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REVIEW

Genomic imprinting mechanisms in embryonic and extraembryonic mouse tissues

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Imprinted genes in mice and humans mainly occur in clusters that are associated with differential DNA methylation of an imprint control element (ICE) and at least one nonprotein-coding RNA (ncRNA). Imprinted gene silencing is achieved by parental-specific insulator activity of the unmethylated ICE mediated by CTCF (CCCTC-binding factor) binding, or by ncRNA expression from a promoter in the unmethylated ICE. In many imprinted clusters, some genes, particularly those located furthest away from the ICE, show imprinted expression only in extraembryonic tissues. Recent research indicates that genes showing imprinted expression only in extraembryonic tissues may be regulated by different epigenetic mechanisms compared with genes showing imprinted expression in extraembryonic tissues and in embryonic/adult tissues. The study of extraembryonic imprinted expression, thus, has the potential to illuminate novel epigenetic strategies, but is complicated by the need to collect tissue from early stages of mouse development, when extraembryonic tissues may be contaminated by maternal cells or be present in limited amounts. Research in this area would be advanced by the development of an *in vitro* model system in which genetic experiments could be conducted in less time and at a lower cost than with mouse models. Here, we summarize what is known about the mechanisms regulating imprinted expression in mouse extraembryonic tissues and explore the possibilities for developing an *in vitro* model.
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Genomic imprinting in mammals

Both the maternal and paternal genomes are required for mammalian development, indicating that they are not equivalent and suggesting the existence of genes showing imprinted expression (Lyon and Glenister, 1977; McGrath and Solter, 1984; Surani *et al*., 1984; Cattanach and Kirk, 1985). This was later confirmed by the identification of imprinted genes expressed only from the maternal or paternal allele, in contrast to the majority of mammalian genes that are equally expressed or repressed from both parental alleles (Barlow *et al*., 1991; Bartolomei *et al*., 1991; DeChiara *et al*., 1991). Imprinted genes are now known to mainly exist in clusters in which individual genes are not autonomously imprinted, but instead imprinted expression of the whole cluster is controlled by a *cis*-acting genomic 'imprint'. The genomic imprint is a DNA methylation mark that is deposited on a CpG-rich DNA sequence (known as the imprint control element or ICE) during oogenesis or spermatogenesis by the DNMT3A/DNMT3L *de novo* methyltransferase complex (Bourc'his *et al*., 2001; Hata *et al*., 2002; Kaneda *et al*., 2004; Jia *et al*., 2007). This gametic DNA methylation mark is then maintained on the maternal or paternal allele by the maintenance DNA methyltransferase DNMT1 (Li *et al*., 1993). Approximately 100 imprinted genes clustered in 25 genomic regions, have been identified in mice and humans (for details see http://www.har.mrc.ac.uk/research/genomic_imprinting/ (Williamson *et al*., 2009)). In spite of this small number, many imprinted genes are developmentally important and require the correct expression level (that is, one copy expressed) for normal growth and development to occur during embryogenesis.

Imprinted genes thus occur in clusters associated with the methyl-sensitive ICE that controls their imprinted expression. The ICE methylation imprint is universal, present in all tissues and at all stages of development (except germ cells), whereas imprinted expression is not always present and may vary during development, differentiation and disease. For example, the *Igf2r* gene is initially biallelically expressed in pre-implantation embryos, with imprinted expression beginning in post-implantation embryos (Szabo and Mann, 1995; Lerchner and Barlow, 1997). This developmental regulation is paralleled in an *in vitro* embryonic stem (ES) cell differentiation system (Latos *et al*., 2009). Imprinted expression can also be lost later during cellular differentiation in a tissue-specific manner. For example, in post-mitotic neurons, imprinted expression of *Igf2r* is lost and it shows biallelic expression (Yamasaki *et al*., 2005). In addition, in the brain, the *Ube3A* gene shows imprinted expression only in the neurons, whereas in glial cells, it gains biallelic expression (Yamasaki *et al*., 2003). In disease states, imprinted expression can also be lost. For example, many cases of human colorectal cancer

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are associated with loss of imprinted expression that results in biallelic *IGF2* expression (Kaneda and Feinberg, 2005). The examples described above involve tissue-specific gain or loss of imprinted expression for genes that show widespread or 'ubiquitous' imprinted expression in embryonic or adult tissues and in extraembryonic tissues, which comprise the short-lived placenta and membranes that support the developing embryo *in utero*. Most genes showing this ubiquitous pattern of imprinted expression are located close to the methyl-sensitive ICE in the cluster. Another pattern is shown by the genes in the cluster that are located further away from the ICE, which show imprinted expression only in extraembryonic tissues, but are biallelically expressed or silenced in embryo or adult soma. Genes expressed in either the embryo or the adult tissues, but showing imprinted expression only in extraembryonic tissues, have mostly been studied in the placenta, and in this review, they are referred to as showing 'placental-specific' imprinted expression. Genes that show imprinted expression in either embryonic or adult tissues, as well as in extraembryonic tissues, will be referred to as showing 'ubiquitous' imprinted expression, although as mentioned above, such genes can show tissue-specific and developmental variation in imprinted expression. It is important to note that as the ICE DNA methylation imprint is universal (that is, present on one parental chromosome in all tissues except the germ line), the imprinting mark is present regardless of imprinted expression.

In a recent review, we described six well-studied imprinted gene clusters: the *Igf2r* cluster, the *Kcnq1* cluster, the *Pws/As* cluster, the *Gnas* cluster, the *Igf2* cluster and the *Dlk* cluster (Koerner *et al*., 2009). Of these clusters, only the *Igf2r* cluster, the *Kcnq1* cluster and the *Igf2* cluster have been shown to contain placental-specific imprinted genes and, hence, we will focus on these clusters in this review (Figure 1). In these clusters, the ubiquitously imprinted genes tend to be the 'inner' genes close to the methyl-sensitive ICE, whereas the placental-specific genes are often the 'outer' genes found further away from the ICE (Fowden *et al*., 2006; Feil and Berger, 2007; Miri and Varmuza, 2009). Epigenetic processes have recently been operationally divided into three different steps: an initial 'epigenator' signal originating outside the cell nucleus, which is read by an 'epigenetic initiator', which then alters the expression state, and this new state is then maintained by 'epigenetic maintainers' (Berger *et al*., 2009). Imprinted gene silencing has been shown to be triggered by two different epigenetic initiators: silencing by an imprinted macro nonprotein-coding RNA (ncRNA) expressed from the nonmethylated allele of the ICE in the *Igf2r* and *Kcnq1* imprinted clusters or by insulator formation induced by CTCF (CCCTC-binding factor) binding the unmethylated ICE allele in the *Igf2* cluster (Bell and Felsenfeld, 2000; Hark *et al*., 2000; Sleutels *et al*., 2002; Mancini-Dinardo *et al*., 2006) Notably, these studies show that within one cluster, the same epigenetic initiator silences genes showing ubiquitous or placental-specific imprinted expression.

