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An in vitro ES cell imprinting model shows that imprinted expression of the *Igf2r* gene arises from an allele-specific expression bias

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Genomic imprinting is an epigenetic process that results in parental-specific gene expression. Advances in understanding the mechanism that regulates imprinted gene expression in mammals have largely depended on generating targeted manipulations in embryonic stem (ES) cells that are analysed in vivo in mice. However, genomic imprinting consists of distinct developmental steps, some of which occur in post-implantation embryos, indicating that they could be studied in vitro in ES cells. The mouse *Igf2r* gene shows imprinted expression only in post-implantation stages, when repression of the paternal allele has been shown to require cis-expression of the *Airn* non-coding (nc) RNA and to correlate with gain of DNA methylation and repressive histone modifications. Here we follow the gain of imprinted expression of *Igf2r* during in vitro ES cell differentiation and show that it coincides with the onset of paternal-specific expression of the *Airn* ncRNA. Notably, although *Airn* ncRNA expression leads, as predicted, to gain of repressive epigenetic marks on the paternal *Igf2r* promoter, we unexpectedly find that the paternal *Igf2r* promoter is expressed at similar low levels throughout ES cell differentiation. Our results further show that the maternal and paternal *Igf2r* promoters are expressed equally in undifferentiated ES cells, but during differentiation expression of the maternal *Igf2r* promoter increases up to 10-fold, while expression from the paternal *Igf2r* promoter remains constant. This indicates, contrary to expectation, that the *Airn* ncRNA induces imprinted *Igf2r* expression not by silencing the paternal *Igf2r* promoter, but by generating an expression bias between the two parental alleles.

KEY WORDS: Embryonic stem (ES) cells, Epigenetics, Genomic imprinting, ES in vitro imprinting model, ncRNA, Histone modifications, DNA methylation, Allele-specific expression, *Airn* (*Air*)

INTRODUCTION

Genomic imprinting is a cis-acting epigenetic mechanism resulting in the parental-specific expression of ~100 mammalian genes (Solter, 2006). Imprinted genes mostly occur in clusters, which are regulated by a cis-acting imprint control element (ICE) that is inactivated by a germline DNA methylation imprint on one parental chromosome (Spahn and Barlow, 2003; Thorvaldsen and Bartolomei, 2007). The unmethylated ICE is active and, in the *Igf2* imprinted cluster, binds CTCF to form an insulator that blocks maternal expression (Bell and Felsenfeld, 2000; Hark et al., 2000). In the *Igf2r* and *Kcnq1* imprinted clusters, the unmethylated ICE contains an active non-coding (nc) RNA promoter that silences multiple genes on the paternal chromosome (Mancini-Dinardo et al., 2006; Sleutels et al., 2002). Thus, as previously noted, genomic imprinting often constitutes the control of cis-regulatory elements by DNA methylation (Mann et al., 2000). Extensive progress has been made in the last decade towards understanding the mechanism, and today genomic imprinting provides one of the best models of mammalian epigenetic gene regulation.

The *Igf2r* imprinted cluster contains three maternally expressed mRNA genes (*Igf2r*, *Slc22a2* and *Slc22a3*) that are silenced on the paternal allele by expression of the *Airn* ncRNA (Sleutels et al., 2002) (formerly named *Air*, now renamed *Airn* by the HUGO Nomenclature Committee) (Fig. 1A). The *Airn* promoter lies in an antisense orientation in *Igf2r* intron 2. The resultant 108 kb *Airn* transcript, which is nuclear localised and largely unspliced, overlaps the 5′ part of *Igf2r* but lies more than 200 kb upstream of *Slc22a2* and *Slc22a3* (Seidl et al., 2006). The maternal *Airn* promoter, which lies in a 3.65 kb *Bam*HI-*Pac*I fragment genetically defined as the ICE, is ubiquitously repressed by a DNA methylation imprint acquired in oocytes (Stoger et al., 1993; Zwart et al., 2001). *Airn*-mediated silencing of *Igf2r* is seen throughout the post-implantation embryo and adult, with the exception of post-mitotic neurons (Yamasaki et al., 2005), but its silencing effects on *Slc22a2* and *Slc22a3* appear to be restricted to the trophoblast placenta (Zwart et al., 2001). Paternal-specific silencing of *Igf2r*, but not of *Slc22a2* and *Slc22a3*, is accompanied by gain of promoter DNA methylation, but, surprisingly, this methylation mark is not necessary for *Igf2r* silencing (Li et al., 1993; Seidl et al., 2006).

