Placenta 30 (2009) 823–834

Contents lists available at ScienceDirect

Placenta

journal homepage: www.elsevier.com/locate/placenta

Current Topic

Unearthing the Roles of Imprinted Genes in the Placenta

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ARTICLE INFO

Article history:
Accepted 22 July 2009

Keywords:
Epigenetics
Genomic imprinting
Knockout
Placentation
Transgenesis

ABSTRACT

Mammalian fetal survival and growth are dependent on a well-established and functional placenta. Although transient, the placenta is the first organ to be formed during pregnancy and is responsible for important functions during development, such as the control of metabolism and fetal nutrition, gas and metabolite exchange, and endocrine control. Epigenetic marks and gene expression patterns in early development play an essential role in embryo and fetal development. Specifically, the epigenetic phenomenon known as genomic imprinting, represented by the non-equivalence of the paternal and maternal genome, may be one of the most important regulatory pathways involved in the development and function of the placenta in eutherian mammals. A lack of pattern or an imprecise pattern of genomic imprinting can lead to either embryonic losses or a disruption in fetal and placental development. Genetically modified animals present a powerful approach for revealing the interplay between gene expression and placental function *in vivo* and allow a single gene disruption to be analyzed, particularly focusing on its role in placenta function. In this paper, we review the recent transgenic strategies that have been successfully created in order to provide a better understanding of the epigenetic patterns of the placenta, with a special focus on imprinted genes. We summarize a number of phenotypes derived from the genetic manipulation of imprinted genes and other epigenetic modulators in an attempt to demonstrate that gene-targeting studies have contributed considerably to the knowledge of placentation and conceptus development.

1. Introduction

In mammals, embryo development and survival, as well as a successful pregnancy, are dependent on the establishment of a functional maternal–fetal interface. This connection is initiated during the primary contact of the embryo, followed by embryo implantation, which is characterized by fetal trophoblast cell invasion into the maternal endometrium, and it culminates with the generation of the chorioallantoic placenta [reviewed by [1]]. Together, these processes are referred to as placentation [2].

The phenomenon of genomic imprinting has been demonstrated extensively to play a key role in fetal development and placentation [3,4]. Although the majority of imprinted genes are expressed in extraembryonic tissues, there is little information available on the mechanisms by which such mono-allelic gene expression regulates placental growth, development and function [5,6].

Continuous research on placentation and the myriad mechanisms controlling this process is needed to clarify the embryonic–endometrial interactions, and the use of animal models has contributed greatly to this study [7]. In particular, genetically modified animals have provided much of the knowledge on the genetic control of placental development [8]. In fact, the use of transgenic models has enabled the creation and analysis of gene regulation assays; the discovery of new roles for genes in placentation; and, most importantly, it has contributed to our understanding of developmental and perinatal pathologies in animals and humans. In the present review, we address the epigenetic events involved in embryogenesis, focusing on imprinted genes and the knowledge generated by transgenic models as tools to increase our understanding of the roles that imprinted genes play in placentation and early development.

2. Epigenetics and development

The placenta is the first organ to be formed during pregnancy. It is responsible for the establishment of vascular connections between mother and conceptus and allows for the exchange of gas,

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doi:10.1016/j.placenta.2009.07.007

nutrients and waste. This organ is involved in immune protection of the fetus and also produces the hormones needed to support fetal development [9].

The creation of an appropriate maternal environment for fetal development depends on the proper functioning and development of the trophoblast cells, which require the well-coordinated expression of many transcription factors, cell cycle regulators, growth factors, cytokines and surface receptors [reviewed by [10,11]]. Embryogenesis and placentation are particularly prone to perturbations in gene expression because these processes depend on a complex cascade of events [12,13]. Any disruption to the well-orchestrated expression of these regulatory factors may lead to placental disorders, causing undesirable phenotypes or even precocious deaths in animals or humans [9].

Following fertilization, a single-cell zygote forms a multicellular organism comprised of more than 200 different cell types [14,15]. The development of lineage-specific cells begins with the differentiation of the trophoblast lineage and the inner cell mass [16]. This event depends on epigenetic modifications that control the expression of particular genes, allowing cells to develop and differentiate into specific cells and tissues [17].

Epigenetics can be defined as the heritable changes in gene expression that are not caused by the changes in DNA sequence [18]. The best studied epigenetic mechanisms are DNA methylation and histone post-translational modifications, which interact with each other and also with regulatory proteins and non-coding RNAs [reviewed by [19]].

The paternal genome is actively demethylated within a few hours of fertilization, while the maternal genome is demethylated passively during the first cleavages in a species-dependent manner. This demethylation, however, spares imprinted genes [20], which must be maintained throughout development without being "de novo" reprogrammed during the pre-implantation stages [21].

Imprinted genes are expressed selectively from either the paternal or maternal allele. This specialized form of gene regulation is necessary for normal development [22,23], as discussed below. In paternally imprinted genes, the paternal allele is epigenetically modified, preventing its transcription and leading to mono-allelic maternal expression [18,24]. The same happens to the maternally imprinted genes, in which the paternal allele is solely expressed. These selectively expressed genes are believed to have an important role in the allocation of maternal resources to fetal growth [25,26].

Imprinted genes are found throughout the mammalian genome, though their occurrence is not random. These genes tend to be found in clusters that contain DNA sequences that are rich in CpG nucleotides. These specific regions, called imprinting control regions (ICRs), are characterized by epigenetic marks, mainly DNA methylation and histone modifications, which influence the binding affinity of transcription activators/suppressors and recruit chromatin remodeling enzymes to locally change the structure and function of chromatin [27]. The existence of control regions suggests that genomic imprinting may be controlled not only at the single gene level but at the level of the chromosome [28].

