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Epigenetic dynamics of the *Kcnq1* imprinted domain in the early embryo

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The mouse *Kcnq1* imprinted domain is located on distal chromosome 7 and contains several imprinted genes that are paternally repressed. Repression of these genes is regulated by a non-coding antisense transcript, *Kcnq1ot1*, which is paternally expressed. Maternal repression of *Kcnq1ot1* is controlled by DNA methylation originating in the oocyte. Some genes in the region are imprinted only in the placenta, whereas others are imprinted in both extra-embryonic and embryonic lineages. Here, we show that *Kcnq1ot1* is paternally expressed in preimplantation embryos from the two-cell stage, and that ubiquitously imprinted genes proximal to *Kcnq1ot1* are already repressed in blastocysts, ES cells and TS cells. Repressive histone marks such as H3K27me3 are present on the paternal allele of these genes in both ES and TS cells. Placentally imprinted genes that are distal to *Kcnq1ot1*, by contrast, are not imprinted in blastocysts, ES or TS cells. In these genes, paternal silencing and differential histone marks arise during differentiation of the trophoblast lineage between E4.5 and E7.5. Our findings show that the dynamics during preimplantation development of gene inactivation and acquisition of repressive histone marks in ubiquitously imprinted genes of the *Kcnq1* domain are very similar to those of imprinted X inactivation. By contrast, genes that are only imprinted in the placenta, while regulated by the same non-coding RNA transcript *Kcnq1ot1*, undergo epigenetic inactivation during differentiation of the trophoblast lineage. Our findings establish a model for how epigenetic gene silencing by non-coding RNA may depend on distance from the non-coding RNA and on lineage and differentiation specific factors.

KEY WORDS: Epigenetics, Imprinting, ES and TS cells, *Kcnq1* domain, Mouse

INTRODUCTION

Imprinted genes are expressed from only one of their parental alleles and regulated by epigenetic marks such as DNA methylation and histone tail modifications. These epigenetic marks are parent-of-origin specific and distinguish between the two parental alleles of an imprinted gene (Fournier et al., 2002; Lewis et al., 2004; Li et al., 1993; Reik and Walter, 2001; Umlauf et al., 2004). Most imprinted genes occur in clusters in the mammalian genome. Within a cluster, the imprinting of multiple genes is often regulated in a coordinated fashion, involving imprinting centres that acquire allele-specific DNA methylation in the parental germ cells. To date, there are two principal mechanisms described by which an allele-specific DNA methylation mark can lead to imprinting of a cluster of genes (Lewis and Reik, 2006). The first involves inactivating a chromatin insulator by DNA methylation; distal enhancers are prevented from accessing promoters on the unmethylated allele by a repressive higher order chromatin structure but can activate transcription on the methylated allele. This mechanism regulates the *Igf2-H19* imprinting cluster (Bell and Felsenfeld, 2000; Hark et al., 2000; Kanduri et al., 2000; Murrell et al., 2004). The second involves a DNA methylation mark that represses a non-coding RNA transcript on one parental allele. On the other allele the non-coding transcript is expressed leading to the repression of flanking genes by targeting polycomb proteins and repressive histone modifications to the region. This mechanism regulates imprinted X inactivation in mouse placenta and is also likely to occur in the *Igf2r* and *Kcnq1* domains (Heard, 2004; Huynh and Lee, 2003; Lewis et al., 2004; Sleutels et al., 2002; Umlauf et al., 2004).

The *Kcnq1* imprinted domain lies on distal mouse chromosome 7 and contains one paternally expressed gene, the non-coding RNA *Kcnq1ot1*, several flanking genes which are paternally repressed in all lineages (we term these ubiquitously imprinted genes) and other flanking genes which are paternally repressed in placental lineages but are not imprinted in embryonic lineages (Engemann et al., 2000; Paulsen et al., 2000). It contains two differentially methylated regions (DMRs): one is a germline imprint which acts as the imprinting centre (IC2) and contains the promoter of the non-coding *Kcnq1ot1* gene; the other is a secondary imprint upstream of the cell cycle regulator *Cdkn1c* which is not established until post-implantation stages of development (Bhogal et al., 2004; Engemann et al., 2000; Fitzpatrick et al., 2002). The other genes in the cluster have no associated differential DNA methylation (Lewis et al., 2004). Allele-specific histone modifications are also present at the locus. In the embryo they are restricted to the DMRs. In extra-embryonic lineages, however, repressive histone modifications mark the entire cluster on the paternal chromosome (with the exception of the *Kcnq1ot1* region), while the maternal chromosome is marked by histone modifications known to be associated with active chromatin (Umlauf et al., 2004). The repressive histone methylation marks on the paternal chromosome depend on the presence of the *Kcnq1ot1* gene (Lewis et al., 2004), and gene silencing in cis of both ubiquitously and placentally imprinted genes indeed requires the *Kcnq1ot1* transcript, or transcriptional elongation at the *Kcnq1ot1* promoter (Mancini-Dinardo et al., 2006).