There are two main possibilities whereby the same epigenetic initiator induces imprinted expression of more genes per cluster in the placenta compared with the embryo. Either, the epigenetic initiator acts differently in the placenta compared with the embryo or epigenetic maintainers, such as DNA methylation and

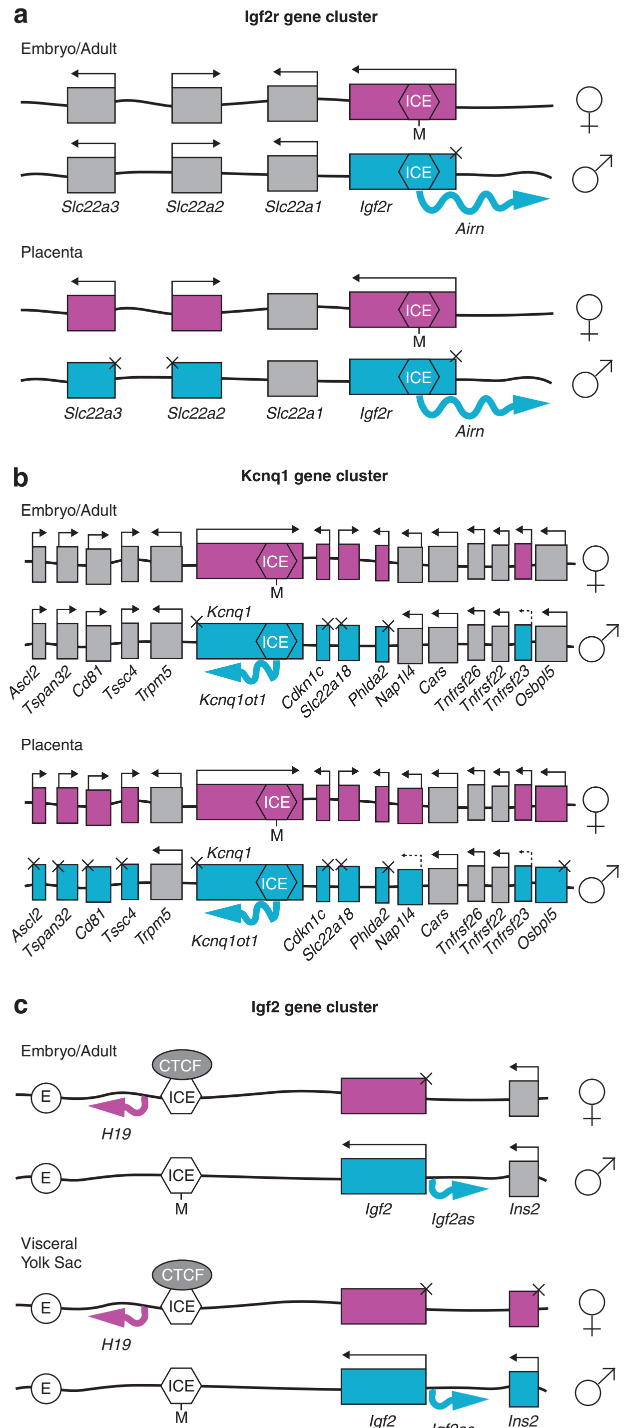

Figure 1 Three examples of mouse imprinted gene clusters showing imprinted expression in the embryo and/or adult versus placenta. (**a**) *Igf2r cluster*: imprinted silencing on the paternal chromosome is initiated by the *Airn* macro ncRNA. (**b**) *Kcnq1 cluster*: imprinted silencing on the paternal chromosome is initiated by the *Kcnq1ot1* macro ncRNA. (**c**) *Igf2 cluster*: imprinted silencing on the maternal chromosome is initiated by CTCF binding the unmethylated ICE forming an insulator that blocks access to enhancers. Protein-coding genes and ncRNAs showing imprinted expression are shown as pink (maternally expressed) and blue (paternally expressed), with genes showing no parental allelic difference in gray. ICE, imprint control element; M, DNA methylation; and E, enhancer.

chromatin modifiers, could function differently in the placenta compared with the embryo. In this review, we examine the evidence for these two possibilities with reference to the developmental origin of embryonic and extraembryonic tissues.

Origin of extraembryonic tissues

The extraembryonic tissues are derived from three embryonic cell types in E4.5 pre-implantation mouse embryos, namely, the trophectoderm, the primitive endoderm and the epiblast (Figure 2). The trophectoderm differentiates only into extraembryonic lineages. In the E6.5 post-implantation embryo, trophectoderm-derived tissues are (i) the ectoplacental cone, which interfaces with the mother and is invaded by maternal blood, (ii) the extraembryonic ectoderm, which surrounds the upper part of the pro-amniotic cavity, and (iii) the primary trophoblast giant cells, which cover the parietal endoderm, surrounding the blastocoel (Figure 2). At E12.5, the primary trophoblast giant cells, together with the parietal endoderm, make up the parietal yolk sac, which is the outer-most extraembryonic membrane. By this stage, the ectoplacental cone and the extraembryonic ectoderm have differentiated to make up a part of the placenta, the spongiotrophoblast, the labyrinth layer and the secondary trophoblast giant cells (Figure 2) (Theiler, 1989; Rossant and Tam, 2002; Watson and Cross, 2005).

The primitive endoderm of the E4.5 embryo differentiates into parietal endoderm and visceral endoderm. The parietal endoderm forms a part of the parietal yolk sac as described above. At E6.5, the visceral endoderm can be divided into the extraembryonic visceral endoderm, which covers the extraembryonic ectoderm, and the embryonic visceral endoderm, which covers the epiblast (Figure 2). Cells from the embryonic visceral endoderm have been shown to contribute to the embryonic gut, whereas extraembryonic visceral endoderm contributes only to visceral yolk sac (VYS) in the E12.5 embryo that forms the middle extraembryonic membrane (Figure 2) (Theiler, 1989; Rossant and Tam, 2002; Kwon *et al*., 2008).

The third source of extraembryonic tissues is the epiblast from which the embryo proper is derived. By the E12.5 stage, epiblast-derived tissues are the visceral mesoderm, which makes up a part of the VYS, the amnion, the innermost extraembryonic membrane and the allantoic embryonic blood vessels of the placenta and the umbilical cord (Figure 2) (Theiler, 1989; Rossant and Tam, 2002; Watson and Cross, 2005). The placenta interfaces with the maternal-derived decidua and maternal blood vessels that have arterial sinuses in the labyrinth layer (Rossant and Tam, 2002; Watson and Cross, 2005) (Figure 2).

The precise site of expression of genes showing extraembryonic-specific imprinted expression is often not determined, but has been broadly characterized as placental or yolk sac specific imprinted expression (Table 1). However, for some imprinted genes, the extraembryonic cell type showing imprinted expression has been determined. For example, *Ascl2* shows imprinted expression in the trophectoderm lineage, beginning in the post-implantation embryo in the ectoplacental cone and continuing in the spongiotrophoblast of the placenta (Tanaka *et al*., 1999; Verhaagh *et al*., 2001; Lewis *et al*., 2006). The placental-specific imprinted gene *Slc22a3* is expressed in the trophectoderm-derived labyrinth layer of the E12.5 placenta (Verhaagh *et al*., 2001). In addition to showing imprinted expression in the spongiotrophoblast of the placenta and in some embryonic tissues, *Phlda2* shows imprinted expression in the primitive endoderm-derived visceral endoderm of the VYS (Frank *et al*., 1999). In summary, genes showing tissue-specific imprinted expression in extraembryonic tissues have been identified in cell lineages derived from the trophectoderm and primitive endoderm, but not from the epiblast.