Epigenetic marks present in single parental copies of imprinted regions are responsible for differential gene expression. Interestingly, the maintenance of imprinting has been recently inferred to depend more on repressive histone methylation than on DNA methylation in the placenta [6,29].

3. Genomic imprinting and placental development

Approximately 200 genes are imprinted in the mammalian genome [30]. More than 70 imprinted genes in mice and at least 50 in humans have already been reported in the current literature (http://www.mgu.har.mrc.ac.uk/imprinting, http://www.geneimprint.com, http://igc.otago.ac.nz). In most genes, the imprinting status is conserved between mouse and human [25] and in some genes the imprinted status is reported to be conserved also in other species, i.e., cattle [31–34]. As summarized in Table 1, imprinted gene expression can be found in the placenta, the fetus, or both, independently of the parental origin of the expressed allele, and may be widespread or specific to certain cell types [4]. Although imprinted gene functions are generally essential for the proper development and function of the placenta, as well as for fetal growth [6], some of these genes have not been reported to be related to development. It is important to note, however, that imprinted genes can show spatial-temporal expression [35]. Their expression window during development, therefore, may be narrow enough to cause the imprinted characteristic to be difficult to recognize.

The placenta is one of the most important sites of imprinted gene action [[36] reviewed by [37]]. Although placentation displays species-specific variation [2], the genomic imprinting phenomenon is conserved amongst eutherian mammals, especially primates, rodents and ruminants [6,38].

According to the conflict hypothesis [39,40], paternally expressed genes enhance fetal growth, while maternally expressed genes suppress fetal growth. One evolutionary explanation for this hypothesis would be that by restricting fetal growth, females can have a longer reproductive lifespan, assuring their reproductive success. In contrast, having more numerous and stronger progeny is advantageous for males. The conflict hypothesis achieved some confirmation through observations made with mouse genome manipulation. Androgenote mice, which contain only paternal DNA, have poorly developed embryonic components but better developed extraembryonic tissues, whereas gynogenotes show the opposite phenotype [41].

It is important to note that both the accurate establishment of genomic imprints and the correct maintenance of genomic imprints during embryogenesis are essential for normal embryonic/placental development [42]. Epimutations affecting imprints can arise during imprint erasure, which occurs when germ cells migrate to the gonads in pre-natal stages, during either the imprint establishment that takes place during gametogenesis or imprint maintenance throughout the life of the organism [43,44].

A clear example of epigenetic disturbance in development is the interference caused by assisted reproductive techniques (ARTs). These techniques likely interfere with imprint establishment (manipulation of gametes) or imprint maintenance (manipulation of pre-implantation embryos; [43]).

4. Imprinted genes control mammalian development

Insulin-like growth factor 2 (*Igf2*) was one of the first imprinted genes to be discovered [45]. *Igf2* and its receptor, *Igf2r*, are essential during fetal–placental development [46]. While the former is a maternally imprinted gene that codes for a growth factor involved in fetal and placental growth in mice and humans, the latter is a maternally expressed gene in mice involved in *Igf2* degradation. Although recent studies demonstrated that *IGF2r* is not imprinted in humans [47,48], the relationship between these genes brings strength to the conflict theory [49,50].

Igf2, together with *H19*, which is an imprinted non-coding transcript, is located in a cluster of imprinted genes in mouse chromosome 7, syntenic to human chromosome 11p15.5 [51,52]. A region upstream of *H19* regulates imprinted expression of both of these genes [53]. The establishment and maintenance of DNA methylation in the *Igf2*/*H19* DMR is acquired during spermatogenesis in the male germ cells; however, the DMR from the female

Table 1
Imprinted gene expression reported in mouse development.

