p21;q12) translocation, the coding sequences of *PLAG1* are brought under the control of the 5' regulatory sequences of the *CTNNB1* gene, and vice versa. The regulation of the expression of both genes is very different. Whereas, the *CTNNB1* gene is highly, ubiquitously, and constitutively expressed, *PLAG1* has high overall developmental expression

levels, which drop shortly after birth (12). As a consequence, the above-sited t(3;8) translocation leads to an aberrant activation of *PLAG1*. In addition to the *CTNNB1-PLAG1* fusion gene, three alternative fusion genes have been identified in pleomorphic adenomas. *LIFR-PLAG1* is found in tumours with t(5;8)(p13;q12) (13), whereas *TCEA1-PLAG1* (also known as *SII*) (14) and *CHCHD7-PLAG1* (15) both result from cryptic rearrangements in tumours with a normal karyotype. Analysis of the structure and function of the *PLAG1* fusion partners have not revealed any obvious similarities besides their constitutive expression pattern, or at least their expression in the cells or tissues from which the tumours are thought to originate. Therefore, it has been suggested that the most important function of the fusion partner genes is most likely to provide an active promoter that induces ectopic expression of *PLAG1* (9).

In addition to pleomorphic adenomas, ectopic expression of *PLAG1* is also found in lipoblastomas (16-18), hepatoblastomas (19), and AML (20,21). The oncogenic capacity of *PLAG1* has been demonstrated *in vitro*, i.e. in cell lines that were retrovirally transduced with *PLAG1* (22), and recently, *in vivo* in mouse models (23,24). Whereas Zhao and co-workers generated classical, non-conditional transgenic mouse strains (24), we developed two independent *PLAG1* transgenic mouse strains, PTMS1 and PTMS2, in which activation of overexpression of the transgene as well as the tissue distribution of such overexpression can be manipulated, by Cre-mediated activation and targeted expression, respectively (23). To study the consequences of genetically engineered *PLAG1* expression in the salivary glands, the two independent *PLAG1* transgenic mouse strains were intercrossed with B6129-Tgn(MMTV-LTR/Cre)1Mam *(MMTV-Cre)* transgenic mice (25,26). The resulting double transgenic offspring mice, referred to as P1-MCre and P2-MCre mice, developed pleomorphic adenomas of the salivary glands with a prevalence of 100% (within 5 weeks) and 6% (after several months), respectively (23).

Previous microarray studies, including comparative gene expression profiling in human and mouse salivary gland tumours (27), revealed genes that are consistently induced by PLAG1. Of interest to note is the upregulation of genes functioning in Igf (mouse) and IGF (human) signalling. In validation experiments, it has already been proven for instance for IGF-II that it constitutes a genuine direct target gene of PLAG1 (28).

The upregulation of *Igf2* in pleomorphic adenomas of the salivary glands thus suggests a contributing role of *Igf2* in the *PLAG1*-induced tumourigenesis. To study the precise impact of Igf2 upregulation on *PLAG1*-induced tumour formation, we intercrossed P1-MCre mice with *Igf2* knockout mice (29,30). The investigation focused on monitoring tumour development, histological analysis of tumour lesions, and upregulation of expression of genes associated with signalling pathways.

Materials and methods

Generation of P1-MCre and various P1-MCre-Igf2 compound transgenic/knockout mice. The generation of the *PLAG1* transgenic founder line, PTMS1, has been reported previously (23). To target *PLAG1* expression to the salivary glands, this founder was crossed with B6129-Tgn(MMTV-LTR/Cre)1Mam transgenic mice *(MMMTV-Cre)* (Jackson's Laboratories, USA) (25), resulting in P1-MCre offspring. In order to generate compound transgenic/knockout mice involving *PLAG1, Cre,* and *Igf2, Igf2*$^{p-/m-}$ mice (29,30) were crossed with PTMS1 mice, and *MMTV-Cre* mice with *Igf2*$^{p-/m-}$ mice to generate *P1-Igf2*$^{p-/m+}$ and *MMTV-Cre-Igf2*$^{p-/m+}$ offspring, respectively. Mating of those compound heterozygous offspring yielded various genotypes, including *P1-MCre-Igf2*$^{p-/m-}$, *P1-MCre-Igf2*$^{p-/m+}$, *P1-MCre-Igf2*$^{p+/m-}$ and *P1-MCre-Igf2*$^{p+/m+}$ mice. These offspring mice were used in further breedings to expand colonies, necessary for further analysis of the impact of *Igf2* inactivation on tumour formation.

Genotyping of genetically modified mice by PCR analysis. Genotyping of candidate *PLAG1* founders was performed by PCR analysis of tail DNA using oligonucleotide primers POS-1599 (5'-TTCTCAAGCATCGTCATCAT-3') and ß-globin (5'-AAAATTCCAACACACTATTGC-3'). For genotyping of the various genetically modified mice, gene-specific primers were designed for the following genes: *Cre* (forward: 5'-CCTG TTTTGCACGTTCACCG-3' and reverse: 5'-ATGCTTCTG TCCGTTTGCCG-3'), *Igf2* wild-type allele (forward: 5'-GTA CCAATGGGGATCCCAGTG-3' and reverse: 5'-GCGGTC CGAACAGACAAACTG-3') and the *Igf2* knockout allele (forward: 5'-TGCTCTGATGCCGCCGTGTT-3' and reverse: 5'-GTGCACTCTCAACCTGGCTGA-3'). All PCR reactions were performed at an annealing temperature of 58˚C and involved 35 cycles except for the *Igf2* knockout allele, which involved 30 cycles. The annealing time was 40, 30, 30, and 50 sec for *PLAG1, Cre,* the *Igf2* wild-type allele, and *Igf2* knockout allele, respectively.

