Emerging role of PLAG1 as a regulator of growth and reproduction

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Abstract

Pleomorphic adenoma gene 1 (PLAG1) belongs to the PLAG family of zinc finger transcription factors along with PLAG-like 1 and PLAG-like 2. The *PLAG1* gene is best known as an oncogene associated with certain types of cancer, most notably pleomorphic adenomas of the salivary gland. While the mechanisms of PLAG1-induced tumorigenesis are reasonably well understood, the role of PLAG1 in normal physiology is less clear. It is known that PLAG1 is involved in cell proliferation by directly regulating a wide array of target genes, including a number of growth factors such as insulin-like growth factor 2. This is likely to be a central mode of action for PLAG1 both in embryonic development and in cancer. The phenotype of *Plag1* knockout mice suggests an important role for PLAG1 also in postnatal growth and reproduction, as PLAG1 deficiency causes growth retardation and reduced fertility. A role for PLAG1 in growth and reproduction is further corroborated by genome-wide association studies in humans and domestic animals in which polymorphisms in the PLAG1 genomic region are associated with body growth and reproductive traits. Here we review the current evidence for PLAG1 as a regulator of growth and fertility and discuss possible endocrine mechanisms involved.

Key Words

- ▶ pleomorphic adenoma gene 1
- ▶ zinc finger transcription factor
- ▶ growth
- ▶ reproduction
- ▶ puberty
- ▶ hypothalamo-pituitary-gonadal axis
- ▶ insulin-like growth factors

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Introduction

The transcription factor pleomorphic adenoma gene 1 (PLAG1) was first discovered via positional cloning when researchers were studying pleomorphic adenomas of the salivary glands (Kas *et al.* 1997). Its role in various types of cancer has been extensively studied since. Apart from salivary gland adenomas, PLAG1 was later shown to be involved in other human cancers such as lipoblastoma, hepatoblastoma, and acute myeloid leukemia (Åström *et al.* 2000, Zatkova *et al.* 2004, Landrette *et al.* 2005) and is also ectopically expressed in uterine leiomyoma and

leiomyosarcoma (Åström *et al.* 1999). One of the causes of PLAG1-induced cancers is a reciprocal chromosomal translocation event, leading to swapping of the *PLAG1* promoter with that of ubiquitously expressed genes. In pleomorphic adenomas of the salivary glands, translocation partners include genes such as *CTNNB1* (which encodes β -catenin), *LIFR* (encoding the leukemia inhibitory factor receptor) and the transcription elongation factor SII gene (Kas *et al.* 1997, Voz *et al.* 1998, Åström *et al.* 1999). The chromosomal breakpoints are located between

the upstream regulatory region and the coding region of both translocation partners, preserving the coding sequence and functionality of the translated proteins involved. Following promoter swapping, *PLAG1* expression comes under the control of strong promoters, leading to overexpression or ectopic activation of *PLAG1*. This in turn results in a deregulation of *PLAG1* target genes and tumor formation (Voz *et al.* 2000, 2004). The tumorigenic capacity of *PLAG1* was confirmed in transgenic mouse models in which targeted *Plag1* overexpression in the salivary and mammary glands resulted in tumor development (Declercq *et al.* 2005).

In contrast to our fairly detailed knowledge about the mechanisms behind *PLAG1*-driven tumorigenesis in various cancer types (reviewed in more detail by Van Dyck *et al.* 2007a), our understanding of the biological role of *PLAG1* in normal physiology is still limited. In this review, we will first summarize what is currently known about the molecular biology of *PLAG1* and then integrate the findings of recent studies that provide clues about the biological role of *PLAG1* in the endocrine systems coordinating growth and reproduction in particular.

Molecular biology of *PLAG1*

Gene and protein structure of *PLAG1*

The *PLAG1* gene is located on human chromosome 8q12. Although the predominant *PLAG1* transcript is 7.3 kb long, there are also three shorter splice variants of 7.2, 7.0 and 6.9 kb (Van Dyck *et al.* 2007a). In humans, *PLAG1* consists of five exons. Exons 4 and 5 contain the coding sequence of 1503 bp, which translates to a 500-amino acid protein of 56 kDa (Fig. 1A). The N-terminus of *PLAG1* contains seven canonical C₂H₂ zinc finger domains responsible for the DNA binding capacity of the protein, and the C-terminus encompasses a serine-rich region that has transcriptional activation activity (Kas *et al.* 1998, Van Dyck *et al.* 2007a). Consistent with its role as a transcription factor, *PLAG1* has been shown to be a nuclear protein. There are two nuclear localization signals present in its structure; one is the N-terminal zinc finger region, and the other is a karyopherin $\alpha 2$ recognition site in the N-terminus involved in the docking of proteins to the nuclear pores (Braem *et al.* 2002, Van Dyck *et al.* 2007a). The predicted mouse *PLAG1* protein is 96% similar to its human homologue. The Ensembl-predicted mouse *Plag1* gene is located on chromosome 4 and consists of six exons, with a coding sequence of 1500 bp that

encompasses the 3' end of exon 5 and the 5' end of exon 6 and translates into a 499-amino acid protein (Fig. 1B).