There are several mechanistic similarities between imprinting in the *Kcnq1* domain and imprinted X chromosome inactivation (Huynh and Lee, 2003; Okamoto et al., 2004). The non-coding RNA *Xist* is paternally expressed in the preimplantation embryo and

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accompanied by exclusion of RNA polymerase II. Inactivation of genes in cis is detected very early and is followed by acquisition of specific repressive histone marks. By the morula-blastocyst stage, the majority of X linked genes have been paternally silenced. This silencing becomes pan-chromosomal and complete in extra-embryonic tissues after implantation. However, in the embryonic lineages, imprinted X inactivation must be reprogrammed. Hence, in the inner cell mass (ICM) of female blastocysts, silencing of the paternal genes on the X is erased and X-linked genes are expressed biallelically in the epiblast and in ES cells, before random X inactivation commences during early differentiation of epiblast cells (Rastan, 1982; Takagi et al., 1982).

By analogy with X inactivation, it has been proposed that genes in the *Kcnq1* domain become paternally silenced in the preimplantation embryo, and placentally imprinted genes are reprogrammed in the ICM to be biallelically expressed in the embryo, but continue to be imprinted in the extra-embryonic tissues (Umlauf et al., 2004). Testing of this model, and detailed mechanistic comparisons with X inactivation, requires the study of the epigenetic dynamics of the *Kcnq1* domain in the preimplantation embryo.

Here, we investigate the establishment of imprinting in the *Kcnq1* domain during preimplantation development and in embryonic stem (ES) and trophoblast stem (TS) cells as a model for the blastocyst stage of development. We show that *Kcnq1ot1* is paternally expressed at the two-cell stage and retains its imprinting throughout preimplantation development. The ubiquitously imprinted genes also show monoallelic expression by the blastocyst stage. Unexpectedly, the placentally imprinted genes are still biallelically expressed in blastocysts. ES and TS cells precisely mirror this pattern of expression and we thus used them as a model system to study allele specific histone modifications. The ubiquitously imprinted genes indeed exhibit differential histone modifications while placentally imprinted genes are not differentially marked. Their silencing and differential histone marking arises during differentiation of the extra-embryonic lineages between E4.5 and E7.5.

MATERIALS AND METHODS

Cell lines and mouse crosses

C57BL/6J×*Mus musculus castaneus* ES cells were kindly provided by E Li (Lei et al., 1996). They were cultured on a layer of feeder mouse embryonic fibroblasts (MEFs) in Dulbecco's modified Eagle medium containing 15% foetal bovine serum and 10^3 U of leukaemia inhibitory factor per ml. Before ES cells were used for RNA or ChIP analysis MEFs were removed by panning. C57BL/6J×*M.m. castaneus* TS cells (Huynh and Lee, 2003) were cultured on a layer of feeder MEFs in RPMI 1640 medium containing 20% fetal bovine serum, 25 ng/ml basic fibroblast growth factor and 1 μg/ml heparin. Before TS cells were used for RNA or ChIP analysis, they were taken through one passage without feeder cells to ensure minimal contamination with MEFs.

RNA analysis

Total RNA was isolated from cells, tissues and staged embryos at E9.5 using the RNeasy mini kit (Qiagen) according to the manufacturer's instructions. The RNA was treated with DnaseI (Roche) and purified by ethanol precipitation. RNA (0.5-1 μg) was reverse transcribed with Superscript II (Invitrogen) or Powerscript single shot (BD Biosciences), according to the manufacturer's instructions. Analogous reactions were performed without reverse transcriptase (RT) to control for DNA contamination. Amplification of cDNA was performed using PCR primers from Table 1. RNA was extracted from preimplantation embryos using Trizol (Sigma) and treated with TURBO DNase (Ambion) according to the manufacturers instructions. RNA (10-20 ng) was reverse transcribed and amplified in a one-step reaction with Superscript III (Invitrogen) using primers from Table 1.

ChIP analysis

ChIP experiments were carried out as previously described (Fournier et al., 2002). Briefly ~5×10^7 cells were collected and washed in PBS. Nuclei were purified through a sucrose cushion and incubated with MNase to obtain fragments of one to five nucleosomes in length. Approximately 20 μg of chromatin was incubated with 5-10 μg of antibody overnight at 4°C. We used the following antibodies: H3AcK9 and K14, H3K4me2 and H3K27me3 from Upstate Biotechnology; and H3K9me2 from Abcam. The antibody chromatin complexes were captured with ProteinA sepharose beads. After washing and elution DNA was extracted from the input chromatin, bound and unbound fractions. We analyzed DNA from the ChIP assays by PCR-SSCP using primers listed in Table 2.