Placental-specific imprinted gene expression in well-characterized imprinted gene clusters

The three imprinted gene clusters shown in Figure 1 show a relationship between distance from the epigenetic

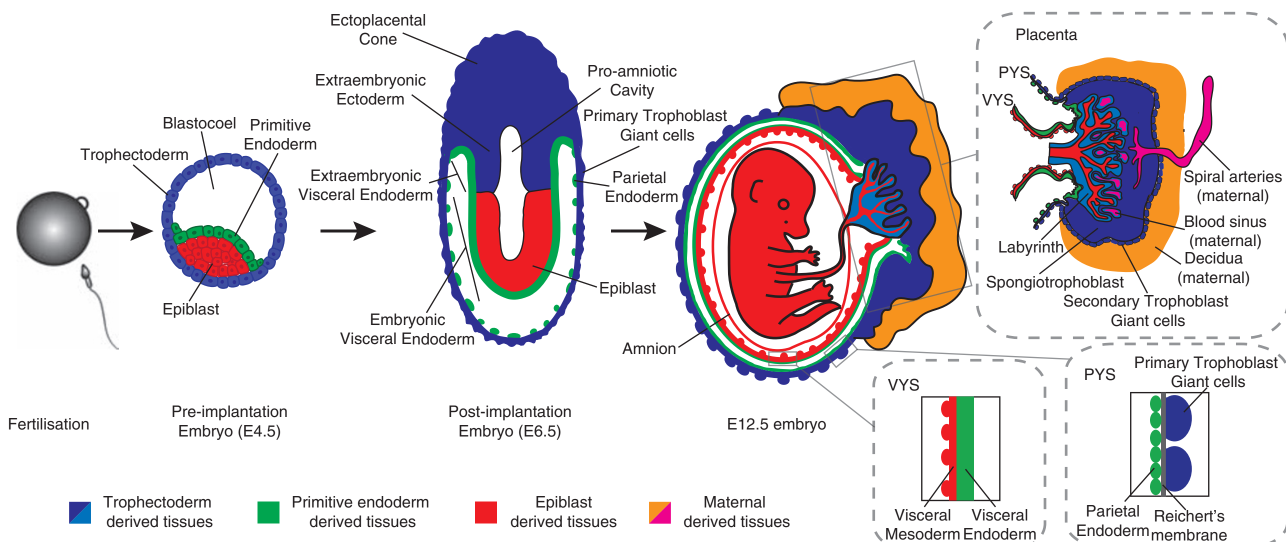

Figure 2 Development of mouse extraembryonic cell lineages. Epiblast-derived tissue is shown in red, trophectoderm-derived tissue in blue and primitive endoderm-derived tissue in green.

Table 1 Mouse imprinted gene clusters that include genes showing imprinted expression only in extraembryonic tissues

Imprinted cluster	*Gene*	*Imprinted expression*	*Expressed allele*	*Chromosome*	*Reference*
Peg10	*Tfpi2*	Pl, YS	Maternal	chr6	(Monk *et al*., 2008)
	Ppp1r9a	Pl, YS	Maternal	chr6	(Ono *et al*., 2003)
	Pon2	Pl, YS	Maternal	chr6	(Ono *et al*., 2003)
	Pon3	Pl, YS	Maternal	chr6	(Ono *et al*., 2003)
	Sgce	Ubiquitous	Paternal	chr6	(Ono *et al*., 2003)
	Peg10	Ubiquitous	Paternal	chr6	(Ono *et al*., 2003)
	Asb4	Ubiquitous	Maternal	chr6	(Mizuno *et al*., 2002)
Igf2	*Ins2*[a]	VYS	Paternal	chr7	(Giddings *et al*., 1994; Duvillie *et al*., 1998)
	Igf2	Ubiquitous	Paternal	chr7	(DeChiara *et al*., 1991)
	Igf2as	Ubiquitous	Paternal	chr7	(Rivkin *et al*., 1993)
	H19	Ubiquitous	Maternal	chr7	(Davis *et al*., 1998)
Kcnq1	*Th*	Pl	Maternal	chr7	(Schulz *et al*., 2006)
	Osbpl5	Pl	Maternal	chr7	(Engemann *et al*., 2000)
	Nap1l4	Pl	Maternal	chr7	(Engemann *et al*., 2000)
	Cd81	Pl	Maternal	chr7	(Caspary *et al*., 1998; Lewis *et al*., 2004b)
	Tssc4	Pl	Maternal	chr7	(Paulsen *et al*., 2000)
	Tspan32	Pl	Maternal	chr7	(Umlauf *et al*., 2004)
	Ascl2	Pl	Maternal	chr7	(Guillemot *et al*., 1995)
	Kcnq1ot1	Ubiquitous	Paternal	chr7	(Smilinich *et al*., 1999)
	Kcnq1	Ubiquitous	Maternal	chr7	(Gould and Pfeifer, 1998; Paulsen *et al*., 1998)
	Cdkn1c	Ubiquitous	Maternal	chr7	(Hatada and Mukai, 1995)
	Slc22a18	Ubiquitous	Maternal	chr7	(Dao *et al*., 1998)
	Phlda2	Ubiquitous	Maternal	chr7	(Dunwoodie and Beddington, 2002; Lewis *et al*., 2004b)
	Tnfrsf23	Ubiquitous	Maternal	chr7	(Clark *et al*., 2002)
Igf2r	*Slc22a2*	Pl	Maternal	chr17	(Zwart *et al*., 2001)
	Slc22a3[b]	Pl	Maternal	chr17	(Zwart *et al*., 2001)
	Igf2r	Ubiquitous	Maternal	chr17	(Barlow *et al*., 1991)
	Airn	Ubiquitous	Paternal	chr17	(Lyle *et al*., 2000)
Single imprinted genes	*Sfmbt2*	Pl, YS	Paternal	chr2	(Kuzmin *et al*., 2008)
	Gatm	Pl, YS	Maternal	chr2	(Sandell *et al*., 2003)
	Dcn	Pl	Maternal	chr10	(Mizuno *et al*., 2002)
	Slc38a4	Pl	Paternal	ch15	(Smith *et al*., 2003)

Abbreviations: Pl, placenta; VYS, visceral yolk sac; YS, yolk sacs (parietal yolk sac, visceral yolk sac and amnion not distinguished).
Genes showing imprinted gene expression in extraembryonic tissues only are shown plus other imprinted genes in their cluster that show ubiquitous imprinted expression. Ubiquitous: imprinted expression in both embryonic and extraembryonic tissues. For details see: http://www.mousebook.org/catalog.php?catalog=imprinting (Williamson *et al*., 2009).
[a]Imprinted expression gained after E14.5.
[b]Imprinted expression lost after E15.5.

initiator and ubiquitous or placental-specific imprinted expression. In the *Igf2r* and *Kcnq1* imprinted gene clusters, the epigenetic initiator of imprinted gene silencing is a macro ncRNA, whose promoter lies in the ICE, whereas in the *Igf2* cluster, it is the insulator function of the ICE. The imprinted genes in the three clusters show a different spatial relationship with the initiator, which may relate to how imprinted silencing occurs in these clusters.