Real-time quantitative PCR (QRT-PCR). Total RNA from selected tissue specimens from normal tissue and tumours of the salivary glands was prepared using the NucleoSpin® RNA L kit, as described by the manufacturer, Macherey-Nagel. Total RNA (5 μg) was reverse transcribed using random hexamer primers and M-MLV reverse transcriptase from Invitrogen (Merelbeke, Belgium). QRT-PCR was performed with the MyIQ system (Biorad). In all cases, 40 cycles of annealing/extension for 1 min at 60˚C were performed. QRT-PCR products were detected using SYBERGreen. QRT-PCR for *mt-Atp6* was used as a reference to correct for sample quantity (forward primer: 5'-AAGCTCACTTGCCCAC TTCCTT-3' and reverse primer: 5'-GCTGTAAGCCGGA CTGCTAATG-3'). Gene-specific primers were designed for the following genes; *PLAG1* (forward: 5'-CCACGTTTCCA TCAAGCTTTTC-3' and reverse: 5'-AGGCAGCCTGC ACCTGAG-3'), *Igf2* (forward: 5'-TGTCTGTTCGGACC GCG-3' and reverse: 5'-GTTGGCACGGCTTGAAGG-3'), *H19* (forward: 5'-AAGAGCTCGGACTGGAGACTAGG-3' and reverse: 5'-GGCACATCCACCTCTGCTG-3'), *Dlk1* (forward: 5'-TGCGCGTCCTCTTGCTC-3' and reverse: 5'-CATTCAGCCCCATAGGTGCT-3'), *Gtl2* (forward: 5'-CTCCAACCCACTGCTTCCTG-3' and reverse: 5'-AGCGA GAGCCGTTCGATG-3'), *Igfbp2* (forward: 5'-CCAACTGT GACAAGCATGGC-3' and reverse: 5'-GAGACATCTT GCACTGCTTAAGGTT-3'), *Igfbp3* (forward: 5'-GCAGG CAGCCTAAGCACC-3' and reverse: 5'-GATGTTTCCT GGAGCAGGTTG-3'), *Cyclin D1* (forward: 5'-CGAGGAG

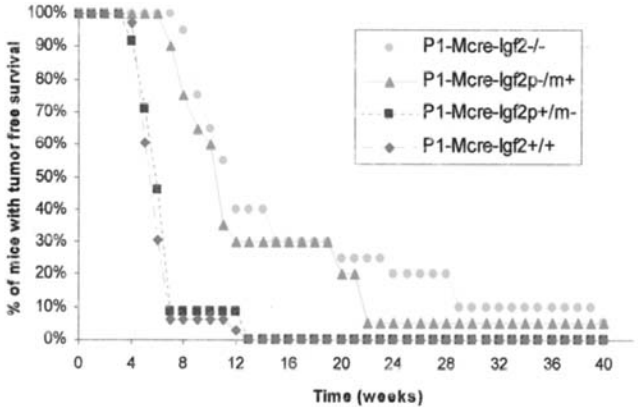

Figure 1. Percentage of *PLAG1/Igf2* genetically modified mice with tumour-free survival as detected by the absence of a macroscopically visible tumour mass in the ventral neck region in function of time. At least 20 mice were studied for each genotype.

CTGCTGCAAATG-3' and reverse: 5'-TTCCACTTGAG CTTGTTCACCA-3'), *Wnt6* (forward: 5'-TGTCAGTTCCA GTTCCGTTTCC-3' and reverse: 5'-GCTGCGGTGATTG CAAACA-3') and *ß-catenin* (forward: 5'-TTCAGATCTTA GCTTATGGCAATCAA-3' and reverse: 5'-TGGCCAGA ATGATGAGCTTG-3').

Western blot analysis. Salivary gland tumours of 8- and 12-week-old P1-MCre mice, control littermate *MMTV-Cre* mice, and 30- and 37-week-old *P1-MCre-Igf2*$^{p-/m-}$ mice were snap-frozen and stored at -80˚C. Cryo-frozen tissue samples were homogenized in 5 volumes of suspension buffer, prepared by combining 0.1 M NaCl, 0.01 M Tris/HCl pH 7.5, 1 mM EDTA pH 8.0 and protease inhibitors cocktail EDTA-free (Roche). An equal volume of 2X SDS sample buffer, prepared by combining 20 ml 10% SDS, 10 ml glycerol, 10 ml 0.5 M Tris/HCl pH 6.8, 60 ml H_2O, was added and the samples were heated for 10 min at 95˚C. The total protein concentration was determined using the BCA method and equal amounts of proteins were size-fractionated by SDS-Page. The proteins were transferred to a nitrocellulose membrane and Western blot analysis was performed, as described previously (23). Active-ß-catenin and total ß-catenin were detected with highly specific antibodies, clone 8E7 (Upstate) to detect active dephosphorylated ß-catenin and antibody 610154 (BD Bioscience) to detect total ß-catenin. As control for equal loading, actin was visualized using antibody A 5060 (Sigma).