E7.5 ectoplacental cone ChIP

Carrier ChIP was adapted from a protocol described by O'Neill et al. (O'Neill et al., 2006). *Drosophila* SL2 cells (5×10^7) were added to one mouse E7.5 ectoplacental cone (EPC) and homogenised. Chromatin was then prepared and the entire sample was used in a precipitation with the H3K4me2 antibodies as above as well as a no-antibody control. The antibody complex was captured with Immunopure immobilized protein A and Handee Spin Cup columns (Pierce). The columns were then washed

Table 1. Details of primers used for RT-PCR analysis

Gene	Primers	PCR conditions	Polymorphism	Gel conditions	Cross
Osbpl5	F: CCACCATCCCAGATCAAGAC R: TTCTCCTGTGTGGCTTTGTG	58°C	*Nco*I	10% polyacrylamide	B6/Cast
Phlda2	F: CGTGATGTCTTCGAAAACCG R: TGACGATGGTGAAGTACACG	57°C	Deletion	0.6×TBE, 0.5×MDE SSCP	B6/Cast
Cdkn1c (C)	F: GCCAAGCGCAAGAGAACT R: CAACAACCATTCCCCTAGAGTC	57°C	*Taq*I	1% agarose	B6/Cast
Cdkn1c (S)	F: TTCAGATCTGACCTCAGACCC R: GACCGGCTCAGTTCCCAGCTCAT	58°C	*Ava*I	10% polyacrylamide	B6/SD7
Kcnq1ot1(C)	F: CTGAATTGGGGGAATAGCA R: CCATTTCTGCACCTGTTTT	57°C	*Stu*I	1% agarose	B6/Cast
Kcnq1ot1(S)	F: TTGCCTGAGGATGGCTGTG R: CTTTCCGCTGTAACCTTTCTG	57°C	*Mwo*I	1% agarose	B6/SD7
Tssc4	F: AGAAGCTGCCCATCCTGAGT R: GACCCACAATTCCCACAGTC	59°C	*Alu*I	10% polyacrylamide	B6/Cast B6/SD7
Cd81(C)	F: GCGTCCTTGCTTCAAAGAGA R: AGGCAAACAGGATCACAAGG	58°C	*Fau*I	1% agarose	B6/Cast
Cd81(S)	F: GGGGACATGGCCTGTGTAT R: GCTCCCAAAAGGTAACAGGA	58°C	Deletion	10% polyacrylamide	B6/SD7
Ascl2	F: TGAGCATCCCACCCCCCTA R: CCAAACATCAGCGTCAGTATAG	55°C	*Sfc*I	1% agarose	B6/Cast

Table 2. Details of primers used for ChIP PCR analysis

Primer set	xTBE gel	Forward sequence	Reverse sequence
Kcnq1ot1	1	CTCAGTTCCACGATACCCTTCC	CTTACAGAAGCAGGGGTGGTCT
Cdkn1c	1	TTCTGTAACCGCACACACC	CAAAGGCTCAGAGAGATTCAGC
Phlda2	0.6	CTCCCTACAGAAACGCACG	CCAGCAGGAACTACACAGACC
Tssc4	0.6	AACACTTGGACCTCCACACC	ACCCTTTAACCACCCAGCTT
Ascl2	0.6	ACAGGTGTGCTCCTGCAATG	GCGGTGACCAAGAGGTAGGA
Cd81	0.6	GAGGTCTATAAAGAGTGAGGAGC	CTTGGATTGGTCCTGGAG
Osbpl5	0.6	CCCTAAAGAAAGTGAAGC	AATAGTCCACTGACCAGC

using the NaCl buffers previously described (Fournier et al., 2002) and eluted. PCR was carried out using primers from Table 2. Using this approach we maximised the amount of material to use in SSCP-PCR analysis.

RESULTS

Imprinted expression of the *Kcnq1* domain in preimplantation development

We and others have previously shown the extent of imprinted expression and differential histone modifications in the *Kcnq1* domain in embryonic and extra-embryonic tissues at embryonic day 9.5 (Lewis et al., 2004; Umlauf et al., 2004). We wished to determine when during preimplantation development *Kcnq1ot1* expression and imprinting arose, and when ubiquitously and placentally imprinted genes were silenced (Fig. 1). Screening of EST library data indicated expression of *Kcnq1ot1* in two-cell embryos. In Fig. 1B we show that the non-coding RNA *Kcnq1ot1* is paternally expressed as early as two-cell embryos. This imprinted expression is maintained throughout preimplantation development to the blastocyst stage (Fig. 1C; data not shown). The ubiquitously imprinted gene *Kcnq1* is paternally repressed at the morula/blastocyst stage (Fig. 1C) confirming previous results. *Cdkn1c* is also imprinted at this stage (Umlauf et al., 2004). By contrast, the placentally imprinted genes *Tssc4* and *Cd81* show biallelic expression in the blastocyst (Fig. 1C), while *Ascl2* expression is not detected until E5.5 (Tanaka et al., 1999; Umlauf et al., 2004). These results show clearly that the non-coding RNA is paternally expressed from the two-cell stage, and that ubiquitously imprinted genes are paternally silenced by the blastocyst stage, whereas the placentally imprinted genes continue to be biallelically expressed.