In the *Igf2r* gene cluster, the paternally expressed *Airn* ncRNA overlaps and silences *Igf2r* in most tissues. In contrast *Slc22a2 and Slc22a3* are not overlapped by *Airn*, their promoters lie 157 and 234 kb upstream, respectively, and they are silenced by *Airn* only in the placenta, but not in adult tissues (Figure 1a). Notably, *Slc22a3*, which lies furthest from the ICE, shows imprinted expression in early and not late placenta, whereas *Slc22a2*, which lies closer to the ICE, shows imprinted expression in early and late placenta (Zwart *et al*., 2001). On the maternal allele *Igf2r*, *Slc22a2* and *Slc22a3* are expressed because *Airn* is silenced by DNA methylation of the ICE (Sleutels *et al*., 2002; Seidl *et al*., 2006). In the *Kcnq1* cluster, the paternally expressed *Kcnq1ot1* ncRNA does not overlap with the promoter of any gene, but silences the paternal allele of the nearby genes *Cdkn1c*, *Slc22a18*, *Phlda2* and *Kcnq1* (which contains the *Kcnq1ot1* promoter and ICE in intron 10) in embryonic and extraembryonic tissues (Figure 1b) (Umlauf *et al*., 2004; Lewis *et al*., 2004a). Imprinted genes that are more distant from *Kcnq1ot1*, the downstream *Tssc4*, *Cd81*, *Tspan32* and *Ascl2* genes, as well as the upstream *Osbpl5* gene, are silenced by this ncRNA only in placenta (Umlauf *et al*., 2004; Lewis *et al*., 2004a). In addition, genes internal to *Osbpl5* in the cluster show partial silencing of the paternal allele *Nap1l4* only in placenta and *Tnfrsf23* in both the embryo and extraembryonic tissues (Engemann *et al*., 2000; Clark *et al*., 2002). On the maternal allele, in which *Kcnq1ot1* expression is silenced by DNA methylation of the ICE, all imprinted genes in the cluster are expressed. In both the *Igf2r* and *Kcnq1* imprinted gene clusters, there are genes that do not show imprinted expression even though they are positioned closer to the ncRNA on the linear chromosome than more distant genes that do show imprinted expression (Figures 1a and b). In some cases, these genes are not expressed in tissues in which flanking genes show imprinted expression. For example, *Slc22a1* is not expressed in placenta in which *Slc22a2* and *Slc22a3* show

imprinted expression. However, in the *Kcnq1* cluster, a number of biallelically expressed genes are present in the cluster, indicating that they are insensitive to silencing initiated by the ncRNA, whereas *Nap1l4* and *Tnfrsf23* that show biased expression may be only partially sensitive to ncRNA-initiated silencing.

In the *Igf2* imprinted gene cluster, the ncRNAs *H19* and *Igf2as* are located separately from the ICE and are not responsible for silencing the imprinted protein-coding genes. Instead, the ICE acts as an insulator element, with CTCF binding the nonmethylated maternal allele and silencing *Igf2* by blocking access to enhancers lying downstream to the ICE and allowing these enhancers to instead promote the expression of *H19* ncRNA. Methylation of the paternal allele prevents CTCF binding to the ICE, which allows the enhancers to interact with the paternal *Igf2* promoter causing it to be expressed, whereas *H19* is silenced (Bell and Felsenfeld, 2000; Hark *et al*., 2000). *Igf2* shows ubiquitous imprinted expression, whereas *Ins2*, which lies further away from the ICE and *H19*, shows imprinted expression only in extraembryonic tissues, and it is unclear how imprinted expression of this gene is controlled.

Table 1 lists single imprinted genes and imprinted clusters that contain genes showing imprinted expression restricted to extraembryonic tissues. This table is incomplete because the imprinted expression pattern of many imprinted genes has not been thoroughly analyzed.

Global gene regulation in extraembryonic tissues

Extraembryonic tissues have a limited lifespan of 16 days in mouse, from setting aside of the trophectoderm linage at E3.0 to birth that occurs between E19 and 21, whereas embryonic tissues develop into adult tissues and the mouse may live for up to 2 years. Embryonic tissues also differentiate into a wider range of cell types compared with extraembryonic tissues. The differences in the function and lifespan of embryonic and extraembryonic tissues may affect the manner in which global gene regulation occurs.

Extraembryonic lineages also tolerate polyploidy more than most embryonic lineages. For example, trophoblast giant cells in the parietal yolk sac and in the placenta are polyploid. Experimentally, it has been shown that tetraploid embryos do not survive past gastrulation, whereas chimeric tetraploid/diploid embryos undergo full development and are born. In these chimeric embryos, tetraploid cells contribute mostly to extra-embryonic tissues, whereas the embryo is almost entirely diploid (Tarkowski *et al*., 1977). The small proportion of tetraploid cells that are detected in the embryo localize to the embryonic gut and appear to be of visceral endoderm and not epiblast origin (Hadjantonakis *et al*., 2002; Kwon *et al*., 2008). This toleration of polyploid cells indicates that extraembryonic tissues are less sensitive to gene dosage than embryonic tissues, which may be a factor explaining the larger number of genes showing imprinted expression in extraembryonic tissues.

Extraembryonic tissues have been shown to have much lower levels of DNA methylation than embryonic tissues (Chapman *et al*., 1984; Rossant *et al*., 1986). This global difference in DNA methylation levels indicates that different epigenetic maintainers of gene silencing may exist in extraembryonic tissues; for example, in the absence of DNA methylation, there may be a greater role for repressive chromatin modifications in maintaining epigenetic gene silencing.

Regulation of imprinted gene expression differs in extraembryonic tissues

Many imprinted genes only show imprinted expression in extraembryonic tissues such as placenta and VYS (Table 1). The epigenetic initiator is known at the *Igf2r*, *Kcnq1* and *Igf2* imprinted clusters as described above and, importantly, the same initiator controls both ubiquitous and placental-specific imprinted expression in each cluster (Figure 1). Ubiquitous and placental-specific imprinted gene expression could be explained by either the epigenetic initiator or epigenetic maintainers, such as DNA methylation and histone modifications, acting differentially in extraembryonic tissues. Here, we review what is known about the differences in the behavior of epigenetic initiators and maintainers of imprinted gene expression in embryonic and extraembryonic tissues and how this may affect imprinted gene silencing.