The *PLAG* family contains two additional zinc finger transcription factors, both with known roles in cancer. One, *PLAG-like 1* (*PLAGL1*, also known as *LOT1* or *ZAC1*), is a maternally imprinted tumor-suppressing gene that inhibits tumor cell proliferation through the induction of apoptosis and cell cycle arrest. Ubiquitously expressed in adult human tissues, *PLAGL1* is a target of growth factor signaling pathways and its expression is lowered or completely abolished in many types of human cancers (Abdollahi 2007). The other, *PLAG-like 2* (*PLAGL2*), is a proto-oncogene structurally and functionally more similar to *PLAG1* (Hensen *et al.* 2002, Landrette *et al.* 2005, Van Dyck *et al.* 2007a), although under certain conditions *PLAGL2* has an apoptotic role (Mizutani *et al.* 2002). The *PLAG* proteins are most similar in their N-terminal domains, with 73% identity between *PLAG1* and *PLAGL1* and 79% between *PLAG1* and *PLAGL2*. The C-terminus of *PLAG1*, on the other hand, shows only 19% identity with that of *PLAGL1* and 35% with *PLAGL2*. In zebrafish, a *plagx* gene was identified in addition to *plag1* and *plag2* (Pendeville *et al.* 2006).

Target genes of *PLAG1*

Being a transcription factor, *PLAG1* acts by altering the transcription rate of target genes. Transient transactivation assays and electrophoretic mobility shift assays have demonstrated that *PLAG1* can bind the embryonic P3 promoter of *insulin-like growth factor 2* (*IGF2*) and stimulate its activity, leading to increased *IGF2* expression (Voz *et al.* 2000). Insulin-like growth factor 2 is a growth factor that is essential for normal embryonic growth and its gene is maternally imprinted (DeChiara *et al.* 1991). Transcripts of *IGF2* derived from the P3 promoter were greatly expressed in salivary gland adenomas overexpressing *PLAG1* but were undetectable in adenomas without abnormal *PLAG1* expression or in normal salivary gland tissue (Voz *et al.* 2000). Similarly, there is a consistent and significant upregulation of *IGF2* expression in *PLAG1*-overexpressing NIH3T3 and human embryonic kidney 293 (HEK293) cells, in murine salivary tumors induced by *Plag1* overexpression, and in hepatoblastomas (Hensen *et al.* 2002, Zatkova *et al.* 2004, Declercq *et al.* 2005). Conversely, *PLAG1* overexpression is unable to transform fibroblasts with a targeted disruption of the insulin-like growth factor 1 receptor (*IGF1R*), required for *IGF2* (but also *IGF1*) function (LeRoith *et al.* 1995, Hensen *et al.* 2002). These data have led to the conclusion that *PLAG1* influences

Figure 1

Schematic representation of the structure of human (A) and predicted mouse (B) *PLAG1/Plag1* genes and their proteins. In the gene structures, introns are represented by lines, and exons by boxes. Shaded boxes are the (parts of) exons that encode the untranslated regions; white boxes refer to the protein-coding sequence. Numbers refer to the number of base pairs. In the protein structures, numbers refer to the number of amino acids and

lightly shaded boxes indicated by 'ZF' represent the seven zinc finger domains. Structures are based on the Ensembl sequences ENSG00000181690.7 (human *PLAG1* gene) and UniProt entry Q6DJT9 (human *PLAG1* protein) and Ensembl ENSMUSG00000003282.7 (mouse *Plag1* gene) and UniProt entry Q9QYE0 (mouse *PLAG1* protein).

tumorigenesis, at least in part, via the mitogenic action of IGF2, most likely via the activation of IGF1R and the Ras/Raf/MAPK signaling pathway (Hensen *et al.* 2002, Van Dyck *et al.* 2007a). It should be noted, however, that the capacity of *PLAG1* to bind the *IGF2* P3 promoter and increase *IGF2* expression is cell type-specific, as overexpression of *PLAG1* in the human choriocarcinoma cell line JEG-3 did not result in significant promoter binding or upregulation of IGF2 (Akhtar *et al.* 2012). An additional role of *PLAG1* in the stimulation of *IGF2* expression appears to be that of promoter/enhancer facilitator, because *PLAG1* induction in these cells was shown to partially overcome the transcription-suppressing effect of the *H19* chromatin insulator on the *IGF2* P3 promoter in reporter constructs (Akhtar *et al.* 2012). Moreover, there is evidence that *Wnt* signaling contributes to *PLAG1*-induced tumorigenesis (Declercq *et al.* 2008).