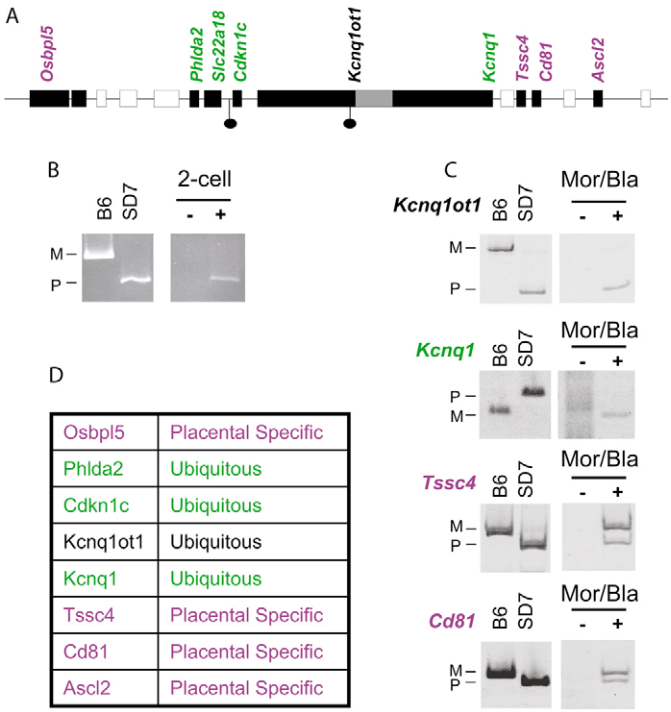

Fig. 1. Allele-specific expression in preimplantation embryos. RT-PCR was used to analyse allele-specific expression of genes within the *Kcnq1* domain in C57BL/6J (B6)×*Mus spretus*-distal chromosome 7 (SD7) two-cell and blastocyst stage embryos. Maternal (M) and paternal (P) alleles were distinguished using RFLP or deletion/insertion polymorphisms described in the Materials and methods. For each gene, a representative sample is shown with its corresponding reverse-transcriptase negative control. (**A**) A schematic of the *Kcnq1* domain, with the non-coding RNA *Kcnq1ot1* labelled in black, ubiquitously imprinted genes labelled in green and placental-specific imprinted genes labelled in purple. DMRs are marked by black circles. (**B**) *Kcnq1ot1* exhibits monoallelic paternal expression at the two-cell stage. (**C**) *Kcnq1ot1* and *Kcnq1* are paternally and maternally expressed, respectively; however, placental specific imprinted genes *Tssc4* and *Cd81* are biallelically expressed at the blastocyst stage. (**D**) Table showing the tissue specific imprinting of genes in the locus.

ES and TS cell lines reflect imprinted expression in the blastocyst

We next wished to determine whether ES and TS cells faithfully reflect the imprinting pattern we see in the blastocyst. In the same way that ES cells have similar properties to the ICM, TS cells reflect properties of the TE lineage from which they are derived and are able to contribute towards all trophoblast cell types in conceptuses when reintroduced into blastocysts (Bradley et al., 1984; Bradley and Robertson, 1986; Tanaka et al., 1998). We used ES and TS cells from C57Bl6/J×*M.m. castaneus* F1 hybrids to study allele-specific expression. First we determined absolute levels of expression of each gene in the cluster by Q-PCR in ES and TS cells, their differentiated derivatives (embryoid bodies and trophoblast giant cells, respectively), and in E10.5 embryos and placentae (see Fig. S1 in the supplementary material). *Ascl2* and *Kcnq1* showed only basal levels of expression in ES and TS cell lines (see Fig. S1 in the supplementary material) making it impossible to reliably assay allelic expression of these two genes. For the remaining genes, we determined that the amplification of each parental allele during RT-PCR was in the linear range.

Similar to our results in blastocysts, we find that the non-coding RNA, *Kcnq1ot1* is largely repressed on the maternal allele in ES and fully repressed in TS cells (Fig. 2). The ubiquitously imprinted genes *Phlda2* and *Cdkn1c* show monoallelic maternal expression. The placentally imprinted genes *Osbpl5* and *Tssc4* show biallelic expression in the ES and TS cells, confirming that they accurately reflect the imprinting status of the blastocyst. *Cd81* shows expression from both alleles in ES and TS cells, although there is some skewing towards the maternal allele in TS cells. We have controlled for primer bias and the final maternal to paternal ratio of *Cd81* is 1:1 in ES cells and has a bias of 3.5: 1 in TS cells. Although this is not the same level of bias seen at later stages, it is possible that