Results

Evaluation of the role of Igf2 in PLAG1-induced tumour development in P1-MCre mice. Previously, we generated two independent *PLAG1* transgenic mouse lines, in which activation of overexpression of the transgene as well as the tissue distribution of such overexpression can be manipulated by Cre-mediated activation and targeted expression, respectively. These founder lines were intercrossed with *MMTV-Cre* mice to target the expression of the transgene mainly to the salivary glands. P1-MCre mice (100%) developed pleomorphic adenomas of the salivary glands at ~5-6 weeks. In contrast, only 6% of the P2-MCre mice developed similar tumours after several months. *Igf2* was highly upregulated in those PLAG1-induced pleomorphic adenomas of the salivary glands. Similarly, *IGF-II* was upregulated in human pleomorphic adenomas that expressed *PLAG1* (28). IGF signalling is also implicated in many tumour types including, breast (31-33), colon (34) and liver (35). Altogether, those data indicate the Igf signalling might be important in PLAG1-induced tumourigenesis.

Table I. Upregulation of PLAG1 target genes in pleomorphic adenomas of the salivary glands.

Genes	P1-MCre-Igf2$^{+/+}$	P1-MCre-Igf2$^{-/-}$
Igf2	3422±1212	0
H19	948±328	276±69
Dlk1	6165±1190	3337±1180
Gtl2	79.5±0.3	78.8±14
Igfbp2	17.4±4.6	11.4±3.5
Igfbp3	16.5±4.4	18.8±4.9
Cyclin D1	3.7±0.6	3.2±0.7
Wnt6	46.8±10.8	43.2±15.1
ß-catenin	2.1±0.4	1.7±0.6[a]

Mean values ± SEM of the fold upregulation of PLAG1 target genes in pleomorphic adenomas of the salivary glands of 5 *P1-MCre-Igf2*$^{p+/m+}$ and 5 *P1-MCre-Igf2*$^{p-/m-}$ mice as compared to normal salivary gland tissue of 5 *MMTV-Cre* and 5 *MMTV-Cre-Igf2*$^{p-/m-}$ littermates, respectively. P-values are <0.05, except for [a]P=0.2.

In this study, the role of *Igf2* on PLAG1-induced tumourigenesis is further investigated. Due to the low tumour incidence and the long latency period in P2-MCre mice, we decided to investigate the role of *Igf2* in PLAG1-induced tumourigenesis only in P1-MCre mice. Therefore, P1-MCre mice were crossed with *Igf2* knockout mice (29,30). Due to imprinting, only the paternal allele of *Igf2* is active, whereas the maternal allele is normally silenced via methylation. P1-MCre offspring, in which the paternal *Igf2* allele is knocked out or both *Igf2* alleles are knocked out, show a significant delay in the development of salivary gland tumours. This was assessed by determing the presence of macroscopically visible tumour masses in the ventral neck region (Fig. 1). As can be deduced from Fig. 1, for example 50% of the *P1-MCre-Igf2*$^{p-/m-}$ mice and *P1-MCre-Igf2*$^{p-/m+}$ mice developed large salivary gland tumours at ~11-12 weeks after birth. In contrast, 50% of the *P1-MCre-Igf2*$^{p+/m-}$ and *P1-MCre-Igf2*$^{p+/m+}$ mice showed macroscopically visible tumour masses already at ~6 weeks after birth (Fig. 1).

We have performed comparative histopathological studies to investigate whether or not particular differences could be found in tumour lesions with and without *Igf2* expression. These studies indicated that the histopathological features of the salivary gland tumours of *P1-MCre-Igf2*$^{p-/m-}$ mice were similar to those observed in corresponding *P1-MCre-Igf2*$^{p+/m+}$ mice (Fig. 2). The salivary gland tumours studied all showed typical characteristics of pleomorphic adenomas. An overview of the pleomorphic character of the tumours of *P1-MCre-Igf2*$^{p-/m-}$