To further unravel the molecular mechanism of *PLAG1*-induced oncogenesis, a microarray containing 12 000 human genes was used to screen transcripts expressed in normal salivary glands vs pleomorphic adenomas and compare them to the expression profile of HEK293 cells overexpressing *PLAG1* (Voz *et al.* 2004). The study revealed that 627 genes were differentially expressed

in the salivary glands, whereby 254 were downregulated and 373 were upregulated at least threefold in the tumors. These genes were grouped into 14 functional categories, including growth factors, growth factor receptors, growth factor-binding proteins, growth regulation proteins, cell division and cell-cycle related proteins, apoptosis-related proteins, proto-oncogenes, tumor suppressors, transcriptional regulators, signaling proteins, extracellular matrix proteins, and metabolism-related proteins. Notably, most of the upregulated genes were growth factors, growth factor binding proteins and growth factor receptors. The comparison of *PLAG1*-overexpressing vs normal HEK cells revealed 47 upregulated genes and 12 repressed genes. The combined data showed that 12 genes were consistently upregulated with *PLAG1* overexpression: those encoding IGF2 (*IGF2*); cytokine-like factor 1 (*CYTL1*); quiescin Q6 sulfhydryl oxidase 1 (*QSOX1*); cellular retinoic acid-binding protein 2 (*CRABP2*); SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily d, member 3 (*SMARCD3*); B-cell CLL/lymphoma 2 (*BCL2*); cyclin-dependent kinase inhibitor 1C (*CDKN1C*); ephrin B1 (*EFNB1*); neuronal pentraxin receptor (*NPTXR*); collagen, type IX, alpha 3 (*COL9A3*); the muscle isoform of filamin μ C, gamma (*FLNC*); and tetraspanin 4 (*TSPAN4*).

In contrast, none of the 12 repressed genes in the *PLAG1*-overexpressing HEK cells were altered in the pleomorphic adenomas (Voz *et al.* 2004). This again emphasizes that *PLAG1* regulates genes in a tissue-specific manner.

Pleomorphic adenoma gene 1 alters the expression levels of its target genes by binding to their regulatory elements. Use of the cyclic amplification and selection of targets (CASTing) strategy revealed a consensus binding motif in *PLAG1* target genes composed of a core GRGGC, followed six to eight nucleotides downstream by a cluster of at least three Gs. The GRGGC core of the bipartite sequence is recognized by zinc fingers 6 and 7 of *PLAG1*, and the G cluster is bound by zinc finger 3 (Voz *et al.* 2000). *In silico* analysis confirmed that the 12 genes identified as direct targets of *PLAG1* in salivary glands and HEK cells all possess the *PLAG1* binding motif (Voz *et al.* 2004). Five such binding motifs were found in the P3 promoter of *IGF2* (Voz *et al.* 2000) and eight in the promoter of *CYTL1*, but most target genes have only one to three (Voz *et al.* 2004). The genes in which the transcription levels were lowered in *PLAG1*-overexpressing HEK cells do not appear to be regulated by *PLAG1* through direct binding to their promoters, because they lack the target sequence (Voz *et al.* 2004).

PLAG1 expression patterns

In general, *PLAG1* expression is elevated during the embryonic/fetal period, whereas expression in most adult organs is low or absent, and this has led to the notion of *PLAG1* being a 'fetal' transcription factor. Zebrafish *Plag* genes have low levels of expression during somitogenesis, which then increase and plateau at 5 days post fertilization (Pendeville *et al.* 2006). Whole-mount *in situ* hybridization revealed that all three zebrafish *plag* genes have similar spatial expression patterns characterized by initial low ubiquitous expression, which becomes restricted to zones of active cell proliferation such as the ventricular zone in the brain and the developing pharyngeal arches. In humans, *PLAG1* is highly expressed in fetal kidney, liver, and lung, whereas in adult tissues expression was reported either as undetectable (in skeletal muscle, brain, heart, pancreas, lung, and placenta; Kas *et al.* 1997), or as detectable (in heart, placenta, spleen, prostate, testis, ovary, and small intestine; Queimado *et al.* 1999) by northern blot analysis. All three *Plag* genes are expressed in both unique and overlapping patterns in early stages of neural development as determined by *in situ* hybridization in mice (Alam *et al.* 2005). Expression in mouse tissues is present until after

birth when levels decline as shown by northern blotting. *Plag1* transcripts in adult mice were below the detection limit in brain, thymus, stomach, intestines, spleen, prostate, skeletal muscle, kidney, salivary gland, uterus, tongue, lung, and liver (Hensen *et al.* 2004). In contrast, *Plag1* has been detected not only in embryonic but also in adult rat liver using quantitative PCR, albeit at greater levels in the embryonic liver compared to the adult organ (Zheng *et al.* 2014). This discrepancy with earlier (mouse) studies may be due to species differences or could point to a lower sensitivity of the northern blot technique to assess *Plag1* expression. Although *Plag1* expression declines drastically after birth to undetectable levels in most mouse tissues, there are some notable exceptions. Expression of *Plag1* mRNA has been detected via *in situ* hybridization and/or PCR in the heart, ovary, testis, vas deferens, and epididymis of adult mice (Hensen *et al.* 2004). In the gonads, *Plag1* expression has been reported in the seminiferous epithelium and isolated Sertoli cells of the testes, as well as in ovarian follicles. Likewise, considerable expression of *Plag1* was shown in the embryonic pituitary primordium, which persists in the anterior pituitary of adult mice (Hensen *et al.* 2004).