Gene	Aliases	Chromosome location	Preferentially imprinted allele	Name	References
Gatm	*AT*	Central 2	Paternal	L-Arginine:glycine amidinotransferase	[140] (Extraembryonic tissues)
Nnat	*Peg 5*	Distal 2	Maternal	Neuronatin	[141] (Fetal brain)
Nesp		Distal 2	Paternal	Neuroendocrine secretory protein	[142,143] (Embryonic and extraembryonic tissues)
Nespas		Distal 2	Maternal	Neuro endocrine secretory protein antisense	[143,144] (embryonic tissues)
Gnas	*Gs-alpha*	Distal 2	Maternal	Guanine nucleotide binding protein, alpha stimulating	[145] (Embryonic tissues, predicted by the embryonic lethality of null mutations)
Gnasxl		Distal 2	Maternal	Guanine nucleotide binding protein, alpha stimulating, 'extra large'	[142,146] (Embryonic tissues)
Mcts2		Distal 2	Maternal	Malignant T-cell amplified sequence 2	[147] (Embryonic tissues)
H13	*SPP*	Distal 2	Paternal	Histocompatibility 13	[147] (Embryonic and extraembryonic tissues)
Sfmbt2		Proximal 2	Maternal	Scm-like with four mbt domains 2	[148] (Early embryos and extraembryonic tissues)
Calcr	*Clr*	Proximal 6	Paternal	Calcitonin receptor	[149](Fetal brain)
Mit1/Lb9		Proximal 6	Maternal	*Mest*-linked imprinted transcript 1	[150] (Fetal brain, partially imprinted in other fetal tissues)
Sgce	*e-SG*	Proximal 6	Maternal	Sarcoglycan, epsilon	[65,151] (Embryonic and extraembryonic tissues)
Peg10	*Edr*, *HB-1*, *Mar2*, *MEF3L*, *Mart2*, *MyEF-3*	Proximal 6	Maternal	Paternally expressed gene 10	[65] (Embryonic and extraembryonic tissues)
Ppp1r9a		Proximal 6	Paternal	Neurabin	[65,152] (Extraembryonic tissues)
Pon3		Proximal 6	Paternal	Paraoxonase 3	[65,152] (Extraembryonic tissues)
Pon2		Proximal 6	Paternal	Paraoxonase 2	[65,152] (Placenta-specific)
Asb4		Proximal 6	Paternal	Ankyrin repeat and suppressor of cytokine signalling	[153] (Embryonic and extraembryonic tissues)
Mest/Peg1		Proximal 6	Maternal	Mesoderm specific transcript	[154,155] (Embryonic and extraembryonic tissues)
Copg2		Proximal 6	Paternal	Coatomer protein complex subunit gamma 2	[150] (Embryonic tissues)
Copg2as		Proximal 6	Maternal	*Copg2* antisense	[150] (Embryonic tissues)
Klf4	*Epfn*, *Klf14*, *epiprofin*, *BTEB5*	Proximal 6	Paternal	Kruppel-like factor 14	[156] (Embryonic and extraembryonic tissues)
Nap1l5		Proximal 6	Maternal	Nucleosome assembly protein 1-like 5	[157] (Embryonic tissues)
Zfp264	*Znf264*	Proximal 7	Maternal	Zinc-finger gene 264	[158] (Embryonic tissues)
Zim3		Proximal 7	Paternal	Zinc-finger gene 3 from imprinted domain	[158] (Embryonic tissues)
Kcnq1ot1	*Kvlqt1as*	Distal 7	Maternal	*Kvlqt1* antisense	[134,159] (Embryonic and extraembryonic tissues)
Zim2		Proximal 7	Paternal	Imprinted zinc-finger gene 3	[160] (Embryonic tissues)
Zim1		Proximal 7	Paternal	Imprinted zinc-finger gene 1	[161] (Embryonic tissues)
Peg3	*Pw1*, *End4*, *Gcap4*, *Zfp102*	Proximal 7	Maternal	Paternally expressed gene 3, probably Pw1	[161,162] (Embryonic tissues)
Usp29	*Ocat*	Proximal 7	Maternal	Ubiquitin-specific processing protease 29	[163,164] (Mid-gestation embryos, fetal brain)
Ube3a	*Hpve6a*, E6-AP ubiquitin protein ligase	Central 7	Paternal	E6-Ap ubiquitin protein ligase 3A	[164] (Fetal brain)
Pwcr1	*snoRNA MBII-85*, *Snord116*	Central 7	Maternal	Prader–Willi chromosome region 1	[165] (Embryonic tissues)
Snrpn/Snurf	*Peg4*, *HCERN3*	Central 7	Maternal	Small nuclear ribonucleoprotein polypeptide N (Snrpn), Snrpn upstream reading frame (Snurf)	[166–168] (Embryonic and extraembryonic tissues)
Pec2		Central 7	Maternal	Paternally expressed in the CNS 2	[164] (Fetal brain)
Pec3		Central 7	Maternal	Paternally expressed in the CNS 3	[164] (Fetal brain)
Ndn	*Peg6*	Central 7	Maternal	*necdin*	[164,169] (Fetal brain)
Magel2	*ns7*, *nM15*, *NDNL1*, *Mage-l2*	Central 7	Maternal	Melanoma antigen, family L, 2	[170] (Extraembryonic tissues and fetal brain)
Mkrn3	*Zfp127*	Central 7	Maternal	Ring zinc-finger encoding gene 127	[171,172] (Embryonic tissues)
Zfp127as/Mkrnas		Central 7	Maternal	Ring zinc-finger encoding gene 127 antisense	[173] (Pre-implantation embryo)
Peg12/Frat3		Central 7	Maternal	Frequently rearranged in advanced T-cell lymphomas	[174] (Embryonic tissues)
Inpp5f_v2		Distal 7	Maternal	Inositol polyphosphate-5-phosphatase, variant 2	[175] (Fetal brain)
Inpp5f_v3		Distal 7	Maternal	Inositol polyphosphate-5-phosphatase, variant 3	[147] (Fetal brain)
H19		Distal 7	Paternal		[176,177] (Embryonic and extraembryonic tissues)
Igf2	*Mpr*, *M6pr*, *Peg2*, *Igf-2*, *Igf-II*	Distal 7	Maternal	Insulin-like growth factor type 2	[45] (Embryonic and extraembryonic tissues)
Ins2	*Mody*, *Ins-2*, *InsII*, *Mody4*, proinsulin, *INS*	Distal 7	Maternal	Insulin 2	[178,179] (Extraembryonic tissues)
Ascl2/Mash2		Distal 7	Paternal	*Mus musculus* achaete-scute homologue 2	[135] (Placenta-specific)

(*continued on next page*)

Table 1 (*continued*)