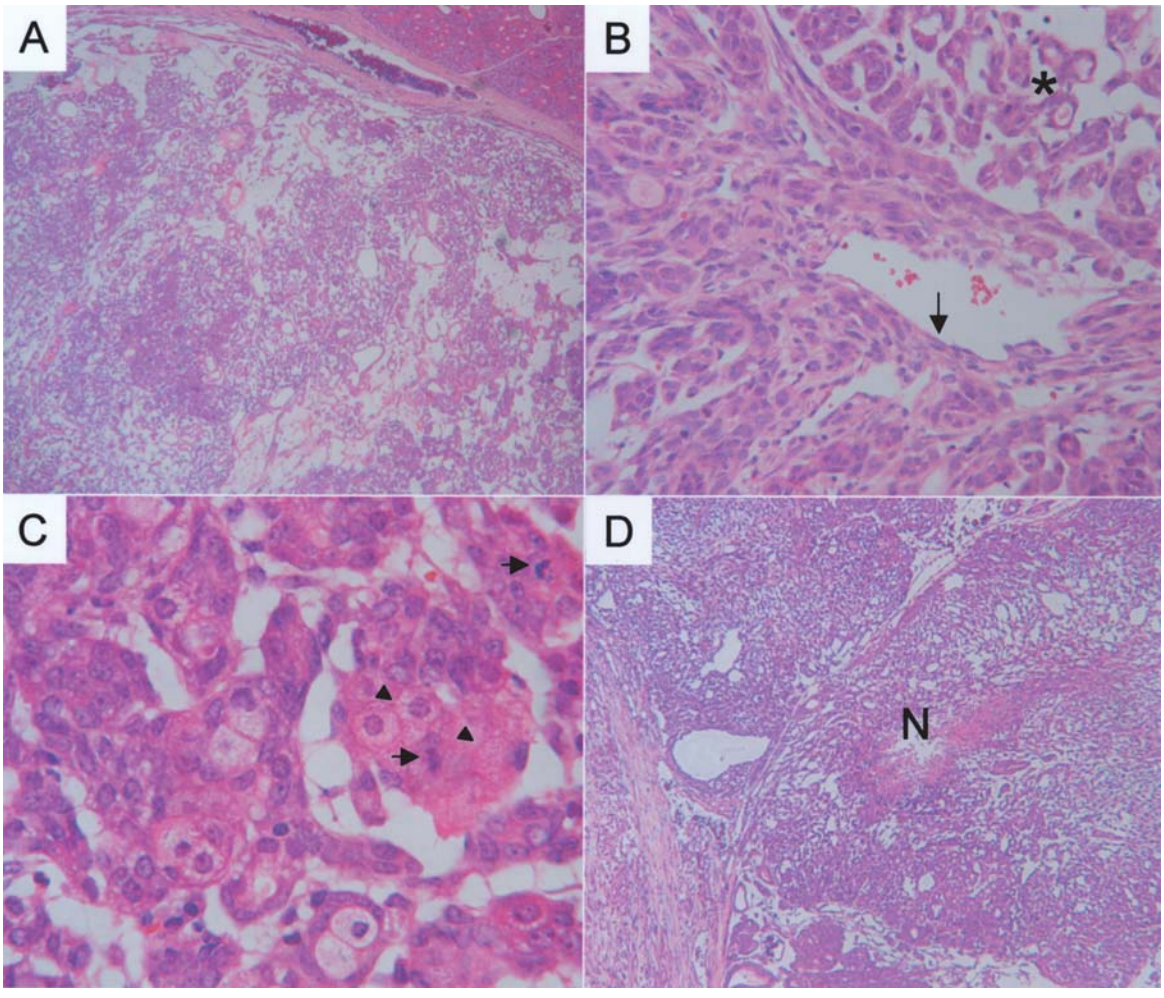

Figure 2. (A) Overview figure of a pleomorphic adenoma of a 12-week-old *P1-MCre-Igf2*$^{p-/m-}$ mouse, demonstrating the pleomorphic character of the tumour. H&E, original magnification of x2.5. (B) Pleomorphic adenoma of a 10-week-old *P1-MCre-Igf2*$^{p-/m-}$ mouse, showing the presence of tubular structures (epithelial cells) (*) as well as spindle, myoepithelial cells (down arrow). H&E, original magnification of x20. (C) Pleomorphic adenoma of a 12-week-old *P1-MCre-Igf2*$^{p-/m-}$ mouse, showing sebaceous/squamous metaplasia (arrowhead) and mitotic figures (right arrow). H&E, original magnification of x40. (D) Pleomorphic adenoma of a 22-week-old *P1-MCre-Igf2*$^{p-/m-}$ mouse, showing malignant characteristics such as the presence of a necrotic region (N). H&E, original magnification of x5.

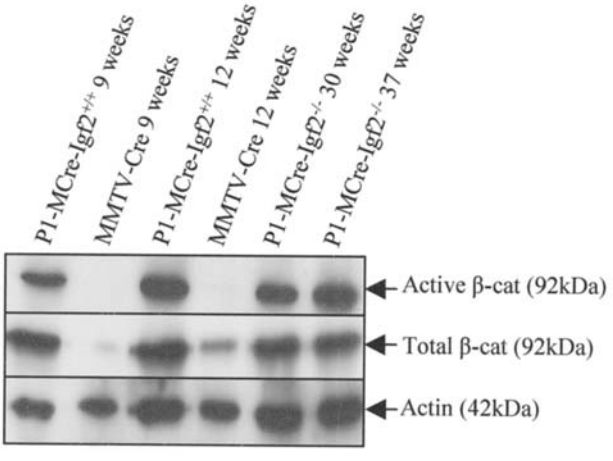

Figure 3. The accumulation of ß-catenin and active-ß-catenin in pleomorphic adenomas of the salivary glands of 9- and 12-week-old *P1-MCre-Igf2*$^{p+/m+}$ mice and 30- and 37-week-old *P1-MCre-Igf2*$^{p-/m-}$ mice as compared to the levels in salivary glands of littermate *MMTV-Cre* mice was demonstrated by Western blot analysis, using different antibodies as described in Materials and methods. An anti-actin antibody was used to visualize equal loading.

mice is illustrated in Fig. 2A. The tumours are composed of regions that contain mainly tubular structures (epithelial cells) and other regions that contain mainly spindle cells (myoepithelial cells) (Fig. 2B). Some of the tumours show sebaceous/squamous differentiation (Fig. 2C). In older tumours malignant features such as numerous mitotic figures (Fig. 2C) and necrotic regions (Fig. 2D) were observed. The same characteristics were found in pleomorphic adenomas of *P1-MCre-Igf2*$^{p+/m+}$ mice (23).

Expression upregulation of PLAG1 target genes in P1-MCre-Igf2$^{p-/m-}$ *mice. P1-MCre-Igf2*$^{p-/m-}$ mice still develop salivary gland tumours, although after a latency period that is about twice as long as in *P1-MCre-Igf2*$^{p+/m+}$ mice. Therefore, it is reasonable to conclude that also other molecular pathways, besides Igf signalling, are involved in PLAG1-induced tumourigenesis. Previously, several other genes, such as *H19, Dlk1, Gtl2, Igfbp2* and *Igfpb3* have been found to be upregulated in PLAG1-induced tumours. In this study, their expression levels were investigated in *P1-MCre-Igf2*$^{p-/m-}$ mice and compared to those in *P1-MCre-Igf2*$^{p+/m+}$ mice (Table I).