Regulation of PLAG1

Transcription of the *PLAG1* gene is itself believed to be controlled by a bidirectional promoter located between the *PLAG1* coding sequence and its neighboring gene *CHCHD7* (Karim *et al.* 2011). In cattle, two sequence variants of the *PLAG1* promoter with different 'strengths' have been described, relating to a variable number tandem repeat (VNTR) immediately upstream of the presumed *PLAG1* transcription start site and a single nucleotide polymorphism (SNP) located 12 bp upstream of the VNTR, in an otherwise highly conserved region (Fig. 2). The VNTR is a (CCG)_n trinucleotide repeat with either nine copies (hereafter referred to as the *q* variant of the promoter) or 11 copies (the *Q* variant), whereas the SNP is an 'A' in the *q* variant or a 'G' in the *Q* variant (Karim *et al.* 2011). Luciferase reporter assays have shown that the *Q* variant of the bovine *PLAG1* promoter is consistently ~1.5× more active than the *q* variant and that both the *Q*-type VNTR and the SNP sequence are needed for maximal transcriptional activity. Concordantly, fetal calves of the *QQ* genotype show significantly higher expression levels of *PLAG1* in the liver, bone, muscle, and brain than *qq* calves (Karim *et al.* 2011). The effects of the VNTR and SNP on promoter strength are most likely

Figure 2

Sequence variants that affect the strength of the bidirectional promoter located in the *PLAG1*–*CHCHD7* intergenic region in cattle. In cattle, the Q variant of the promoter, in which the 'CCG' sequence in the VNTR is repeated 11 times and the SNP is 'G', leads to higher transcriptional activity of the promoter compared to the q variant in which the VNTR is repeated nine times and the SNP is 'A' (as shown, shaded). This genomic region is

otherwise highly conserved, as demonstrated by the comparison of the cattle (*Bos taurus*) sequence with that of human (*Homo sapiens*), little brown bat (*Myotis lucifugus*), nine-banded armadillo (*Dasypus novemcinctus*), and mouse (*Mus musculus*). Reprinted by permission from Macmillan Publishers Ltd: *Nature Genetics* (Karim *et al.* 2011), copyright 2011.

caused by differential binding of trans-acting factors, as suggested by electrophoretic mobility shift assays. A number of complexes of nuclear proteins and promoter probes was seen in all tissues and cell lines tested, indicating that widely expressed trans-acting factors are involved. Some complexes, however, were tissue specific, and with nuclear extracts from the mouse myoblast C2C12 cell line, a Q-specific complex was observed that did not form with the q promoter variant (Karim *et al.* 2011). It is not known whether the same *PLAG1* promoter polymorphisms exist in species other than cattle.

In addition to transcriptional regulation, *PLAG1* activity is also controlled by different posttranscriptional mechanisms. Posttranslational modification of transcription factors is known to affect their transactivation capacity. Posttranslational deacetylation and SUMOylation, the reversible attachment of a small ubiquitin-like modifier (SUMO) protein, have been shown to inhibit the transactivation function of *PLAG1*, whereas acetylation activates it (Van Dyck *et al.* 2004, Zheng & Yang 2005). It has been postulated that these regulatory mechanisms change the affinity of co-repressors and co-activators (Van Dyck *et al.* 2007a). SUMOylation may also influence the nuclear localization of *PLAG1*, but contradictory results have been reported (Van Dyck *et al.* 2004, Zheng & Yang 2005). Furthermore, the C-terminal serine-rich transactivation domain of *PLAG1* possesses phosphorylation sites, raising the possibility that phosphorylation may regulate the transactivation capacity of *PLAG1* (Van Dyck *et al.* 2007a).