Gene	Aliases	Chromosome location	Preferentially imprinted allele	Name	References
Tapa1/Cd81	*Tspan28*	Distal 7	Paternal	cd 81 antigen	[133] (Extraembryonic tissues)
Tssc4		Distal 7	Paternal	Tumor-suppressing subchromosomal transferable fragment 4	[134,180] (Placenta-specific)
Kcnq1	*Kvlqt1*	Distal 7	Paternal	Potassium voltage-gated channel, subfamily Q, member 1	[134,180,181] (Embryonic and extraembryonic tissues)
Cdkn1c	$p57^{Kip2}$	Distal 7	Paternal	Cyclin-dependent kinase inhibitor 1C	[135,182] (Embryonic and extraembryonic tissues)
Slc22a18	*HET*, *ITM*, *Impt1*, *TSSC5*, *Orctl2*, *Slc22a1l*, *Slc22a1*, *BWR1A*	Distal 7	Paternal	Solute carrier family 22, member 18	[183,184] (Embryonic and extraembryonic tissues)
Phlda2	*Ipl*, *Tssc3*	Distal 7	Paternal	Pleckstrin homology-like domain, family A, member 2 (Phlda2), Imprinted in placenta and liver (*Ipl*)	[185,186] (Weakly in embryonic, mainly in extraembryonic tissues)
Nap1l4	*Nap2*	Distal 7	Paternal	Nucleosome assembly protein 1-like 4	[181] (Mainly in placenta; however, reported not imprinted by [187])
Tnfrsf23	*Tnfrh1*	Distal 7	Maternal	Tumor necrosis factor receptor superfamily, member 23	[188] (Embryonic and extraembryonic tissues)
Obph1	*Osbpl5*	Distal 7	Paternal	Oxysterol-binding protein 1 (*Obph1*), oxysterol binding protein-like 5 (*Osbpl5*)	[181,189] (Placenta-specific)
Plagl1	*Lot1*, *Zac1*	Proximal 10	Maternal	Pleomorphic adenoma gene-like 1	[151] (Embryonic tissues)
Dcn	*DC*, *PG40*, *PGII*, *PGS2*, *mDcn*, *DSPG2*, *SLRR1B*	Central 10	Paternal	Decorin	[153] (Placenta)
Ddc	*Aadc*	Proximal 11	Maternal	Dopa decarboxylase (Ddc); aromatic L-amino acid decarboxylase (Aadc)	[190] (Embryonic heart)
Grb10	*Meg 1*	Proximal 11	Paternal	Growth factor receptor bound protein	[191] (Embryonic tissues)
U2afl1-rs1	*SP2*, 35 kDa, *Irlgs2*, *D11Ncvs75*, *U2afbp-rs*, *Zrsr1*	Proximal 11	Maternal	U2 small nuclear ribonucleoprotein auxiliary factor (U2AF), 35 kDa, related sequence 1	[192] (Embryonic tissues)
Mirg	*Meg9*	Distal 12	Paternal	miRNA containing gene	[193] (Embryonic and extraembryonic tissues)
Dlk1	*FA1*, *ZOG*, *pG2*, *Peg9*, *SCP1*, *Ly107*, *pref-1*	Distal 12	Maternal	Delta-like 1	[194,195] (Embryonic and extraembryonic tissues)
Gtl2	*Meg 3*	Distal 12	Maternal	Gene trap locus 2	[195,196] (Embryonic and extraembryonic tissues)
Rtl1	*Mar*, *Mor1*, *Mart1*, *Peg11*	Distal 12	Maternal	Retrotransposon-like 1	[196] (Embryonic and extraembryonic tissues)
Dio3		Distal 12	Maternal	Deiodinase, iodothyronine type III	[197] (Embryonic tissues and weakly imprinted in extraembryonic tissues)
Antipeg11/Rtl1as	Hosts several miRNAs	Distal 12	Paternal	Antisense to *Rtl1/Peg11*	[198] (Embryonic and extraembryonic tissues)
Htr2a	*Htr2*, *Htr-2*, *5-HT2A* receptor	Distal 14	Paternal	5-Hydroxytryptamine (serotonin) receptor 2 A	[199] (Embryonic eye)
Kcnk9	*Task3*	Distal 15	Paternal	Potassium channel, subfamily K, member 9	[200] (Embryonic tissues)
Peg13		Distal 15	Maternal	Paternally expressed 13	[157] (Embryonic and extraembryonic tissues)
Slc238a4	*Ata3*, *mATA3*	Distal 15	Maternal	Solute carrier family 38, member 4/amino acid transport system A3	[153] (Embryonic and extraembryonic tissues)
Slc22a3	*EMT*, *Oct3*, *Orct3*, *Slca22a3*	Proximal 17	Paternal	Solute carrier family 22 (organic cation transporter), member 3	[201] (Placenta-specific)
Slc22a2	*Oct2*, *Orct2*	Proximal 17	Paternal	Solute carrier family 22 (organic cation transporter), member 2	[202] (Placenta-specific)
Igf2r	*CD222*, *CI-MPR*, *Mpr300*, *M6P/IGF2R*	Proximal 17	Paternal	Insulin-like growth factor type 2 receptor	[203,204] (Embryonic and extraembryonic tissues)
Airn	*Air*, *Igf2ras*	Proximal 17	Maternal	Insulin-like growth factor 2 receptor antisense RNA	[57,205] (Embryonic and extraembryonic tissues)

germline cell is protected against methylation by the zinc-finger protein CTCF [52]. Such protection prevents interactions between the *Igf2* gene and enhancers located downstream of *H19* in the maternal allele, thus preventing *Igf2* transcription. When CTCF does not bind to the paternal allele, on the other hand, *Igf2* is expressed, and DNA is methylated within the *H19* promoter region, resulting in *H19* transcriptional silencing. The different methylation status of the *Igf2–H19* locus, therefore, guarantees the exclusive paternal *Igf2* expression and maternal *H19* expression [51].