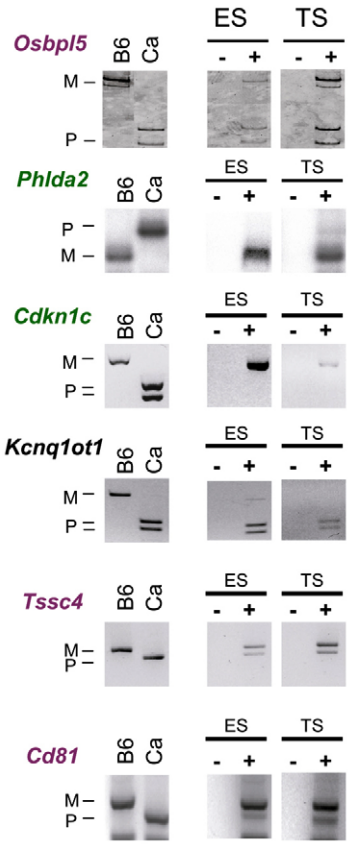

Fig. 2. Allele-specific expression in ES and TS cells. RT-PCR was used to analyse the allele-specific expression of genes within the *Kcnq1* domain in undifferentiated C57BL/6J (B6)×*M.m. castaneus* (Ca) ES and TS cells. Maternal (M) and paternal (P) alleles were distinguished using RFLP or deletion/insertion polymorphisms described in the Materials and methods. The non-coding RNA *Kcnq1ot1* is labelled in black, ubiquitously imprinted genes are labelled in green and placental specific imprinted genes are labelled in purple. For each gene, a representative sample is shown with its corresponding reverse transcriptase-negative control. *Phlda2*, *Cdkn1c* and *Kcnq1ot1* exhibit monoallelic expression, and *Tssc4* and *Osbpl5* show biallelic expression in both cell types. *Cd81* shows expression from both alleles although there is a bias towards the maternal allele. We corrected for primer bias by densitometry and normalisation to a mix of 50% B6 cDNA and 50% Cast cDNA. The parental ratio of *Cd81* in ES cells is 1:1, whereas TS cells show a ratio of 3.5:1.

imprinting at each gene in the domain is established at slightly different times, with *Cd81* imprinting occurring earlier than other placental specific genes.

Allele specific histone modifications in ES and TS cells

We next examined allelic histone modifications in ES and TS cells by ChIP and SSCP (Fig. 3A). At the *KvDMR1* (overlapping the *Kcnq1ot1* promoter region), there is enrichment of activating histone marks (acetylation and H3K4me2) on the paternal allele, and enrichment of repressive modification (H3K9me2) on the maternal allele in both cell types. No bias in K27me3 is observed in either ES or TS cells, which differs from published data by Umlauf et al. (Umlauf et al., 2004) in ES cells. This is possibly due to the different genotype of the cell lines used (C57Bl/6×*M.m. castaneus* versus C57Bl/6×*Mus spretus*) or to different K27me3 antibodies. The ubiquitously imprinted genes *Phlda2* and *Cdkn1c* are enriched for acetylation and H3K4me2 on the maternal chromosome, and for H3K27me3 on the paternal chromosome both in TS and ES cells. Previous observations show that in E9.5 placenta, H3K9me2 is also present on the paternal allele (Lewis et al., 2004; Umlauf et al., 2004), which suggests that the chromatin-based repression continues to be established during placental development. This is similar to imprinted X chromosome inactivation, where K27me3 along the X chromosome is observed in preimplantation embryos before K9me2 (Huynh and Lee, 2003; Okamoto et al., 2004). We note that in our ES and TS cells, K9me2 is associated only with regions that also exhibit differential DNA methylation, as is commonly observed in regions of heterochromatin and in vitro assays (Fuks et al., 2003; Lehnertz et al., 2003).

In the placentally imprinted genes, no major differences in histone modifications are observed between the parental alleles in either ES or TS cells, with the exception of *Cd81*, which shows a paternal bias for K27me3 in TS cells, reflecting the skewed expression seen in this cell type. These results in stem cells, which are representative of the ICM and TE lineages, suggest that gene silencing and histone marks of ubiquitously imprinted genes are established during preimplantation development.

Placentally imprinted genes are silenced and epigenetically marked during differentiation of extra-embryonic lineages

The finding that placentally imprinted genes are biallelically expressed in blastocysts and TS cells and lack allelic histone marks suggests that gene silencing arises during differentiation of the trophectoderm lineage. We thus investigated allelic expression and histone marks during differentiation in vitro and in vivo. Upon differentiation of TS cells to trophoblast giant cells there is no change in allele-specific expression or in histone modifications along the locus (see Fig. S2 in the supplementary material). This may be due to cell culture effects or a specific property of isolated trophoblast giant cells (this has never been studied).

By contrast, allelic silencing and histone modification is observed in vivo. Fig. 3B shows that at E7.5 in the ectoplacental cone (EPC, a derivative of the trophectoderm), the paternal allele of *Tssc4* has been silenced. Similarly, *Ascl2* and *Cd81* also show imprinted expression by this stage (Tanaka et al., 1999) (data not shown). The maternal allele of *Tssc4* is enriched for the active modification H3K4me2, revealing that allelic silencing and histone modifications are established between E4.5 and E7.5. The small numbers of cells in the EPC at this stage (~10,000) did not allow a more comprehensive ChIP analysis.