Recently, it was shown that expression of *PLAG1* is also regulated by microRNAs (miRNAs). These short (~22 bp), non-coding, double-stranded RNA molecules bind to target mRNAs, most often to a binding site located in their 3' untranslated regions (3'UTRs), leading to translational repression or mRNA decay. Target prediction of miRNAs found to be deregulated in B cells from chronic lymphocytic leukemia patients led to the discovery of nine highly conserved and eight less conserved putative miRNA binding sites in the 3'UTR of *PLAG1* (Pallasch *et al.* 2009). It was subsequently confirmed that *PLAG1* expression is regulated by miR-107, -141, -155, -181a, -181b, and -424. Transcriptional silencing of some of these miRNAs due to hypermethylation of their promoters is thought to result in overexpression of *PLAG1*, ultimately leading to chronic lymphocytic leukemia. In B cells of chronic lymphocytic leukemia patients, *PLAG1* mRNA levels were not different to those in healthy donor B cells, but *PLAG1* protein levels were indeed upregulated, suggesting that the miRNAs control *PLAG1* levels primarily by translational repression rather than mRNA breakdown (Pallasch *et al.* 2009). Upregulation of miR-141 in the placentas of human fetuses suffering intrauterine growth restriction, on the other hand, was found to be associated with down-regulation of placental *PLAG1* mRNA and protein levels (Tang *et al.* 2013). Based on a significant correlation between *PLAG1* and *IGF2* expression levels also seen in these placentas, the authors of this study proposed that development of fetal growth restriction may be due in part to a deregulation of a miR-141–*PLAG1*–*IGF2* pathway.

Role of PLAG1 in growth

Evidence from *in vivo* studies

To elucidate the role of PLAG1 in embryonic development, a *Plag1* knockout (KO) mouse strain was generated in which both alleles of *Plag1* are inactivated by targeted disruption by homologous recombination (Hensen *et al.* 2004). One of the most prominent features of the KO phenotype is reduced body size (Fig. 3). Although *Plag1*-deficient mice are viable, they are significantly smaller at birth compared to WT mice (Hensen *et al.* 2004). A size difference of 18% was detectable as early as embryonic day 11.5, and by the end of gestation, mutants were ~30% smaller. This difference in body size augmented during postnatal growth with mutants being up to 50% smaller at postnatal day 21. After weaning, the growth rate of the *Plag1*-deficient animals increased such that the difference in body weight at postnatal day 60 was back to 30%

smaller than the WT mice. Notably, the weight of most organs of KO mice was proportionate to body weight, apart from the ventral prostate and seminal vesicles, which were significantly smaller. Despite detection of *Plag1* expression in the placenta and testis of WT mice, no significant difference in organ weight relative to body weight was noted in these organs in mutants (Hensen *et al.* 2004). It is possible that some of the effects of *Plag1* deficiency were compensated for by functionally redundant genes like *Plagl2*. Pleomorphic adenoma gene 1 and PLAGL2 both possess similar DNA binding affinities, have common downstream target genes such as *Igf2*, and, at least in some tissues, have overlapping spatial and temporal expression patterns (Kas *et al.* 1997, 1998, Hensen *et al.* 2002, Landrette *et al.* 2005). Surprisingly, the *Plagl2* KO mouse has a much more severe phenotype. KO animals exhibit high neonatal lethality due to a failure of fat absorption, and this prevents studies of the specific role of PLAGL2 in adult mice. It is believed that PLAG1 and PLAGL2 are functionally redundant during embryonic/fetal development but not postnatally, when PLAG1 levels are very low (Van Dyck *et al.* 2007b).

Evidence from genome-wide association studies

The importance of PLAG1 in growth is further supported by a range of recent genome-wide association studies (GWASs) in humans and cattle. A SNP in the chromosome fragment containing *PLAG1* and its neighboring genes was identified as 1 of 27 loci that showed the strongest correlation with adult human height in a meta-analysis of 39 510 (mostly Caucasian) individuals (Gudbjartsson *et al.* 2008). Similarly, SNPs in the *PLAG1* chromosomal region were found to correlate with human height in Asian populations (Cho *et al.* 2009, Okada *et al.* 2010). Prior to these discoveries in humans, a myriad of genomic studies had identified a region on bovine chromosome 14 as having a significant effect on growth-related traits in cattle (Wibowo *et al.* 2008). It was subsequently shown that the loci associated with height in humans are also associated with stature in cattle (Pryce *et al.* 2011). The region on chromosome 14 that was consistently found to affect cattle stature (or related traits such as hip height, live weight at birth and at various other ages, growth rate, body condition score, and carcass weight) contains the bovine *PLAG1* homologue, among other genes (Karim *et al.* 2011, Pausch *et al.* 2011, Pryce *et al.* 2011, Hawken *et al.* 2012, Littlejohn *et al.* 2012, Nishimura *et al.* 2012, Fortes *et al.* 2013a, Utsunomiya *et al.* 2013). The association of body weight with the chromosome 14

Figure 3

(A) Phenotype of a female *Plag1* knockout mouse (right) compared to a heterozygous female littermate (left) at the age of ~45 days. (B) Body weights (in grams) of male and female WT (black bars), heterozygous (dotted bars) and knockout (white bars) mice at weaning (P E Damdimopoulou, unpublished results). Values are means $\pm$ S.E.M. ($n=6-13$). Data without a common letter are significantly different ($P<0.05$, ANOVA and *post-hoc* Tukey's honest significant difference (HSD) test). Two-way ANOVA revealed an effect of both sex and genotype on body weight but no sex $\times$ genotype interaction.