The importance of the parental origin of *Igf2*/*H19* genes was elegantly demonstrated when Kono and collaborators (2004, [54]) successfully produced viable parthenogenetic offspring in mice by correcting the *Igf2*/*H19* dosage. In this experiment, one of the maternal alleles was derived from a non-growing oocyte (ng), while the other was derived from a fully grown (fg) oocyte. The process of imprinting in the maternal germline occurs at late stages of oogenesis. Therefore, ng oocytes are considered to be "imprint-neutral", and both *H19* and *Igf2* genes are expressed [43,55,56]. By introducing a deletion in the *H19* gene and its flanking regions in the ng oocyte and consequently disrupting the imprinting of *Igf2* gene, the authors demonstrated both that parthenogenetic development to term could be achieved and also that the proper

expression of *Igf2*/*H19* likely drove modifications of other genes that allow parthenote survival.

The *Igf2r* cluster, which contains *Slc22a2* and *Slc22a3* genes, a solute carrier family 22 that codifies imprinted genes, is also regulated by methylation-sensitive elements. Unlike most imprinted genes, the methylated allele is expressed in this cluster. In this gene, the maternally methylated allele leads to paternal *Igf2r* repression. The paternal non-methylated allele expresses a non-coding RNA (ncRNA), called *Airn* (previously named *Air*), which is responsible for preventing paternal *Igf2r* expression [52,57].

Other important imprinted loci display the same behavior. The *Gnas* and *Kcnq1* loci, for example, contain ncRNAs believed to contribute to genomic imprint control, i.e., *Nespas*/*Gnas-as* and *Kcnq1ot1*, respectively. Therefore, in addition to DNA methylation and post-translational histone modification, ncRNAs also control imprinted gene expression [58].

The mechanisms by which ncRNAs are responsible for the epigenetic changes observed in these imprinted loci are still not well characterized. Numerous ncRNAs are located in clusters regulated by ICRs [59]. In fact, each imprinted region expresses at least one ncRNA [58,60]. Although their function and mechanisms are not well understood, it is known that ncRNAs regulate imprinted clusters that recruit chromatin remodeling complexes to nearby genomic regions. The expression of specific ncRNAs, i.e., long ncRNAs, is associated with the acquisition of genomic imprinting and the silencing of imprinting clusters [61,62].

A recently discovered imprinted retrotransposon-derived gene, *Peg10* [63], showed an essential function as an endogenous gene in placental development [64]. *Peg10* is highly conserved among mammalian species [65], raising questions about its importance in mammalian evolution. Ono and collaborators [64] highlighted the possibility that ancestral mammals may have developed placenta from newly acquired retrotransposon-derived genes or by modification of endogenous genes present in oviparous animals millions of years ago. The understanding of the physiological roles of *Peg10* and the other imprinted retrotransposon homologue *Rtl1* is definitely important to improving our understanding of placental evolution.

Disrupting the normal regulation of imprinted genes is decisive throughout gestation and post-natal life, often leading to lethal phenotypes in early development, as described in Table 2. Not surprisingly, these phenotypes are related to several human syndromes and disorders in post-natal life.

The *IGF2* gene, for example, is involved in Russell–Silver syndrome (RSS), which is characterized by the loss of methylation in *IGF2–H19* ICR, reduction in *IGF2* expression, and biallelic expression of *H19*, resulting in intrauterine and post-natal growth retardation [66]. Beckwith–Wiedemann syndrome (BWS), on the other hand, is characterized by the loss of *IGF2* imprinting, causing biallelic overexpression and a lack of expression of *H19*, leading to overgrowth of the fetus, among other symptoms. Both BWS and RSS phenotypes include pronounced growth disorders [67].

Table 2
Imprinted genes knockout and their phenotypes.

Imprinted gene	Mouse KO phenotype	References
Nesp	Development without any obvious phenotype – behavior linked	[206]
Gnas	Embryonic lethality. Heterozygous disruption is associated with significant early post-natal lethality. When maternal allele is disrupted mice become obese. When paternal allele is disrupted, mice are hypermetabolic and thin	[145,207]
Sgce	Increased myoclonus and deficits in motor coordination and balance	[208]
Peg10	Growth retardation and early embryonic lethality due to incomplete placenta formation	[64]
Ppp1r9a	Reduction in contextual fear memory, loss of hippocampal long-term potentiation	[209]
Mest/*Peg1*	Embryonic and placental growth retardation	[210]
Klf4	Neonatal lethality within 15 hours of birth, selective perturbation of late-stage differentiation structures in the epidermis	[211]
Kcnq1ot1	Reduction of 10–20% of weight	[212]
Peg3	Embryonic and placental growth retardation, impairment of normal maternal behavior	[213]
Ube3a	Motor dysfunction, inducible seizures, context-dependent learning deficit	[214]
Pwcr1	Severe post-natal growth retardation, delayed sexual maturation, but fertile. Elevated level of anxiety or fear. Motor learning deficiency, hyperphagie	[215]
Snrpn	Viable offspring, with no obvious phenotypic or histopathologic defects. However, KO of its IC leads to increase in neonatal mortality and underweight newborns showing hypotonia	[216]
Ndn	Neonatal lethality and respiratory distress, underweight at birth	[217,218]
Magel2	Reduced viability at embryonic day 12.5. Offspring showing disregulation of sleep and food intake, growth retardation soon after birth	[219]
Peg12/*Frat3*	Viable, healthy and fertile. No obvious phenotype. Triple *Frat* knockout (*Frat1*, *Frat2* and *Frat3*) shows the same normal phenotype	[220]
H19	Increase in placental weight, fetal overgrowth	[221]
Igf2	P0 and null mutants showed reduced placental growth, followed by fetal growth restriction. Phenotypes more severe in *Igf2* null mutants at later stages of gestation	[26,46]
Ins2	Viable and fertile, without major metabolic disorders. *Ins1* and *Ins2* double homozygous knockout, however, were growth-retarded, developed diabetes mellitus and died within 48 h	[222,223]
Ascl2/*Mash2*	Death at 10 d post-coitum, placental failure	[224]
Tapa1/*Cd81*	Reduction of female fertility, increase in post-natal lethality	[225]
Kcnq1	Deafness, circular movement and repetitive falling. Gastric hyperplasia. Severe anatomic disruption of cochlear and vestibular end organs. Phenotypes unrelated to BWS	[226]
Cdkn1c	Divergent phenotypes in offspring. Abnormal placental development (placentomegaly and trophoblast dysplasia), morphological defects in neonates	[227–229]
Phlda2	Placental overgrowth, consequent reduction of fetal-to-placental weight ratio	[186]
Plagl1	Intrauterine growth restriction, altered bone formation, increased neonatal lethality	[230]
Dcn	Skin fragility, tumor development	[231,232]
Grb10	Embryo and placenta overgrowth	[233]
Dlk1	Pre- and post-natal growth retardation, eyelid and skeletal abnormalities, smaller litter size, increased neonatal mortality	[234]
Gtl2	Fetal and post-natal growth reduction	[235]
Rtl1	Placental abnormalities and functional deficiencies, pre- and post-natal growth retardation, placental growth retardation, increased late-fetal or neonatal lethality	[196]
Slc238a4	Placental and fetal growth restriction	[236]
Slc22a3	Impairment of neurotransmitters release. No obvious phenotypes, viable and fertile offspring	[202]
Slc22a2	No obvious phenotypes, viable and fertile offspring	[237]
Igf2r	Lethality at birth, embryo overgrowth	[238]
Air	Reduction in birth weight	[57,239]