DISCUSSION

We have carried out a systematic analysis of epigenetic modifications and allele-specific expression in the *Kcnq1* imprinting cluster in TS and ES cells. Our expression data in preimplantation embryos confirms that these cell lines provide a good model with which to study the establishment of imprinting in this cluster. Our findings are summarised and compared with imprinted X inactivation in Fig. 4. We find that in the *Kcnq1* imprinted domain, the non-coding RNA *Kcnq1ot1* is expressed from the paternal allele from the two-cell stage onwards. The ubiquitously imprinted genes in the cluster are monoallelically expressed at the morula to blastocyst stage and in ES and TS cells, and allelically marked by histone modifications in both types of stem cell. These genes thus acquire their imprinting early on during preimplantation development, prior to lineage determination, and as a result are

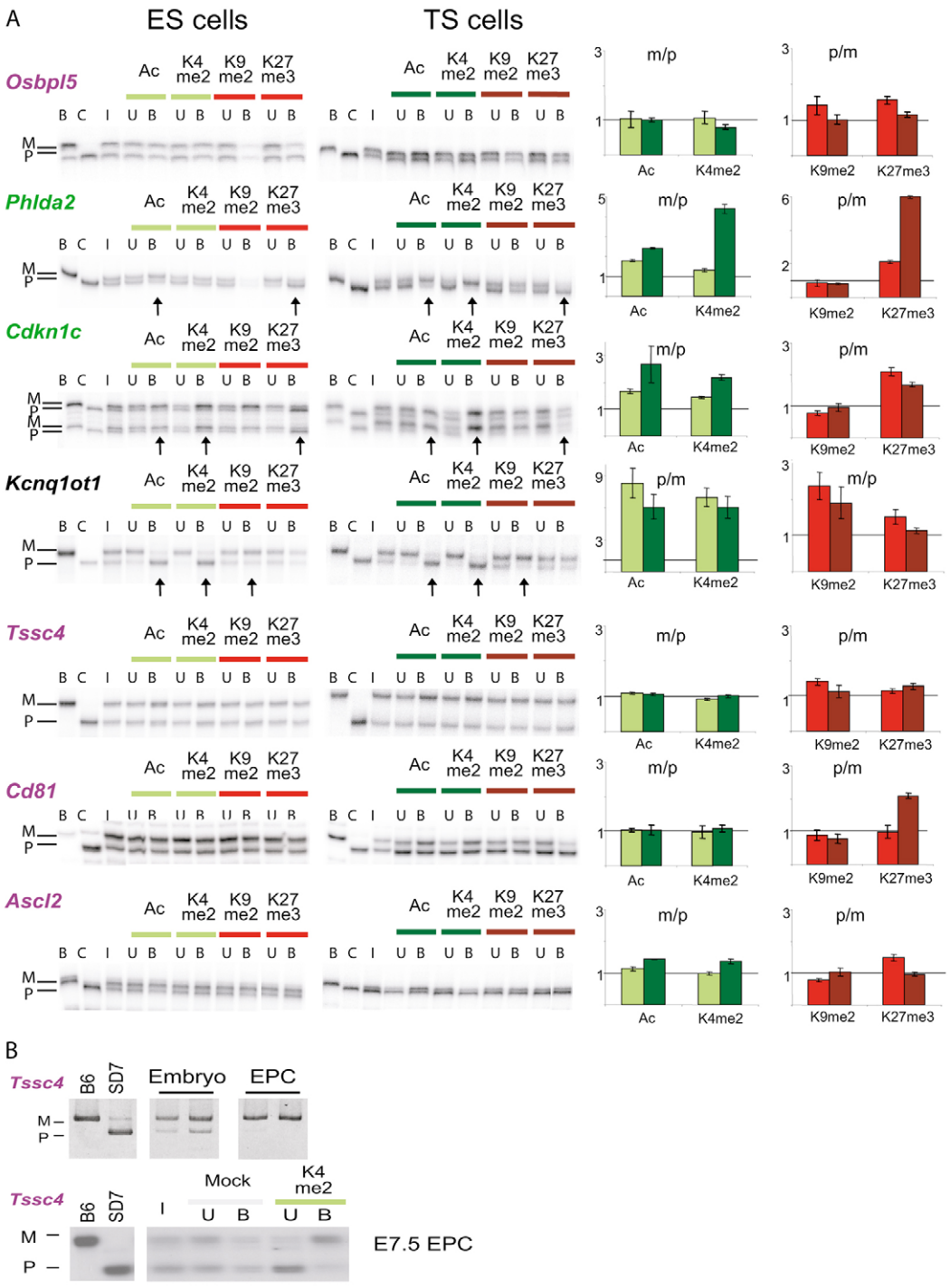

Fig. 3. Histone modifications in ES cells, TS cells and E7.5 EPC. (**A**) ChIP analysis of the previously mentioned ES and TS cell lines was carried out to analyse allele-specific histone modifications with antibodies against H3Ac, H3K4me2, H3K9me2 and H3K27me3. The modifications associated with active chromatin regions are marked in green (light green for ES, dark green for TS cells), while those associated with repressive chromatin are marked in red (light red for ES, dark red for TS cells). The parental alleles are distinguished by SNPs which are separated on SSCP gels. The non-coding RNA *Kcnq1ot1* is labelled in black, ubiquitously imprinted genes are labelled in green and placental specific imprinted genes are labelled in purple. *Phlda2*, *Cdkn1c* and *Kcnq1ot1* show an allelic bias in histone modifications (marked by arrows) and *Osbpl5*, *Tssc4*, *Cd81* and *Ascl2* show no bias in both cell types. Each panel is a representative example of the ChIP, beside it is a graphical representation of the ratio of the bound maternal allele to the bound paternal allele (normalised according to the input) shown for the