region was already detectable at birth (Karim *et al.* 2011). A co-localized effect on height was observed, but because no association was found with the weight:height ratio, it was concluded that the *PLAG1*-containing chromosomal region affects stature rather than weight *per se*. Karim *et al.* (2011) were able to narrow down the stature-affecting sequences on bovine chromosome 14 to a small number of polymorphisms in the vicinity of the *PLAG1* gene, most notably the VNTR and SNP in the *PLAG1* promoter (the Q and q variants) mentioned earlier, as well as a SNP in the 3'UTR of *PLAG1*. For each Q variant of the promoter present (i.e., the sequence variant proven to result in greater *PLAG1* transcription levels; see above), an additional ~20 kg in body weight and ~2 cm in body height were observed in the cattle under investigation, resulting in a difference between full-grown QQ and qq animals of ~40 kg and ~4 cm (Karim *et al.* 2011). Similarly, newborn homozygous QQ and heterozygous Qq dairy calves were found to be 18.8 and 10.4% heavier, respectively, compared to homozygous qq calves (Littlejohn *et al.* 2012). Variants of *PLAG1* have also been suggested to contribute to body size in European domestic pigs and horses (Rubin *et al.* 2012, Metzger *et al.* 2013), indicating that this role of *PLAG1* is likely to be conserved across mammals. It should be emphasized, however, that although *PLAG1* was found to be the most plausible candidate on chromosome 14 as the causative gene for stature differences in cattle, other genes in the vicinity of *PLAG1*, such as *RPS20*, *MOS*, *RDHE2*, *SDR16C6*, and *PENK*, some of which having established links with growth, were also differentially expressed in the fetal tissues of QQ and qq animals (Karim *et al.* 2011).

Possible mechanisms linking *PLAG1* to growth

Whereas evidence for an important role of *PLAG1* in mammalian growth is increasing, the underlying mechanisms are somewhat elusive. This is likely to stem, in part, from the demonstrated effects of *PLAG1* on IGF2 expression levels in the cell culture and cancer studies mentioned before. Insulin-like growth factors regulate pathways involved in cell proliferation and differentiation and are well known as key regulators of normal fetal development and growth. The control of fetal growth by IGF2 occurs both directly at the level of cell proliferation in the fetus and indirectly at the level of the placenta, where, because of its effects on placental structure and physiology, the supply of maternal nutrients to the fetus is affected (Reik *et al.* 2001). Insulin-like growth factor 2 also functions in cardiac development, myogenesis,

vasculogenesis, and bone growth and development (Minuto *et al.* 2004, Wilson & Rotwein 2006, Harris & Westwood 2012). Therefore, it is generally assumed that the role of *PLAG1* in growth is mainly a reflection of its capacity to increase the expression of IGF2 (at least in some cell types; see above) and other growth factors. Indeed, like murine IGF2 (but unlike human IGF2) (LeRoith & Roberts 2003), *PLAG1* is predominantly expressed in fetal tissues and declines thereafter. In addition, *Plag1*-deficient mice show remarkable phenotypic similarities with *Igf2*^{+/p-} mice in which the disruption of the paternal *Igf2* allele resulted in an ~40% size reduction compared to WT littermates (DeChiara *et al.* 1990). In both mouse lines, the size differences were first detected at embryonic day 11.5 and the growth retardation was maintained throughout life (or the observation period). Furthermore, the absence of an association between *PLAG1* polymorphisms and growth rate relative to body weight in dairy cattle GWASs indicates that the differences in the growth rate observed in QQ and qq calves are partly due to initial differences in size, meaning that the effect of *PLAG1* on growth is likely to occur mainly during fetal development (Karim *et al.* 2011, Littlejohn *et al.* 2012). For the trait 'post-weaning live weight' in beef cattle, epistatic interactions of the *PLAG1* polymorphisms with other genes were noted, including *IGF2* and *Insulin* (Bolormaa *et al.* 2015). Taken together, these observations make a strong case for the involvement of IGF2 in the growth effect of *PLAG1*, but direct evidence is nevertheless lacking. Intriguingly, *Igf2* expression levels (at least as measured by northern blot in whole animals) were not significantly altered in *Plag1* KO embryos and pups, nor were placenta weights as compared WT embryos (Hensen *et al.* 2004). It remains to be investigated whether local IGF2 mRNA or protein levels within tissues differ between *Plag1* KO and WT animals. In addition, possible effects in tissues such as placenta should be studied in more detail through histology and gene expression analyses. It is also possible that *PLAG1* affects growth in additional ways that do not involve IGF2, but these pathways are currently unknown and unexplored.