In this way, we wanted to see whether or not the expression of these genes is dependent on *Igf2* expression, and presumably Igf2 signalling. As might be expected, no expression of *Igf2* was observed when *Igf2* was inactivated in P1-MCre mice and the expression of *Igf2* was significantly decreased (P=0.02) in *P1-MCre-Igf2*$^{p-/m-}$ mice.

The expression levels of all the other genes investigated were not significantly different in *P1-MCre-Igf2*$^{p-/m-}$ mice as compared to littermate *P1-MCre-Igf2*$^{p+/m+}$ mice and are as such independent of *Igf2* expression. The *H19* gene of the imprinted gene cluster *Igf2/H19* is still significantly upregulated when both *Igf2* alleles are knocked out in P1-MCre mice. Similarly, the genes from the imprinted gene cluster *Dlk1/Gtl2* and the *Igfbp2* and *Igfbp3* genes are also significantly upregulated under those conditions. Zhao and co-workers reported about the involvement of Wnt signalling in PLAG1-induced pleomorphic salivary gland adenomas in non-inducible *MMTV-PLAG1* transgenic mice (24). Therefore, we decided to investigate whether we could confirm this also in *P1-MCre-Igf2*$^{p-/m-}$ mice. Several genes involved in Wnt signalling, such as Wnt6, cyclin D1 and ß-catenin, were found to be upregulated in *P1-MCre-Igf2*$^{p-/m-}$ and *P1-MCre-Igf2*$^{p+/m+}$ mice. This upregulation was low and not significant for ß-catenin in *P1-MCre-Igf2*$^{p-/m-}$ mice.

Wnt signalling in pleomorphic adenomas of P1-MCre-Igf2$^{p+/m+}$ *and P1-MCre-Igf2*$^{p-/m-}$ *mice*. To further substantiate Wnt signalling in PLAG1-induced pleomorphic adenomas of the salivary glands of *P1-MCre-Igf2*$^{p+/m+}$ and *P1-MCre-Igf2*$^{p-/m-}$ mice, Western blot analysis was performed. In absence of Wnt, GSK-3 is constitutively active and is believed to promote degradation of ß-catenin by N-terminal phosphorylation, ubiquitination, and proteasomal targeting subsequently. Upon Wnt signalling, the activity of GSK-3 is inhibited. As a consequence, ß-catenin can no longer be phosphorylated and accumulates to form nuclear complexes with TCF/LEF transcriptional co-activators (36). In order to demonstrate the presence of active Wnt in *PLAG1*-induced pleomorphic adenomas of the salivary glands of *P1-MCre-Igf2*$^{p+/m+}$ and *P1-MCre-Igf2*$^{p-/m-}$ mice, Western blot analysis was performed on pleomorphic adenomas of these mice and on control salivary glands of *MMTV-Cre* littermate mice. Accumulation of ß-catenin in pleomorphic adenomas of P1-*MCre-Igf2*$^{p+/m+}$ and *P1-MCre-Igf2*$^{p-/m-}$ mice was clearly demonstrated (Fig. 3). Furthermore, the presence of active-ß-catenin was demonstrated in those pleomorphic adenomas, using a monoclonal antibody that specifically recognizes the non-phosphorylated residues Ser-37 and Thr-41 of ß-catenin (36). Western blot analysis for actin was performed to demonstrate equal loading. These data all point towards the presence of active Wnt in the *PLAG1*-induced pleomorphic adenomas, independent of *Igf2* expression.

Discussion

IGF signalling has been reported to be involved in many different human tumour types (31-35). Furthermore, the oncogenic capacity of *IGF-II* has been demonstrated in mouse models [reviewed by Werner and co-workers (37)]. For example, ectopic expression of *IGF-II* in the mammary glands leads to the development of mammary gland tumours after a long latency period (38,39). In contrast, *MMTV-IGF-II* transgenic mice do not develop overt mammary and salivary gland tumours. Nevertheless, they do develop focal areas of epithelial hyperplasia in mammary glands as well as sporadic tumours in other organs, such as in the lungs. Overexpression of a constitutively active IGF-IR whose expression was driven by the MMTV-promoter resulted in rapid appearance of mammary and salivary gland tumours, further pointing towards a contributing role of IGF signalling in tumourigenesis. For those reasons, we decided to investigate the impact of Igf expression on PLAG1-induced tumourigenesis in the salivary glands, via inactivation of *Igf2* in P1-MCre mice. The results clearly demonstrated an effect of *Igf2* expression on PLAG1-induced tumourigenesis. If *Igf2* is disrupted in P1-MCre mice, the appearance of macroscopically visible salivary gland tumours was delayed significantly. The latency period for tumour appearance about doubled. This observation suggests that Igf2 signalling in these PLAG1-induced tumours is similarly important as in the human salivary gland pleomorphic adenomas with *PLAG1* activation.

Given the above-suggested importance of Igf signalling in PLAG1-induced tumourigenesis, neutralization of Igf2 or other downstream components of this signalling pathway could be an effective therapeutic strategy in combination with other therapies to treat those tumours. Pharmacological antagonism of IGF ligands has been accomplished by neutralizing monoclonal antibodies (40-42). Most therapies target the IGF-IR (43). Several monoclonal antibodies, directed against IGF-IR (44,45) and several small molecule inhibitors have been created (46,47). Other therapies target factors even more downstream in the IGF-IR signalling pathway, such as mTOR signalling. mTOR inhibitors have shown promising clinical efficacy in subsets of cancers, with low toxicity profiles (48). In previous studies (22), we have shown that *Igf-1r*- NIH3T3 cells cannot be transformed *in vitro* by *PLAG1*. Therefore, the *PLAG1* transgenic mouse model could constitute a tool to validate anti-cancer therapies that target Igf-1r *in vivo* in mice. Since the same molecular pathway also plays an important role in different human cancers, the generated data might have an impact relevant to many human cancers in case the data from the various mouse model systems that can be generated can be translated accordingly.