Differential maternal or paternal DNA methylation of the ICE of imprinted gene clusters is established during oogenesis or spermatogenesis, and maintained after fertilization and during development. Deletion of the unmethylated ICE allele results in loss of imprinted expression for all genes in a cluster that show either ubiquitous or placental-specific imprinted expression, in all clusters examined so far (Wutz *et al*., 1997; Thorvaldsen *et al*., 1998; Bielinska *et al*., 2000; Fitzpatrick *et al*., 2002; Lin *et al*., 2003; Williamson *et al*., 2006). DNA methylation is required for establishing and maintaining imprinted gene expression as described above. However, studies of mice lacking the maintenance DNA methyltransferase DNMT1 have shown that some genes showing placental-specific imprinted gene expression do not require DNA methylation to maintain imprinted expression. In the *Kcnq1* cluster, genes showing placental-specific imprinted gene expression, *Ascl2*, *Cd81*, *Osbpl5* and *Tssc4*, maintain imprinted expression in the absence of DNA methylation (Caspary *et al*., 1998; Tanaka *et al*., 1999; Lewis *et al*., 2004b). In contrast, genes closer to the ICE showing ubiquitous imprinted expression (*Cdkn1c*, *Slc22a18*, *Phlda2*, *Kcnq1ot1*), do require DNA methylation to maintain imprinted expression in both the placenta and the embryo (Lewis *et al*., 2004b). However, the relationship is not clear for the *Kcnq1* gene, which contains the ICE within its gene body, and in the absence of DNA methylation, shows a loss of imprinted expression in the embryo, but maintains imprinted expression in the placenta (Lewis *et al*., 2004b). A maternal protein store of the oocyte-specific DNMT1 isoform DNMT1O transiently translocates to the nucleus at the eight-cell stage and is required for maintaining the correct methylation pattern at imprinted loci (Howell *et al*., 2001). Therefore, imprinted DNA methylation patterns are maintained in early DNMT1-null embryos, as the full-length nuclear-localized somatic DNMT1 isoform is not detected until the post-implantation

stage at E7.0 (Mertineit *et al*., 1998). In embryos from *DNMT1O*-null mothers, loss of DNA methylation leads to upregulation of *Kcnq1ot1* on the maternal allele, resulting in downregulation of both placental-specific and ubiquitous imprinted genes in the *Kcnq1* cluster (Green *et al*., 2007). This shows that maintenance of DNA methylation is required in the pre-implantation embryo to maintain imprinted expression of the placental-specific imprinted genes in the *Kcnq1* cluster, but in the post-implantation embryo, it is not required for maintaining imprinted expression of these genes. Further studies are required to determine whether DNA methylation is also not required for maintenance of placental-specific imprinted expression at other loci or whether this is a phenomenon restricted to the *Kcnq1* cluster.

It has been speculated that histone modifications may have a more important role in maintaining imprinted gene expression in extraembryonic tissues than DNA methylation (Umlauf *et al*., 2004; Lewis *et al*., 2004b). At the *Kcnq1* cluster, it has been shown that in the absence of DNA methylation, placental-specific imprinted genes maintain imprinted expression and the silent parental alleles are marked by the repressive histone modifications, histone H3 lysine 9 dimethylation (H3K9me2) and H3 lysine 27 trimethylation (H3K27me3) (Lewis *et al*., 2004b). The expressed parental alleles are marked by the active histone modifications H3 lysine 4 dimethylation (H3K4me2) and H3 acetylation of lysine 9 and 14 (H3K9ac and H3K14ac). In the embryo, these differential histone modification marks are lost on all imprinted genes except on the *Kcnq1* and *Cdkn1c* genes that show ubiquitous imprinted expression (Umlauf *et al*., 2004; Lewis *et al*., 2004b).

The genes showing placental-specific imprinted expression in the *Kcnq1* cluster are also associated with the polycomb repressive complex 2 (PRC2), responsible for adding the H3K27me3 mark to the silent allele (Umlauf *et al*., 2004). PRC2 is required to maintain imprinted expression of the placental-specific imprinted genes *Cd81* and *Tssc4* in extraembryonic tissue of E6.5 post-implantation embryos (many other placental-specific genes are not expressed at this stage) (Terranova *et al*., 2008). In addition, in the E6.5 embryo, PRC2 appears to be required to maintain imprinted expression of the ubiquitous imprinted gene *Cdkn1c* in extraembryonic tissues, but not in embryonic tissues (Terranova *et al*., 2008). *Tfpi2*, which shows placental-specific imprinted expression in the *Peg10/Sgce* cluster, also requires PRC2 for silencing, whereas other genes in the cluster showing ubiquitous imprinted expression do not (Monk *et al*., 2008). Recently, it has been shown that PRC1 is also required for silencing of genes showing placental-specific imprinted expression in the *Kcnq1* cluster. The PRC1 repressive mark H2A lysine 119 monoubiquitination (H2aK119u1) is associated with the repressed allele of imprinted genes in undifferentiated trophoblast stem (TS) cells. In the E6.5 post-implantation embryo, PRC1 is required to maintain placental-specific imprinted expression of *Cd81* and *Tssc4* (Terranova *et al*., 2008).

The H3K9me2 methyltransferase G9A was shown to be required for genes showing placental-specific imprinted expression in the *Kcnq1* cluster (Wagschal *et al*., 2008). Similarly, it has been shown in the *Peg10/Sgce* cluster that placental-specific imprinted expression of *Tfpi2* requires G9A, whereas it is not required for other genes showing ubiquitous imprinted expression (Monk *et al*., 2008). In the *Igf2r* cluster, the silent allele of *Slc22a3* is associated with G9A and requires G9A for imprinted expression in the placenta, whereas *Igf2r* does not require G9A for imprinted expression in the embryo or placenta (Nagano *et al*., 2008).

These studies suggest that the repressive histone modifications H3K27me3, H2aK119u1 and H3K9me2 deposited by PRC2, PRC1 and G9A, respectively, are required for imprinted expression in the placenta. However, it remains unclear whether these repressive histone modifications are required for the initiation or maintenance of imprinted expression in the placenta, or for both.

Macro ncRNAs are associated with imprinted gene clusters and are usually expressed in a reciprocal pattern to protein-coding genes, that is, they are expressed from the parental chromosome carrying the repressed imprinted mRNA genes. These ncRNAs can be epigenetic initiators responsible for initiating imprinted gene silencing such as *Kcnq1ot1* in the *Kcnq1* cluster and *Airn* in the *Igf2r* cluster, or not be involved in silencing such as the *H19* ncRNA in the *Igf2* cluster (Sleutels *et al*., 2002; Mancini-Dinardo *et al*., 2006; Shin *et al*., 2008). The *Kcnq1ot1* ncRNA shows imprinted expression from the two-cell embryo stage, whereas genes showing ubiquitous imprinted expression are expressed monoallelically by the blastocyst stage. However, genes showing placental-specific imprinted expression are first expressed in the ectoplacental cone of the E6.5 post-implantation embryo (Lewis *et al*., 2006; Terranova *et al*., 2008). In the *Igf2r* cluster, *Igf2r* is biallelically expressed in the pre-implantation embryo from the four-cell to the blastocyst stage, and then shows imprinted expression only after implantation by E6.5 (Szabo and Mann, 1995; Lerchner and Barlow, 1997). This gain of imprinted expression is paralleled in undifferentiated and differentiated ES cells, respectively (Latos *et al*., 2009). The onset of *Airn* expression in the embryo has not been determined, but it is not expressed in undifferentiated ES cells; it is paternally expressed upon differentiation together with the onset of imprinted *Igf2r* expression (Latos *et al*., 2009). In extraembryonic tissues, imprinted expression of *Slc22a2* and *Slc22a3* in relation to *Airn* ncRNA expression has not been tested before E11.5 (Zwart *et al*., 2001).

Truncation of the 108 kb-long *Airn* and the 91 kb-long *Kcnq1ot1* to less than 3 kb, causes loss of imprinted expression of all protein-coding genes in both embryonic and extraembryonic tissues in the *Igf2r* and *Kcnq1* clusters, respectively (Sleutels *et al*., 2002; Mancini-Dinardo *et al*., 2006; Shin *et al*., 2008). An exception to this is the *Cdkn1c* gene in the *Kcnq1* cluster, which retains imprinted expression in some embryonic tissues after truncation of *Kcnq1ot1* (Shin *et al*., 2008). This indicates that either a short form of *Kcnq1ot1* ncRNA is sufficient for silencing *Cdkn1c* in some tissues or that that two distinct mechanisms operate in this cluster. The function of macro ncRNAs in other well-studied imprinted gene clusters, such as the *Pws/As*, *Gnas* and *Dlk* clusters, has not yet been tested by truncation studies (Koerner *et al*., 2009).