Abnormal imprinting patterns are also associated with neurodevelopmental disorders, such as Prader–Willi (PWS) and Angelman (AS) syndromes, which are associated with the loss of paternal or maternal imprinting on chromosome 15q11–q13, respectively [reviewed by [14,23,68]].

5. Imprinting alterations and implications

In humans, pregnancy losses are extremely common and not completely understood. In fact, 25% of spontaneous abortions remain unexplained [69]. The majority of these losses occur during the pre-implantation period, though after implantation, approximately 15–20% of pregnancies are also lost spontaneously [70,71]. In farm animals, embryonic mortality is also the major cause of reproductive wastage, where a dysfunctional placenta accounts for 80% of this mortality [72,73].

ARTs have been widely used in an attempt to correct fertility impairment in humans and animals and to provide a higher reproductive efficiency in farm animals. In 2003, almost 4% of the total number of human births in developed countries was estimated to have been produced with *in vitro* procedures [74]. This scenario is not different for farm species. The last report of the IETS (International Embryo Transfer Society), released in 2006, announced that in the previous year, nearly 266,000 bovine embryos were produced *in vitro* and transferred worldwide.

Despite its wide use, ARTs, such as IVF or cloning in animals, increase the incidence of abnormalities in the morphology and function of the placenta [75]. Hydroallantois, poor vascularization and abnormal (mostly reduced but also enlarged) placentomes are some of the most common pathological alterations [76–78]. Overall growth of the placenta and other particular structures (such as the labyrinthine trophoblast), as well as regulation of specific transporters and channels needed for nutrient supply to the fetus, are frequently regulated or affected by imprinted genes [reviewed by [25]].

Placental perturbations also lead to high birth weights and reduced survival rates, a condition known in ruminants as large offspring syndrome (LOS, [79,80]). This condition is reminiscent of the BWS in humans and is correlated with *IGF2R* imprinting disruption [81]. The incidence of placental failures is especially important in cloning by nuclear transfer because such failures represent the major cause of pregnancy failure in these animals [76,82–85]. Placental abnormalities in cloned animals are evident and appear frequently even in gestations carried to term [86,87].

Furthermore, the use of ARTs and their *in vitro* culture conditions changes the methylation and expression patterns of imprinted genes [81,88]. In laboratory animals, 5–10% of non-manipulated embryos undergoes abnormal methylation reprogramming and fails to develop. However, embryos derived from some kinds of manipulation, for example, superovulation and *in vitro* culture, undoubtedly present a higher rate of methylation and/or imprinting abnormalities when compared to non-manipulated embryos [89,90]. When nuclear transfer is considered, methylation patterns are also abnormal and highly variable between individuals [91,92].

Imprinted loci disruption has been observed in a number of human developmental disorders and cancers [reviewed by [93]]. For example, a loss of imprinting (LOI) has been found in patients with PWS (at a frequency of approximately 1%), patients with AS (at a frequency of 3%), patients with BWS (50% of patients), and nearly 50% of the transient neonatal diabetes mellitus [reviewed by [44,67]].

The observation that epigenetic abnormalities are present in normal or manipulated pregnancies has made the animal model suitable for a more profound study of these perturbations. In this context, the generation of *in vivo* gene function assays is vital for understanding the biological roles of developmental genes and their interactions with each other and with environmental stimuli.

6. Transgenic strategies to study mammalian development

Understanding the genetic control of fetal–maternal interactions has dramatically improved with the introduction of genome modifications in animal models. In fact, gene-targeting strategies are the most widely accepted models used to provide reliable and accurate information on the mechanisms of implantation and placentation, given their ability to provide definitive evidence for the *in vivo* function of a specific gene.