Since tumour development was not fully abrogated by inactivation of *Igf2*, it is likely that additional signalling pathways contribute to PLAG1-induced tumourigenesis as well. Zhao and co-workers (24), previously demonstrated that Wnt signalling is involved in the formation of pleomorphic adenomas of the salivary glands in *MMTV-PLAG1* transgenic mice. Immunohistochemical staining demonstrated that expression of ß-catenin was highly upregulated with overexpression of the *PLAG1* transgene in tumour and normal transgenic salivary gland tissue. They suggest that PLAG1 activates the transcription of mouse ß-catenin in *MMTV-PLAG1* transgenic mice. They demonstrated by luciferase experiments that PLAG1 can activate the mouse but not the human ß-catenin promoter. As such, a direct upregulation of ß-catenin in *MMTV-PLAG1* transgenic mice is a plausible explanation for the possible involvement of

Wnt signalling in PLAG1-induced pleomorphic adenomas in mice but not in humans. Nevertheless, also in human salivary gland pleomorphic adenomas WNT signalling seems to be implicated (49). One mechanism leading to the activation of the WNT signalling pathway in human pleomorphic adenomas is expected to be the downregulation of the WNT inhibitory factor 1 *(WIF1)*.

In our studies, we provide evidence for the possible involvement of Wnt signalling in PLAG1-induced pleomorphic adenomas of the salivary glands in *P1-MCre-Igf2*$^{p+/m+}$ as well as *P1-MCre-Igf2*$^{p-/m-}$ mice. In contrast to Zhao and coworkers, we propose another mechanism involving Wnt-signalling in PLAG1-induced pleomorphic adenomas in mice. We establish that Wnt6 is significantly upregulated in PLAG1-induced pleomorphic adenomas as compared to normal salivary gland tissue of *MMTV-Cre* transgenic mice. As such, Wnt6 may bind to the frizzled receptor and activate Wnt signalling directly and not necessarily via upregulation of ß-catenin itself. In contrast to the mechanism proposed by Zhao and co-workers, this mechanism might also be involved in human pleomorphic adenomas. Nevertheless, this hypothesis still needs to be confirmed in human pleomorphic adenomas of the salivary glands, which overexpress PLAG1.

In conclusion, we clearly demonstrate that Igf signalling is involved in PLAG1-induced pleomorphic adenomas of the salivary glands in mice. Inactivation of Igf2 in P1-MCre mice leads to a delay in tumour development. Furthermore, we also demonstrated that Wnt signalling might be involved in those tumours in P1-MCre mice as well as in P1-MCre mice in which Igf2 is disrupted.

Acknowledgements

This work was supported in part by the Geconcerteerde Onderzoekacties (GOA) 2008/016, the 'Industrieel Onderzoeksfonds' (IOF) of the Flemish government, the 'Fonds voor Wetenschappelijk Onderzoek Vlaanderen' (FWO) grants G.0099.02, G.0441.06 and G.0234.07, the Cancer Research Program of Fortis Bank Insurance 2002-2005, the Foundation for Biochemical, Pharmacological Research and Education, and the Foundation against Cancer, foundation of public interest. J.D. is an aspirant fellow of the 'Fonds voor Wetenschappelijk Onderzoek Vlaanderen' (FWO), whereas FVD is an aspirant fellow of the 'Institute for the Promotion of Innovation through Science and Technology in Flanders (IWT-Vlaanderen)'.