The macro ncRNAs, *Airn* and *Kcnq1ot1*, silence protein-coding genes *in cis*, but their mechanism of action is not yet fully understood and it is unclear whether they function in the same manner to silence

gene showing ubiquitous and placental-specific imprinted expression. An important question is whether the macro ncRNA is required at all for silencing or whether transcription itself induces silencing. These possibilities cannot be distinguished by truncation experiments, as both the RNA and transcription length are shortened. The macro ncRNA in an imprinted cluster is often positioned antisense and partially overlapping one of the imprinted genes, as is the case for *Igf2r*, in which *Airn* overlaps its promoter, and *Kcnq1*, in which internal exons are overlapped by *Kcnq1ot1*. This suggests the possibility of transcriptional interference (Pauler *et al*., 2007), wherein transcription of the ncRNA directly inhibits transcription of the protein-coding gene, although this has not yet been shown for any imprinted cluster. For transcription of a macro ncRNA to regulate nonoverlapped genes, which includes genes showing placental-specific imprinted expression, it would have to do so indirectly, for example, by disrupting an enhancer or inducing a repressor element. Such an affect has not yet been shown at imprinted clusters. Imprinted macro ncRNAs, such as, *Airn* (108 kb) and *Kcnq1ot1* (91 kb), are mostly unspliced and nuclear localized (Seidl *et al*., 2006; Pandey *et al*., 2008; Redrup *et al*., 2009). RNA interference acts in the cytoplasm to target RNA for degradation and therefore cannot be used against nuclear-localized RNA species (Zeng and Cullen, 2002). Therefore, RNA interference cannot be used to distinguish whether transcription of imprinted macro ncRNAs or the RNA product is responsible for initiating epigenetic silencing. Using an episome system, the first 5 kb of *Kcnq1ot1* was able silence a nonoverlapped *Hygromycin* gene *in cis*, an effect that was abolished when the highly unstable *c-fos* 3′-untranslated region was inserted after the *Kcnq1ot1* fragment, reducing the RNA half-life by threefold. Transcription was not affected by the *c-fos* 3′-untranslated region insertion, indicating that the RNA product was responsible for silencing (Pandey *et al*., 2008). Destabilization of an endogenous full-length imprinted macro ncRNA has not yet been shown, but would provide an important tool to distinguish whether imprinted silencing is mediated by ncRNA transcription or by the RNA itself (Pauler *et al*., 2007).

A number of studies have presented evidence for a role for RNA-mediated silencing of imprinted genes. Under this model, the imprinted ncRNA itself would target, *in cis*, the imprinted genes in a cluster, recruiting repressive epigenetic marks (Pauler *et al*., 2007). In an episome system, it has been shown that a 890-bp silencing domain in the 5′ region of the *Kcnq1ot1* ncRNA is able to silence nonoverlapped genes *in cis*, implying a role for this element in the endogenous ncRNA (Mohammad *et al*., 2008; Pandey *et al*., 2008). This evidence conflicts with an *in vivo* truncation experiment in which the truncated *Kcnq1ot1* still includes this domain, but is unable to silence imprinted genes in the *Kcnq1* cluster (Mancini-Dinardo *et al*., 2006; Shin *et al*., 2008). Many imprinted macro ncRNAs contain small ncRNAs that may have a potential post-transcriptional silencing role such as microRNAs (for example, *H19*) and small interfering RNAs (for example, *Airn*) (reviewed in Koerner *et al*., 2009). However, using a conditional *Dicer* mutant, the RNA interference pathway was shown not to be involved in imprinted gene silencing at the *Kcnq1* cluster in the embryo, although extraembryonic imprinted expression was not examined in this experiment (Redrup *et al*., 2009).

Macro ncRNAs have been suggested to recruit repressive chromatin marks to induce imprinted gene silencing (Kanduri *et al*., 2009). At the *Kcnq1* cluster, *Kcnq1ot1* has been shown to associate with a repressive chromatin domain excluding RNA polymerase II, and enriched in PRC2 and PRC1 complexes and their repressive marks H3K27me3 and H2AK119u1. The same association with a repressive compartment was also shown for *Airn* (Terranova *et al*., 2008). In the *Kcnq1* cluster, *Kcnq1ot1* was shown to interact with chromatin at the promoters of imprinted genes, but not with non-imprinted genes in the placenta (Pandey *et al*., 2008). In placenta, *Airn* was shown to be associated with the repressed promoter of *Slc22a3*, which was also associated with G9A, implicating the ncRNA in recruiting repressive histone modifications in placental-specific imprinted expression. In contrast, *Airn* and G9A were not associated with the promoter of *Igf2r* in the placenta, a gene showing ubiquitous imprinted expression (Nagano *et al*., 2008). Using a Histone 3 antibody, *Kcnq1ot1* was also shown to be more associated with chromatin in placenta than in fetal liver (Pandey *et al*., 2008). The *Kcnq1ot1* RNA fluorescence *in situ* hybridization signal was also shown to occupy a larger volume in placenta than in embryo and to frequently overlap genes that it epigenetically silences *in cis* (Redrup *et al*., 2009). These results suggest that imprinted macro ncRNAs are involved in recruiting repressive chromatin marks to genes showing placental-specific imprinted expression to induce silencing of the repressed allele, whereas silencing of genes showing ubiquitous imprinted expression may be induced by a different downstream mechanism (Figure 3).

Models explaining extraembryonic imprinted gene expression

Models explaining the existing data on imprinted expression need to take into account the differences in imprinted gene expression between extraembryonic and embryonic tissues. In addition, different models are required to explain imprinted clusters regulated by an insulator mechanism (for example, the *Igf2* cluster) and clusters regulated by a macro ncRNA (for example, the *Igf2r* and *Kcnq1* clusters). Models to explain how imprinted gene expression could be regulated have been proposed previously (Pauler *et al*., 2007). On the basis of this, we propose models to specifically explain the difference between imprinted expression in embryonic and extraembryonic tissues (Figure 4).