Genes that are candidates to have a role in early development can have their biological effects analyzed *in vivo* in one of the two ways: gain of function or loss of function studies. The first method is based on gene overexpression, achieved by the random integration of a transgene into the genome or a targeted insertion of the transgene into a specific locus (a knock-in). On the contrary, the loss of function gene assay relies on the suppression of a gene function. Mainly, it is achieved by gene-trapping in ES cells or targeted gene deletion (a knockout, KO). The first method, although relatively inexpensive, has the significant limitation of being only effective for genes that are expressed in ES cells, whereas gene targeting can be used for any gene, either permanently or in a conditional manner [reviewed by [94–96]].

The gain of function strategy is especially interesting for characterizing placental features that are not fully described. The transfer of transgenic embryos expressing a reporter gene, such as green fluorescent protein (GFP) or the β-galactosidase enzyme (*LacZ*), to wild-type recipients enables the precise discrimination of uterine and trophoblast contributions to placental defects [97]. The inverse is also valid when wild-type blastocysts are introduced into mutant uterine tract [71]. This technique has been used for several purposes, such as elucidating trophoblast invasion in hemochorial placentas [98], demonstrating the spatial-temporal pattern of imprinted gene expression in embryos [99] or revealing the X inactivation mechanism [100,101].

KO mice model is another strategy that has greatly contributed to the understanding of several diseases and different biological processes [reviewed by [94]], usually revealing a gene role by comparing the knockout phenotype with that of wild-type mice. For example, it has been used to uncover basic mechanisms of DNA repair [102], cancer research [103], diabetes [104], behavioral analysis [105], and developmental related processes [106,107], among several others.

Despite differences between mice and human morphology and endocrine function, the mouse is the most popular model organism for studying mammalian genomic imprinting and other processes in eutherian animals [108]. Great advantages of mice when compared to other animals are the availability of maternal- or paternal-only derived embryos and the characteristics of these animals, such as uniparental chromosomal duplications (UPD), high fertility, low costs to maintain feeding and housing facilities, and responsiveness to a range of assisted reproductive technologies [94,109]. Most importantly however, is the availability of a fully sequenced genome for this species [110] and the technology available for the manipulation of embryonic stem cells, allowing the use of these cells for the production of genetically altered offspring [111,112].

The generation of KO mice relies on several *in vitro* procedures that, although specific, are technically simple to perform. The first step consists of the design and construction of the desired vector. Circular sections of bacterial DNA (plasmids) are frequently used to manipulate the genome of embryonic stem cells by introducing

a DNA sequence flanked by homologous sequences into the gene to be inactivated [113]. Reporter genes, as well as antibiotic resistance genes, are introduced into the center of the target gene, causing interference with expression and also allowing for the positive selection of the transgene in the cell genome [114].

Homologous recombination of plasmid and DNA sequences is obtained with a very low and variable efficiency rate [115]. Normally, it consists of the recombination of similar chromosome sections derived from each parent [116]. Gene-targeting technologies exploit this characteristic by recombining transgenes containing a disrupted gene with a similar DNA sequence, leading to targeted gene disruption.

Successfully modified embryonic stem cells are injected in preimplantation blastocoels, contributing to the tissues of the developing animal, including the germline [117,118]. Embryonic and adult tissues are composed of transgenic and non-transgenic cells called chimeras. Once these embryonic stem cells are integrated into germ cells, the newly inserted gene alteration may be passed on to the next generations. As a result, the chimeras produced are able to generate mouse strains that are heterozygous for the altered genes, and, most importantly, homozygous offspring can be obtained by planned matings [reviewed by [94]].

7. Developmental studies based on knockout models

Transgenic approaches in mice have provided reliable means of investigating complex biological phenomena or diseases by allowing gene products to be expressed in a controlled manner in a whole organism where the majority of the genes have a human counterpart [119,120].

Indeed, an International Mouse Knockout Consortium composed of four groups, the Knockout Mouse Project (KOMP, http://knockoutmouse.or), the European Conditional Mouse Mutagenesis Program (EUCOMM, http://www.eucomm.org), the North American Conditional Mouse Mutagenesis Program (NorCOMM, http://norcomm.phenogenomics.ca/index.htm), and the Texas Institute for Genomic Medicine (TIGM, http://tigm.org), was created in 2007 to obtain a mutation of all protein-encoding genes in the mouse using a combination of gene-targeting and gene-trapping strategies [96,121].

Regarding developmental process, mouse mutants have been created for the broad study of gene expression and developmental interactions not only throughout the peri-implantation and gestation periods [reviewed by [1,9]] but also for different stages of reproduction [reviewed by [107,122]].

KO models have been used for more than a decade to investigate gene function, including the role of certain genes for epigenetic patterning and embryogenesis. Trasler and collaborators in 1996 [123] showed that DNA methyltransferase (Dnmt−/−) KO embryos failed to develop past the 25-somite stage and were developmentally delayed and asynchronous. The authors concluded that DNA methylation is vital for embryo development. Five main mammalian DNA methyltransferases (Dnmt) have been characterized and are related to the establishment and maintenance of genomic imprinting: Dnmt1, Dnmt1o, Dnmt3a, Dnmt3b and Dnmt3L [124]. Dnmt1 and the oocyte isoform Dnmt1o are responsible for the maintenance of the imprinted methylation patterns [125,126], Dnmt3a and Dnmt3b are required for de novo methylation and are essential for paternal and maternal methylation imprints during germline development [127]. Most recent studies have shown that Dnmt3-like (Dnmt3L) cooperates with Dnmt3a and is necessary for the establishment of genomic imprinting during gametogenesis [128–130]. By constructing KO mice models, it was possible to show that these methyltransferases are indispensable for embryogenesis, as summarized in Table 3.