References

1. Speight PM and Barrett AW: Salivary gland tumours. Oral Dis 8: 229-240, 2002.
2. Silvers AR and Som PM: Salivary glands. Radiol Clin North Am 36: 941-966, 1998.
3. Lee KC, Chan JK and Chong YW: Ossifying pleomorphic adenoma of the maxillary antrum. J Laryngol Otol 106: 50-52, 1992.
4. Olsen KD and Lewis JE: Carcinoma ex pleomorphic adenoma: a clinicopathologic review. Head Neck 23: 705-712, 2001.
5. Mizui T, Ishimaru JI, Miyamoto K and Toida M: Malignant transformation of a gigantic pleomorphic adenoma of the submandibular gland: a case report. J Oral Maxillofac Surg 58: 1422-1424, 2000.
6. Roijer E, Nordkvist A, Strom A-K, Ryd W, Behrendt M, Bullerdiek J, Mark J and Stenman G: Translocation, deletion/amplification, and expression of HMGIC and MDM2 in a carcinoma ex pleomorphic adenoma. Am J Pathol 160: 433-440, 2002.
7. Sandros J, Stenman G and Mark J: Cytogenetic and molecular observations in human and experimental salivary gland tumors. Cancer Genet Cytogenet 44: 153-167, 1990.
8. Bullerdiek J, Raabe G, Bartnitzke S, Boschen C and Schloot W: Structural rearrangements of chromosome Nr 8 involving 8q12: a primary event in pleomorphic ademona of the parotid gland. Genetica 72: 85-92, 1987.
9. Stenman G: Fusion oncogenes and tumor type specificity: insights from salivary gland tumors. Semin Cancer Biol 15: 224-235, 2005.
10. Schoenmakers EF, Wanschura S, Mols R, Bullerdiek J, Van den Berghe H and Van de Ven WJ: Recurrent rearrangements in the high mobility group protein gene, HMGI-C, in benign mesenchymal tumours. Nat Genet 10: 436-444, 1995.
11. Kas K, Voz ML, Roijer E, Astrom AK, Meyen E, Stenman G and 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 15: 170-174, 1997.
12. Hensen K, Braem C, Declercq J, Van Dyck F, Dewerchin M, Fiette L, Denef C and Van de Ven WJ: Targeted disruption of the murine Plag1 proto-oncogene causes growth retardation and reduced fertility. Dev Growth Differ 46: 459-470, 2004.
13. Voz ML, Astrom AK, Kas K, Mark J, Stenman G and 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 16: 1409-1416, 1998.
14. Astrom AK, Voz ML, Kas K, Roijer E, Wedell B, Mandahl N, Van de Ven W, Mark J and 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 59: 918-923, 1999.
15. Asp J, Persson F, Kost-Alimova M and Stenman G: CHCHD7-PLAG1 and TCEA1-PLAG1 gene fusions resulting from cryptic, intrachromosomal 8q rearrangements in pleomorphic salivary gland adenomas. Genes Chromosomes Cancer 45: 820-828, 2006.
16. Astrom A, D'Amore ES, Sainati L, Panarello C, Morerio C, Mark J and Stenman G: Evidence of involvement of the PLAG1 gene in lipoblastomas. Int J Oncol 16: 1107-1110, 2000.
17. Hibbard MK, Kozakewich HP, Dal Cin P, Sciot R, Tan X, Xiao S and Fletcher JA: PLAG1 fusion oncogenes in lipoblastoma. Cancer Res 60: 4869-4872, 2000.
18. Morerio C, Rapella A, Rosanda C, Tassano E, Gambini C, Romagnoli G and Panarello C: PLAG1-HAS2 fusion in lipoblastoma with masked 8q intrachromosomal rearrangement. Cancer Genet Cytogenet 156: 183-184, 2005.
19. Zatkova A, Rouillard JM, Hartmann W, Lamb BJ, Kuick R, Eckart M, von Schweinitz D, Koch A, Fonatsch C, Pietsch T, Hanash SM and Wimmer K: Amplification and overexpression of the IGF2 regulator PLAG1 in hepatoblastoma. Genes Chromosomes Cancer 39: 126-137, 2004.
20. Castilla LH, Perrat P, Martinez NJ, Landrette SF, Keys R, Oikemus S, Flanegan J, Heilman S, Garrett L, Dutra A, Anderson S, Pihan GA, Wolff L and Liu PP: Identification of genes that synergize with Cbfb-MYH11 in the pathogenesis of acute myeloid leukemia. Proc Natl Acad Sci USA 101: 4924-4929, 2004.
21. Landrette SF, Kuo YH, Hensen K, Barjesteh van Waalwijk van Doorn-Khosrovani S, Perrat PN, Van de Ven WJ, Delwel R and Castilla LH: Plag1 and Plagl2 are oncogenes that induce acute myeloid leukemia in cooperation with Cbfb-MYH11. Blood 105: 2900-2907, 2005.
22. Hensen K, Van Valckenborgh IC, Kas K, Van de Ven WJ and Voz ML: The tumorigenic diversity of the three PLAG family members is associated with different DNA binding capacities. Cancer Res 62: 1510-1517, 2002.
23. Declercq J, Van Dyck F, Braem CV, Van Valckenborgh IC, Voz M, Wassef M, Schoonjans L, Van Damme B, Fiette L and Van de Ven WJ: Salivary gland tumors in transgenic mice with targeted PLAG1 proto-oncogene overexpression. Cancer Res 65: 4544-4553, 2005.
24. Zhao X, Ren W, Yang W, Wang Y, Kong H, Wang L, Yan L, Xu G, Fei J, Fu J, Zhang C and Wang Z: Wnt pathway is involved in pleomorphic adenomas induced by overexpression of PLAG1 in transgenic mice. Int J Cancer 118: 643-648, 2006.