In the *Igf2* imprinted cluster, *Igf2* shows ubiquitous imprinted expression, whereas *Ins2* shows imprinted expression only in the VYS from E14.5 (Giddings *et al*., 1994; Deltour *et al*., 1995; Duvillie *et al*., 1998). It is known that silencing of *Igf2* is initiated on the maternal allele by CTCF binding to the unmethylated ICE, inducing insulator function that blocks access to enhancers (Hark *et al*., 2000). Silencing of *Ins2* could be explained by the insulator enhancer blocking function extending over a larger distance in the VYS than in the embryo (Figure 4a). How this can be achieved is unknown, but one possibility is that differences in chromatin structure

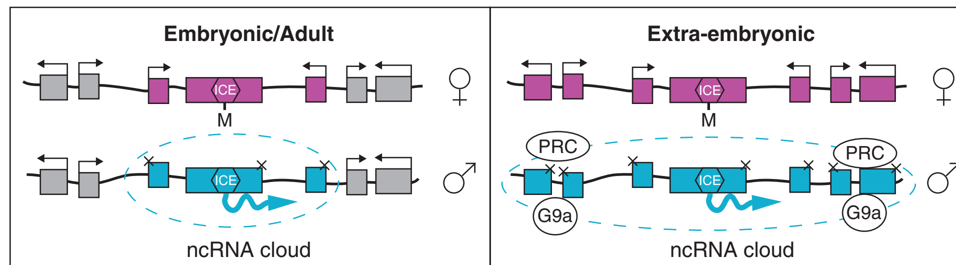

Figure 3 A summary of published work on imprinted macro ncRNA function in the embryo and in extraembryonic tissues. Genes showing imprinted expression in embryo/adult tissues tend to be close to the ICE that contains the macro ncRNA promoter. DNA methylation is important to maintain imprinted expression in the embryo. In extraembryonic tissues, genes located further away from the ICE/ncRNA promoter will show imprinted expression. DNA methylation may not be required to maintain imprinted expression of genes showing extraembryonic-specific imprinted expression, instead repressive chromatin modifications such as H3K27me3, H2AK119u1 and H3K9me2 that are deposited by polycomb complexes (PRC) and G9A are required to maintain imprinted expression. The macro ncRNA is implicated in recruiting these repressive complexes to extraembryonic-specific imprinted genes. The ncRNA forms a cloud that is larger in extraembryonic tissues and often covers the genes that are silenced (dotted blue line). ICE, imprint control element; M, DNA methylation; and E, enhancer.

and histone modifications in the *Igf2*-imprinted gene cluster in the VYS, compared with the embryo, could affect the range of the insulator. If such differences exist, this hypothesis could be tested by assessing the insulator function in the VYS of conditional knockouts of the relevant chromatin-modifying complexes.

Imprinted gene silencing by macro ncRNAs can be explained by transcription-mediated silencing or RNA-mediated silencing models (Pauler *et al*., 2007). Direct interference of ncRNA transcription with the promoter of the protein-coding gene can be proposed as a silencing mechanism for genes such as *Igf2r* that show ubiquitous imprinted expression and in which the ncRNA *Airn* overlaps the promoter. Genes showing placental-specific imprinted expression tend to be further away from and not overlapped by the silencing macro ncRNA. Transcriptional interference disrupting an essential placental enhancer element may prevent upregulation of genes showing placenta-specific imprinted expression, a model that could explain, for example, imprinted expression of *Slc22a2* and *Slc22a3* in the *Igf2r* cluster (Figure 4b). In addition, transcriptional interference with an enhancer may also explain how the *Kcnq1ot1* ncRNA induces ubiquitous imprinted expression of the *Kcnq1* gene without overlapping its promoter. Alternatively, macro ncRNA transcription could induce a repressor element or induce formation of a repressive chromosome loop, silencing genes showing placenta-specific imprinted expression (Pauler *et al*., 2007).

Macro ncRNAs may also induce silencing by forming a repressive chromatin domain, recruiting repressive chromatin-modifying complexes and excluding RNA polymerase II (Terranova *et al*., 2008). This domain may be larger in placental tissues than in the embryo, silencing more genes (Redrup *et al*., 2009) (Figure 4c). Genes showing placental-specific imprinted expression have been shown to be more sensitive to silencing by repressive histone modifications than genes showing ubiquitous imprinted expression (Monk *et al*., 2008; Nagano *et al*., 2008; Terranova *et al*., 2008; Wagschal *et al*., 2008). This supports a suggestion that the mechanism by which genes showing ubiquitous imprinted expression are silenced by macro ncRNAs is different from those showing placental-specific imprinted expression.

In vitro model systems for extraembryonic imprinting

In vitro model systems of imprinted expression that mimic *in vivo* development provide a method to study the imprinting mechanism that is more flexible, quicker and cheaper than mouse models. The use of ES cell-based *in vitro* model systems that parallel some developmental stages also allows the effect of genetic modifications on imprinted expression to be tested before generating mutant mouse models. If results in such an *in vitro* model system indicate that a mouse is required, the already available genetically modified ES cells can be used to generate chimeras, from which mutant mice can be derived. The development of *in vitro* model systems of embryonic and extraembryonic imprinted gene expression is therefore a priority.

The transition from biallelic *Igf2r* expression in the pre-implantation embryo to the onset of imprinted expression in the post-implantation embryo is paralleled by the differentiation of ES cells, providing a model for embryonic imprinted gene expression (Latos *et al*., 2009). A number of models of extraembryonic imprinted gene expression have been proposed (Figure 5). TS cells are stem cells derived from trophectoderm in the pre-implantation embryo, a cell linage that develops into the ectoplacental cone and extraembryonic ectoderm *in vivo*, and then into the placental tissues that display placental-specific imprinting (Tanaka *et al*., 1998; Sleutels *et al*., 2002; Lewis *et al*., 2004b, 2006; Terranova *et al*., 2008). In the early trophectoderm, genes that show placental-specific imprinted expression later in development are either biallelically expressed or are not yet expressed, and in culture, TS cells that differentiate into trophoblast giant cells do not display imprinted expression of these genes (Lewis *et al*., 2006). Recent work has shown that TS cells can be differentiated toward a labyrinth cell fate (Hughes *et al*., 2004; Natale *et al*., 2009). *In vivo*, the labyrinth cell layer of the placenta shows extraembryonic-specific imprinted gene expression of genes such as *Slc22a3* and *Ascl2* (Tanaka *et al*., 1999; Verhaagh *et al*., 2001). Therefore, differentiation of TS cells into labyrinth cells could provide an *in vitro* system to study extra-embryonic genomic imprinting, but validation that