Similar to DNA methylation, histone modifications, mainly acetylation and methylation, also influence gene expression [131,132]. In contrast to embryo formation, placentation seems to be more dependent on repressive histone methylation than DNA methylation, as stated earlier. Some imprints in extraembryonic tissues directly correlated with histone H3 repressive methylation but not with DNA methylation [133,134]. In placenta, several genes maintain imprinting status in the absence of Dnmt1 [135,136]. These genes probably have their DNA-methylated allele enriched with histone H3-lysine-9 methylation, together with other histone lysine methylation. Using KO models, the histone methyltransferase (HMT) *G9a* was shown to contribute to the allelic repression of genes that are imprinted only in the trophoblast. The dependence of histone post-translational modification in the parental origin-specific expression probably prevents imprint erasure during the genome-wide demethylation wave that occurs after fertilization [29,136].

KO studies of other HMTs or histone deacetylases (HDACs) have shown that deletions of its encoding genes (i.e., HMTs *Eset* and *G9a* and Polycomb-group genes *Ezh2* and *Suz12*) lead to embryonic lethality [131,137–139]. The mechanisms by which histone modifiers regulate the maintenance of differentially allelic chromatin organization in imprints require further investigation.

From more than 70 imprinted genes in which expression was already reported in developmental stages, roughly half have been analyzed through KO studies, which are summarized in Table 2. The phenotypes observed in the KOs ranged from increased embryonic or post-natal lethality (i.e., *Gnas*, *Peg10*, *Klf4*, *Ascl2*, *Tapa1*/*CD81*) to no obvious phenotypes (i.e., *Nesp*, *Peg12*/*Frat3*, *Slc22a2*). Most phenotypes evaluated by KO experiments confirm the preferential allelic gene expression and its importance for fetoplacental growth. For example, Table 2 shows that the deletion of the paternally expressed genes *Peg10*, *Mest*/*Peg1*, *Peg3*, *Igf2*, *Dlk1*, *Gtl2*, *Rtl1* and others suppresses growth, whereas the deletion of the maternally expressed genes *H19*, *Grb10*, *Igf2r* and others increases fetoplacental growth. Some mutations, although apparently unrelated to nutrition allocation and fetal growth, are essential for fetal development, i.e., the deletion of *Ube3a*, *Sgce*, *Ppp1r9a* and *Pwcr1*, among

Table 3
DNA and histone methyltransferases knockout consequences.

Methyltransferases	Knockout consequences	References
Dnmt1	Embryonic extensive demethylation	[240]
Dnmt1o	Embryos from Dnmt1o−/− females lose half of their imprints during one cell cycle	[126]
Dnmt3a	Apparently normal at birth, increased lethality at about 4 weeks of age, presenting runted phenotype	[127,241]
Dnmt3b	Embryonic lethality probably due to multiple developmental defects	[241]
Dnmt3a and Dnmt3b	Impaired de novo methylation. Embryonic lethality before 11.5 dpc	[241,242]
Dnmt3L	Null mutations reveal disruption of maternal methylation imprints. Heterozygous progeny of homozygous females fail to develop beyond 10.5 dpc due to abnormal development of extraembryonic structures	[128–130]
HMT *G9a*	Decrease in H3-K9 methylation in placenta, embryonic lethality before/at 10 dpc	[29,137]

others, and mainly result in impairments related to the nervous system during the post-natal period.

Interestingly, the deletion of *Peg10*, a paternally expressed gene, as well as the deletion of *Ascl2*, a maternally expressed gene, both leads to embryonic lethality due to placental defects. Fetal growth and placentation are now seen as complex processes dependent on very particular gene expression networks. By generating animals lacking a specific gene, it was possible to evaluate a variety of reproductive parameters in controlled experiments, turning transgenesis into an extremely valuable tool for imprinted gene expression studies.

8. Conclusions and perspectives

In 2007, the Nobel Prize in physiology or medicine was awarded Drs. Mario Capechi, Martin Evans and Oliver Smithies for their work on genetic modifications in mice using embryonic stem cells. Great progress in several fields of basic and medical science was made possible with the use of animals harboring genetic modifications. Undoubtedly, this technology has greatly contributed to the understanding of the mechanisms that regulate genomic imprinting and development in mammals.

For a long time, the KO approach has been the method of choice in placentation and early development studies, allowing for the evaluation of specific phenotypes *in vivo* throughout gestation. Although this technique is well established in mice, its technical unavailability in other animal species is a considerable drawback. Moreover, animals other than the mice have increasingly been accepted as research models because they may be better correlated with human characteristics such as birth weight, organ morphology or genome similarity (i.e., ewe, swine or primate models). We believe that in the near future, epigenome interferences, i.e., targeted epimutations in numerous animal models, may allow the "knockout" technique to become the basis for several other new and valuable techniques in science.

By reviewing the importance of genomic imprinting in early development in mammals and the genes involved, we have emphasized the role of imprinted genes in successful placental and fetal development. Moreover, we have highlighted the regulation of some important genes, which may turn into future targets of genetic therapies.

Because the acquisition and evolution of genomic imprinting are among the most fundamental biological questions, further use of gene transfer techniques to improve the understanding of this process in mammals is warranted. In particular, gaining insight into the regulation of epigenetic mechanisms during early development would greatly contribute to the improvement of ARTs and their outcomes.

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