25. Wagner KU, McAllister K, Ward T, Davis B, Wiseman R and Hennighausen L: Spatial and temporal expression of the Cre gene under the control of the MMTV-LTR in different lines of transgenic mice. Transgenic Res 10: 545-553, 2001.
26. Wagner KU, Wall RJ, St-Onge L, Gruss P, Wynshaw-Boris A, Garrett L, Li M, Furth PA and Hennighausen L: Cre-mediated gene deletion in the mammary gland. Nucleic Acids Res 25: 4323-4330, 1997.
27. Voz ML, Mathys J, Hensen K, Pendeville H, Van Valckenborgh I, Van Huffel C, Chavez M, Van Damme B, De Moor B, Moreau Y and Van de Ven WJ: Microarray screening for target genes of the proto-oncogene PLAG1. Oncogene 23: 179-191, 2004.
28. Voz ML, Agten NS, Van de Ven WJ and Kas K: PLAG1, the main translocation target in pleomorphic adenoma of the salivary glands, is a positive regulator of IGF-II. Cancer Res 60: 106-113, 2000.
29. DeChiara TM, Efstratiadis A and Robertson EJ: A growth-deficiency phenotype in heterozygous mice carrying an insulin-like growth factor II gene disrupted by targeting. Nature 345: 78-80, 1990.
30. DeChiara TM, Robertson EJ and Efstratiadis A: Parental imprinting of the mouse insulin-like growth factor II gene. Cell 64: 849-859, 1991.
31. Sachdev D and Yee D: The IGF system and breast cancer. Endocr Relat Cancer 8: 197-209, 2001.
32. Helle SI: The insulin-like growth factor system in advanced breast cancer. Best Pract Res Clin Endocrinol Metab 18: 67-79, 2004.
33. Hadsell DL and Bonnette SG: IGF and insulin action in the mammary gland: lessons from transgenic and knockout models. J Mammary Gland Biol Neoplasia 5: 19-30, 2000.
34. Tricoli JV, Rall LB, Karakousis CP, Herrera L, Petrelli NJ, Bell GI and Shows TB: Enhanced levels of insulin-like growth factor messenger RNA in human colon carcinomas and liposarcomas. Cancer Res 46: 6169-6173, 1986.
35. Martin DC, Fowlkes JL, Babic B and Khokha R: Insulin-like growth factor II signalling in neoplastic proliferation is blocked by transgenic expression of the metalloproteinase inhibitor TIMP-1. J Cell Biol 146: 881-892, 1999.
36. van Noort M, Meeldijk J, van der Zee R, Destree O and Clevers H: Wnt signalling controls the phosphorylation status of beta-catenin. J Biol Chem 277: 17901-17905, 2002.
37. Werner H and Le Roith D: The insulin-like growth factor-I receptor signalling pathways are important for tumorigenesis and inhibition of apoptosis. Crit Rev Oncog 8: 71-92, 1997.
38. Bates P, Fisher R, Ward A, Richardson L, Hill DJ and Graham CF: Mammary cancer in transgenic mice expressing insulin-like growth factor II (IGF-II). Br J Cancer 72: 1189-1193, 1995.
39. Pravtcheva DD and Wise TL: Metastasizing mammary carcinomas in H19 enhancers-Igf2 transgenic mice. J Exp Zool 281: 43-57, 1998.
40. Van Den Berg CL, Cox GN, Stroh CA, Hilsenbeck SG, Weng C-N, Mcdermott MJ, Pratt D, Osborne CK, Coronado-Heinsohn EB and Yee D: polyethylene glycol conjugated insulin-like growth factor binding protein-1 (IGFBP-1) inhibits growth of breast cancer in athymic mice. Eur J Cancer 33: 1108-1113, 1997.
41. Miyamoto S, Nakamura M, Shitara K, Nakamura K, Ohki Y, Ishii G, Goya M, Kodama K, Sangai T, Maeda H, Shi-Chuang Z, Chiba T and Ochiai A: Blockade of paracrine supply of insulin-like growth factors using neutralizing antibodies suppresses the liver metastasis of human colorectal cancers. Clin Cancer Res 11: 3494-3502, 2005.
42. Goya M, Miyamoto S, Nagai K, Ohki Y, Nakamura K, Shitara K, Maeda H, Sangai T, Kodama K, Endoh Y, Ishii G, Hasebe T, Yonou H, Hatano T, Ogawa Y and Ochiai A: Growth inhibition of human prostate cancer cells in human adult bone implanted into nonobese diabetic/severe combined immunodeficient mice by a ligand-specific antibody to human insulin-like growth factors. Cancer Res 64: 6252-6258, 2004.
43. Yee D: Targeting insulin-like growth factor pathways. Br J Cancer 94: 465-468, 2006.
44. Sachdev D, Li SL, Hartell JS, Fujita-Yamaguchi Y, Miller JS and Yee D: A chimeric humanized single-chain antibody against the type I insulin-like growth factor (IGF) receptor renders breast cancer cells refractory to the mitogenic effects of IGF-I. Cancer Res 63: 627-635, 2003.
45. Wang Y, Hailey J, Williams D, Lipari P, Malkowski M, Wang X, Xie L, Li G, Saha D, Ling WL, Cannon-Carlson S, Greenberg R, Ramos RA, Shields R, Presta L, Brams P, Bishop WR and Pachter JA: Inhibition of insulin-like growth factor-I receptor (IGF-IR) signalling and tumor cell growth by a fully human neutralizing anti-IGF-IR antibody. Mol Cancer Ther 4: 1214-1221, 2005.
46. Haluska P, Carboni JM, Loegering DA, Lee FY, Wittman M, Saulnier MG, Frennesson DB, Kalli KR, Conover CA, Attar RM, Kaufmann SH, Gottardis M and Erlichman C: *In vitro* and *in vivo* antitumor effects of the dual insulin-like growth factor-I/insulin receptor inhibitor, BMS-554417. Cancer Res 66: 362-371, 2006.
47. Wittman M, Carboni J, Attar R, Balasubramanian B, Balimane P, Brassil P, Beaulieu F, Chang C, Clarke W, Dell J, Eummer J, Frennesson D, Gottardis M, Greer A, Hansel S, Hurlburt W, Jacobson B, Krishnananthan S, Lee FY, Li A, Lin TA, Liu P, Ouellet C, Sang X, Saulnier MG, Stoffan K, Sun Y, Velaparthi U, Wong H, Yang Z, Zimmermann K, Zoeckler M and Vyas D: Discovery of a (1H-benzoimidazol-2-yl)-1H-pyridin-2-one (BMS-536924) inhibitor of insulin-like growth factor I receptor kinase with *in vivo* antitumor activity. J Med Chem 48: 5639-5643, 2005.
48. Thomas GV: mTOR and cancer: reason for dancing at the crossroads? Curr Opin Genet Dev 16: 78-84, 2006.
49. Queimado L, Lopes CS and Reis AM: WIF1, an inhibitor of the Wnt pathway, is rearranged in salivary gland tumors. Genes Chromosomes Cancer 46: 215-225, 2007.