Role of *PLAG1* in fertility and reproduction

Evidence from *in vivo* studies

In addition to growth retardation, both male and female *Plag1* KO mice were found to have reduced fertility (Hensen *et al.* 2004). Males deficient in *Plag1* were able to fertilize only 7% of WT females compared to 20% by

WT males. KO females, on the other hand, had normal conception rates, but up to 50% reduced average litter size compared to WT females when mated to WT males. The mating of male and female *Plag1* mutants with each other resulted in small litters and infrequent conception (Hensen *et al.* 2004). *Pleomorphic adenoma gene 1* is expressed in both male and female gonads in the adult mouse, as well as in the pituitary gland (Hensen *et al.* 2004). It will be important to study where along the hypothalamo–pituitary gonadal (HPG) axis the effects of *Plag1* primarily take place, leading to the observed reduced fertility in both sexes. In the chicken, *PLAG1* was identified among nine genes differentially expressed in the hypothalamo–pituitary system of high- and low-producing layer strains. Greater *PLAG1* expression levels were associated with a higher number of eggs up to 50 weeks of age, a higher frequency of egg laying after the first egg, and/or a shorter average pause length between laying (Shiue *et al.* 2006, Chen *et al.* 2007a,b). Because these studies used a mixture of hypophyseal and hypothalamic RNA, it is impossible to determine whether the differential expression relates to pituitary *PLAG1*, hypothalamic *PLAG1*, or both and thus may involve gonadotrophs, gonadotropin-releasing hormone (GnRH) neurons and/or other cell types. Because no functional studies were carried out, the mechanism connecting greater *PLAG1* expression to increased egg production in the chicken is unknown, and even though the hormones involved in the reproduction in chicken have, in general terms, similar functions to those in mammals, one should be careful to extrapolate these findings over the species borders.

Evidence from GWAS studies

While a role for *PLAG1* in human fertility is yet to be established, GWASs have found links between puberty onset and *PLAG1* (or chromosome 14) polymorphisms in cattle (Fortes *et al.* 2012a,b, 2013a,b,c, Hawken *et al.* 2012, Bolormaa *et al.* 2015). A number of genetic markers for the onset of puberty mapped to the *PLAG1*-containing region on chromosome 14 in Brahman cows (determined as the age at which the first corpus luteum was detected) and bulls (determined as scrotal circumference or age at a scrotal circumference of 26 cm) (Fortes *et al.* 2012a,b, 2013b, Hawken *et al.* 2012). Interestingly, a number of GWASs have shown that sequence variants in the *PLAG1* chromosomal region in cattle are associated not only with the growth- and reproduction-related traits discussed above but also with circulating IGF1 concentrations. The Q variant, which is associated with increased body size and

higher *PLAG1* expression, correlates to lower IGF1 levels in the blood and later onset of puberty (Fortes *et al.* 2012a,b, 2013b, Hawken *et al.* 2012). Other reproduction-related traits, such as plasma inhibin levels at 4 months of age, plasma luteinizing hormone (LH) levels following GnRH challenge at 4 months, and sperm quality, showed no obvious associations with the *PLAG1* region (Fortes *et al.* 2012b, 2013b). Like always in the case of GWASs, one should bear in mind that the causality between detected polymorphisms and a phenotype cannot be deduced with certainty. For example, it is possible that the polymorphisms in bovine chromosome 14 do not affect only *PLAG1* but also other genes in the vicinity of *PLAG1*. Some of the genes neighboring *PLAG1* do indeed have known effects on reproduction (Fortes *et al.* 2012b).

Possible mechanisms linking *PLAG1* to reproductive physiology

The presence of *PLAG1* is required for normal fertility in both male and female, as suggested by the *Plag1* KO mice. At present it is not known through which mechanisms *PLAG1* regulates reproduction, but clues in the literature suggests that regulation on multiple levels of the HPG axis is possible (Fig. 4).

Expression of *Plag1* is observed in the urogenital ridge and ovaries of 11.5- and 16.5-day-old mouse embryos respectively (Hensen *et al.* 2004), which may suggest that *PLAG1* has a role in the development and differentiation of the reproductive tract or in the establishment of the ovarian germ cell pool. Spermatogenesis and ovarian follicle maturation have not been studied in the KO animals so far but were assumed to be normal in KO mice because no gross morphological changes were observed in the gonads (Hensen *et al.* 2004). The KO males have smaller relative ventral prostate and seminal vesicle weights compared to WT littermates, which could suggest that *Plag1* is required for normal androgen balance in the male. Neither ventral prostate nor seminal vesicles express detectable levels of *Plag1* in adult animals, which support an indirect mode of action on these organs. Involvement of *Plag1* in, for instance, steroidogenesis should be evaluated in future studies.

High expression levels of *Plag1* were noted in the embryonic and adult anterior pituitary of mice (Hensen *et al.* 2004). The gonadotrophs, producing LH and follicle-stimulating hormone, reside in this part of the pituitary gland. However, immunostaining of LH cells in pituitaries from *Plag1* KO and WT mice did not reveal obvious differences in the number of LH cells (Hensen *et al.* 2004).