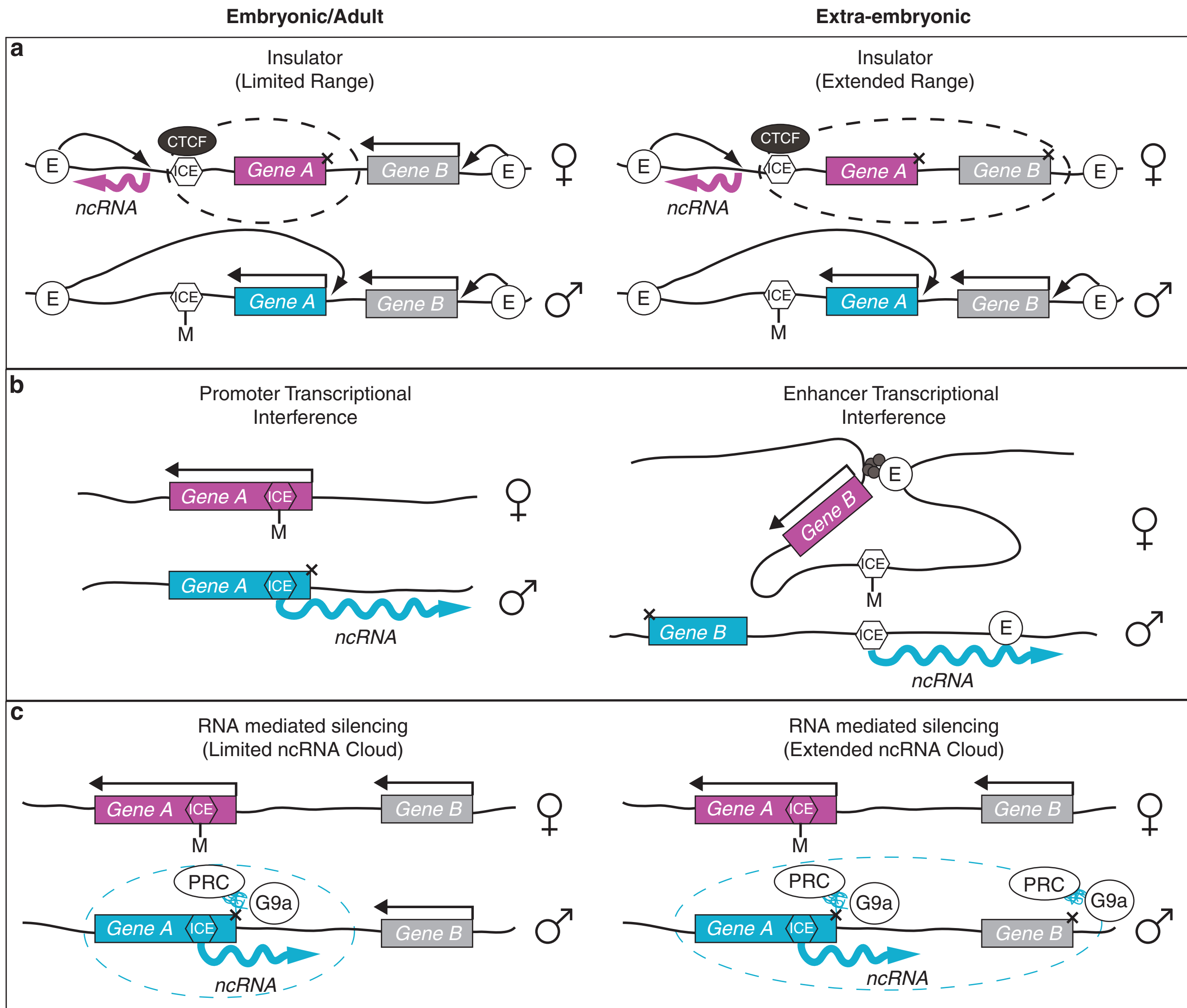

Figure 4 Models explaining embryonic and/or adult versus extraembryonic imprinted expression. (**a**) *Insulator model*: in the embryo/adult model, CTCF binds the unmethylated ICE allele and acts as an insulator, blocking access of enhancers to Gene A, whereas expression of the more distant Gene B is unaffected. In extraembryonic tissues, the insulator action extends over a greater distance also blocking enhancer access to Gene B. (**b**) *Transcriptional interference model*: in the embryo/adult model, transcription of the ncRNA overlaps the promoter of Gene A preventing upregulation of expression on this chromosome. In extraembryonic tissues, expression of the ncRNA disrupts an enhancer interaction with a distant Gene B preventing upregulation of expression on this chromosome. (**c**) *RNA-meditated silencing model*: in the embryo/adult model, the ncRNA cloud covers a limited area and the ncRNA recruits repressive chromatin-modifying complexes PRC and G9A to nearby Gene A. In extraembryonic tissues, the ncRNA cloud covers a larger area and recruits repressive chromatin modifiers to both Gene A and the more distant Gene B. ICE, imprint control element; M, DNA methylation; and E, enhancer.

placental-specific imprinted genes show imprinted expression in this system is required.

Extraembryonic endoderm (XEN) cells are stem cells derived from the blastocyst that have a cellular identity similar to primitive endoderm (Kunath *et al*., 2005). *In vivo* primitive endoderm differentiates into tissues, including the visceral endoderm that becomes part of the VYS and can show extraembryonic-specific imprinted expression for a number of genes (Table 1). However, it has not been shown that XEN cells can differentiate into yolk sac visceral endoderm in culture; hence, using currently known culture conditions, XEN cells cannot be used as a model of extraembryonic imprinted gene expression.

Embryonic stem cells cultured in leukemia inhibitory factor-free media on nonadhesive surfaces form aggregates and differentiate into structures known as embryoid bodies (EBs), which initially most closely resemble early post-implantation embryos with an outer endoderm layer and inner ectoderm layer (Doetschman *et al*., 1985). Following prolonged differentiation of EBs, multiple differentiated cell types can arise, including cardiomyocytes, endothelial cells, adipocytes, neuronal cells and hematopoietic cells (Desbaillets *et al*., 2000). It has been reported by a number of studies that EBs can differentiate into VYS-like structures expressing visceral endoderm markers (Doetschman *et al*., 1985; Abe *et al*., 1996; Koike *et al*., 2007). As VYS is known to express

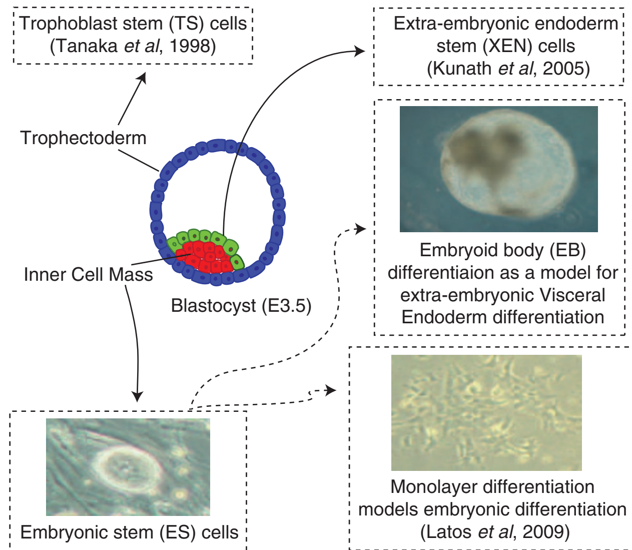

Figure 5 *In vitro* models to study the imprinting mechanism. ES cells derived from the ICM can be differentiated *in vitro* providing a model of embryonic imprinted expression. Models of extraembryonic imprinted expression are TS cells derived from trophectoderm and XEN cells derived from primitive endoderm. Differentiation of ES cells into embryoid bodies (EBs) can form tissues expressing markers of visceral endoderm and resembling VYS, suggesting that this system may provide a model of extraembryonic imprinted expression.

genes showing extraembryonic-specific imprinted expression, a robust EB culture system for deriving VYS-like visceral endoderm tissue could provide an *in vitro* model for extraembryonic imprinting and therefore warrants further investigation.

Conclusions

Imprinted gene expression is controlled by differential DNA methylation of an ICE, which is read by an epigenetic initiator that silences genes in the surrounding imprinted gene cluster *in cis*. Imprinted genes close to the epigenetic initiator tend to show ubiquitous imprinted expression, whereas genes further away can show placental-specific imprinted expression. Evidence so far indicates that, compared with genes showing ubiquitous imprinted expression, genes showing placental-specific imprinted expression are less sensitive to DNA methylation; instead, repressive histone modifications seem to have a more important role in imprinted gene expression in the placenta. An *in vitro* model system for extraembryonic imprinted expression would complement *in vivo* studies, but currently, a good model is lacking.

Conflict of interest

The authors declare no conflict of interest.

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