active modifications and the ratio of paternal over maternal for repressive modification, except for *Kcnq1ot1* where the ratios are reversed. (**B**) RT-PCR was used to analyse allele specific expression of *Tssc4* in C57BL/6J (B6)×*M. spretus*-distal chromosome 7 (SD7) E7.5 embryos and EPCs. Maternal (M) and paternal (P) alleles were distinguished using an RFLP polymorphism described in the Materials and methods. Embryos show biallelic expression while EPCs show maternal expression. Carrier ChIP analysis was used to analyse the histone modification H3K4me2 at *Tssc4* in C57BL/6J (B6) × SD7 (SD7) E7.5 EPC. The parental alleles are distinguished by a SNP which is separated on an SSCP gel. There is a clear bias in the distribution of K4me2 with the majority associated with the active maternal allele.

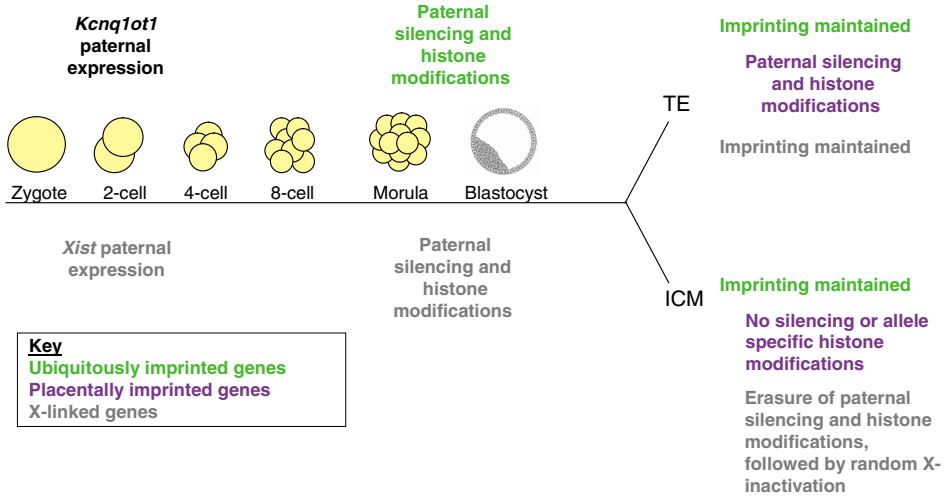

Fig. 4. Epigenetic dynamics in the *Kcnq1* domain during preimplantation development and comparison with imprinted X inactivation. *Kcnq1ot1* and *Xist* RNAs are paternally expressed at the two-cell stage. X-linked genes and ubiquitously imprinted genes are monoallelically expressed and repressive histone modifications are established by implantation. Monoallelic expression of ubiquitously imprinted genes is maintained in both lineages but X-linked genes only retain imprinting in the TE lineage and undergo erasure of paternal silencing in the ICM. Placental-specific imprinted genes are still biallelically expressed in blastocysts but become imprinted and marked by allele specific histone modifications exclusively in the TE lineage after implantation. The non-coding RNA *Kcnq1ot1* is labelled in black, ubiquitously imprinted genes are labelled in green, placental specific imprinted genes are labelled in purple and X linked genes are shown in grey.

imprinted in both embryonic and extra-embryonic tissues. The placentally imprinted genes, by contrast, acquire monoallelic expression after the blastocyst stage, accompanied by the establishment of differential histone methylation exclusively during trophoblast development.