Figure 4

Interactions of PLAG1 with the hypothalamo-pituitary-gonadal axis, displaying known interactions on the left and possible mechanisms that remain to be investigated on the right. FSH, follicle-stimulating hormone; GH, growth hormone; KO, knockout. Reproduced, with permission, from

Pinilla L, Aguilar E, Dieguez C, Millar RP & Tena-Sempere M (2012) Kisspeptins and reproduction: physiological roles and regulatory mechanisms. *Physiology Reviews* **92** 1235–1316. Copyright 2012, American Physiological Society.

Measurement of a wider selection of hormones, in both the male and the female, would reveal more about *Plag1* as a regulator of the HPG axis. In addition, the striking growth retardation phenotype of the KO animals calls for an evaluation of the growth hormone (GH) levels in the animals. Like gonadotropins, GH is secreted by the anterior pituitary where *Plag1* is expressed. In addition, GH has a direct effect on the IGF system that regulates reproductive physiology (Baker *et al.* 1996, Zhou *et al.* 1997, Hull & Harvey 2000, 2001, Pitetti *et al.* 2013, Zhou *et al.* 2013, Wolfe *et al.* 2014) and that has been systematically identified as a target of *Plag1*. However, *Plag1* KO mice do not seem to have a lower number of hypophyseal GH cells (Hensen *et al.* 2004).

As discussed earlier in this review, *IGF2* is a direct target of *PLAG1*, but *IGF1* also appears to have a connection to *PLAG1*. Bovine QQ genotypes, which show a higher expression of *PLAG1*, are significantly associated with lower circulating IGF1 levels in the animals (Fortes *et al.* 2012a,b, 2013b, Hawken *et al.* 2012). It is generally accepted that IGF1, be it of peripheral or central origin, affects the onset of puberty, which in

endocrinological terms equates to the re-activation of the GnRH neurons after adolescence (DiVall *et al.* 2010, Wolfe *et al.* 2014). In addition to a direct effect at the level of the GnRH neurons, IGF1 may also stimulate GnRH neuronal activity through the kisspeptin neurons upstream of the GnRH neurons (Hiney *et al.* 2009, Pinilla *et al.* 2012). The later onset of puberty in cattle that display both a higher expression of *PLAG1* and lower IGF1 levels is an intriguing finding, and the mechanism that connects these two molecules should be studied in experimental systems. To start with, IGF1 levels and puberty onset could be studied in the existing KO mouse model.

Effects of *PLAG1* on puberty onset through GH signaling should be further studied too. In prepubertal beef heifers, immunization against GH-releasing hormone causes a reduction in serum IGF1 concentrations and delays puberty (Simpson *et al.* 1991). It seems paradoxical that the same Q polymorphism that leads to higher *PLAG1* expression levels is associated with both delayed puberty and increased growth. Body size and growth rate are the two major factors affecting the onset of puberty in cattle: puberty is delayed until the heifer possesses an adequate

body size to successfully reproduce (Velazquez *et al.* 2008). One would expect the animals with higher levels of PLAG1 to reach this 'threshold' body size earlier and thus commence puberty faster. It has been proposed that IGF1R expression levels may be higher in these individuals, thus increasing the sensitivity of target tissues to IGF1 stimulation (Fortes *et al.* 2013a). In this scenario, however, PLAG1 may have to increase IGF1R levels in certain target tissues only, but not in others (such as the GnRH and kisspeptin neurons). Finally, the many negative feedback loops of the endocrine system could lead to a situation in which a measurement of a single hormone or growth factor does not show the whole picture. Measurement of hypothalamic and pituitary hormones along with the IGF1 and sex steroids at different stages of sexual maturity will be essential in understanding the reproductive effects of *PLAG1* in more detail.

Summary and future perspectives

In summary, there is increasing evidence that the transcription factor PLAG1 plays a role in both growth and reproduction. The role of PLAG1 in mammalian growth is supported by a KO mouse model as well as numerous GWASs in human and particularly in cattle, where one study (Karim *et al.* 2011) has shown that genetic polymorphisms in the *PLAG1* gene promoter lead to higher expression levels of *PLAG1* in fetal tissues, such as muscle and bone, and correlates with a larger stature. It is likely that the effect of PLAG1 on stature and related traits is the result of its transcriptional control over a range of genes involved in growth and cell proliferation, most notably IGF2. A role for PLAG1 in both male and female fertility has been suggested by the KO mouse model and GWASs in cattle. The mechanisms have not been studied in detail thus far but could be related to GH and IGF1/IGF2 signaling as well as steroidogenesis. In other words, PLAG1 is likely to have effects on more than one level of the HPG axis, and the same mechanisms could be involved in the reported effects of PLAG1 on growth and fertility. More functional studies should be carried out to reveal the exact role of PLAG1 as a regulator of growth and reproductive physiology.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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