Recent publications have demonstrated that the *Kcnq1ot1* RNA or the process of transcription is required for the silencing of neighbouring genes (Kanduri et al., 2006; Mancini-Dinardo et al., 2006). Truncation of the transcript to just a few kilobases demonstrates that either transcription or the full length RNA itself is necessary to silence the paternal chromosome in cis. Other imprinting clusters show similar characteristics. *Air* is a 108 kb non-coding RNA transcribed in an antisense direction to *Igf2r* on chromosome 17 (Lyle et al., 2000). *Air* is inactivated on the maternal allele by oocyte-derived methylation and is essential for silencing of genes at the *Igf2r/Air* locus (Wutz et al., 1997; Zwart et al., 2001). Truncation of the *Air* transcript to 3 kb also results in loss of imprinting at the locus (Sleutels et al., 2002) and therefore, *Air* RNA or its transcription is a key element of the imprinting control at the *Igf2r/Air* cluster. We have previously proposed that the *Kcnq1ot1* RNA silences by coating of the chromosome followed by RNA polymerase II exclusion and by recruitment of repressive histone modifications using a mechanism similar to that described for X-chromosome inactivation (Lewis et al., 2004; Umlauf et al., 2004). Consistent with this proposal, *Kcnq1ot1* RNA is expressed from the two-cell stage, in parallel with *Xist*. Our observation that ubiquitously imprinted genes are paternally silenced by the blastocyst stage, and have acquired differential histone marks in ICM- and TE-derived pluripotent cell lines, adds further weight to the suggestion that the epigenetic dynamics of the *Kcnq1* cluster and of imprinted X inactivation share a number of significant features.

Our initial expectation for the placentally imprinted genes was therefore that they would be monoallelically expressed in the TE and in TS cells. This expectation was compatible with the model by Umlauf et al. (Umlauf et al., 2004), who proposed that this group of genes were paternally silenced during preimplantation development, and reprogrammed to biallelic expression in the epiblast, in further analogy with X-linked genes. Our results show clearly that this is not the case, and that silencing and histone modifications of these genes arise during early differentiation of the extra-embryonic lineages. The *Igf2r/Air* cluster is comparable in size to the *Kcnq1* imprinted region cluster and encodes a mixture of ubiquitous and tissue specific maternally expressed genes and biallelically expressed genes along the locus (Zwart et al., 2001; Lyle et al., 2000). In this cluster imprinted expression of surrounding genes is also established after initiation of expression of the non-coding RNA, *Air*, although this occurs at a later stage of development than *Kcnq1ot1* (Lerchner and Barlow, 1997; Szabo and Mann, 1995).

How can ubiquitously imprinted genes in the *Kcnq1* region be silenced early on, yet placentally imprinted ones are silenced late and only in the trophoblast lineage? Mancini-DiNardo et al. (Mancini-DiNardo et al., 2006) showed that the *Kcnq1ot1* RNA is required to silence both groups of genes, and they also acquire the same repressive histone modifications, albeit with different kinetics. Because ubiquitously imprinted genes are located closer to the *Kcnq1ot1* transcription unit than placentally imprinted ones (Fig. 1A), we suggest that the RNA represses (in cis) the nearest genes initially and then spreads to more-distant, placental-specific, genes in the trophoblast after implantation. Repetitive elements in the region and/or higher-order chromatin structures that differ between the embryo and placenta may influence putative RNA coating and gene repression in cis. The different epigenetic response of embryo and extra-embryonic tissues may also involve lineage-specific transcription factors or epigenetic marks. The PRC2 proteins Eed and Ezh2 are located at specific foci with *Xist* RNA in late blastocysts in the TE, where imprinted X inactivation has occurred (Mak et al., 2004). In the ICM where there is random X inactivation, Eed and Ezh2 are present but a homogeneous staining is observed in the nucleus. The PRC2 complex might be a good candidate for establishing lineage-specific imprinting; indeed, imprinting of some

genes in the *Kcnq1* cluster is partially lost in extra-embryonic tissues in the *Eed* mutant (Mager et al., 2003). However Eed, Ezh2 and Suz12 are associated with the repressed paternal allele at many regions along the locus in ES cells (Umlauf et al., 2004). Given the similarity in expression and allele specific histone modifications between TS and ES cells, we would expect that distribution of these PRC2 proteins would be similar in TS cells and in the blastocyst. Therefore any lineage specific differences would occur after implantation.

Although the distance between the *Kcnq1ot1* transcription unit and the placentally imprinted genes may partly explain the relatively slow kinetics of their inactivation, these genes are still relatively close to *Kcnq1ot1* when compared with the distance between *Xist* and distal genes on the X chromosome. There are several possible explanations for the difference in timing between *Xist* and *Kcnq1ot1* mediated silencing. *Kcnq1ot1* may be expressed at a lower level than *Xist* during preimplantation development causing a slower accumulation of RNA and a delay in coating. Alternatively, sequence features that promote spreading of *Xist* and of repressive chromatin may occur at a higher frequency on the X chromosome. It is known that Line 1 elements are enriched twofold on the X chromosome compared with autosomes (Bailey et al., 2000), and there may be other features that affect the time required to establish silencing. The *Kcnq1ot1* transcript is longer than the *Xist* transcript and, unlike *Xist*, there are no known introns (L.R. et al., unpublished). These differences must affect the secondary structure and possibly the stability of the RNAs.

Data shown here and in other recent publications demonstrate that X-inactivation and autosomal imprinting do indeed have mechanistic similarities. This strengthens the hypothesis that these two processes may have evolved together (Lee, 2003; Reik and Lewis, 2005).

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/133/21/4203/DC1

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