Genomic imprinting consists of distinct developmental stages: imprint acquisition in gametes, onset of imprinted expression in early embryos, maintenance of imprinted expression in differentiated cells and, finally, imprint erasure in germ cells of early embryos (Barlow and Bartolomei, 2007). Most studies investigating these processes have involved targeted manipulations in an in vivo mouse model – a long-term and laborious procedure. However, some stages in genomic imprinting are potentially amenable to in vitro analysis. Undifferentiated embryonic stem (ES) cells are a cell culture derivative of the pluripotent blastocyst inner cell mass that can provide an in vitro model of early embryonic development (Evans, 2005). In vitro differentiation of female ES cells has been used to study X-chromosome inactivation in mammals (Heard et al., 2004; Wutz, 2007). Changes in *Xist* ncRNA expression, coating of the inactive X-chromosome by *Xist*, gain of histone modifications

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and DNA methylation, are all recapitulated in correct temporal order during ES cell differentiation. Similarities between X-inactivation and imprinted expression indicate that ES cells may also provide a model in which to investigate genomic imprinting (Reik and Lewis, 2005). ES cells might be particularly useful to analyze the onset of *Igf2r* imprinted expression because undifferentiated ES cells express *Igf2r* biallelically and lack *Airn* ncRNA expression (Braidotti et al., 2004; Wang et al., 1994). This mimics the in vivo situation, as pre-implantation embryos express *Igf2r* biallelically and lack *Airn* in the blastocyst inner cell mass, whereas post-implantation embryos gain imprinted *Igf2r* expression between 4.5 and 6.5 days post-coitum (dpc) (Lerchner and Barlow, 1997; Szabo and Mann, 1995; Terranova et al., 2008). Thus, ES cell in vitro differentiation could provide a reliable model in which to examine the developmental onset and maintenance of imprinted *Igf2r* expression.

Recent progress in the reprogramming of somatic cells to pluripotent embryonic-like cells has focussed interest on understanding epigenetic gene regulation in ES cells (Jaenisch and Young, 2008). Histone modifications and DNA methylation have been shown to undergo dynamic changes upon ES cell differentiation (Meshorer and Misteli, 2006). For example, ES cells are globally deficient in DNA methylation and appear insensitive to a lack of DNA methyltransferases, but show the reverse behaviour upon differentiation (Meshorer and Misteli, 2006). However, the role played by epigenetic modifications in regulating ES cell pluripotency is not fully understood because, in contrast to differentiated cells, they lack a clear correlation between expression status and the epigenetic modifications typically associated with expressed or silent genes. For example, when silent, key developmental genes are marked both by repressive H3K27me3 histone modifications and by active H3K4me3 modifications (so-called bivalent domains) (Azuara et al., 2006; Lee et al., 2007). It was also shown recently that in undifferentiated ES cells, peaks of H3K4me3 mark all CpG island promoters irrespective of expression state (Guenther et al., 2007). Genomic imprinting is one of the clearest examples of epigenetic gene regulation and could provide a platform to better understand the role played by epigenetic modifications during in vitro reprogramming and differentiation of ES cells.

Here, we follow the dynamics of *Igf2r* and *Airn* imprinted expression during mouse ES cell differentiation. Because several reports have shown that imprinted expression can be disturbed in inter- and intraspecies crosses, possibly owing to cis-regulatory polymorphisms (Jiang et al., 1998; Shi et al., 2005), we used inbred ES cells with a polymorphism introduced into our gene of interest. We show that *Igf2r* is biallelically expressed in undifferentiated ES cells and that activation of *Airn* ncRNA expression in differentiating ES cells coincides with gain of imprinted *Igf2r* expression. This is accompanied by characteristic epigenetic changes, including gain of DNA methylation and H3K9me3. Unexpectedly, even though the paternal *Igf2r* promoter gains repressive epigenetic modifications it continues to be expressed at similar levels throughout ES cell differentiation, while expression of the maternal *Igf2r* promoter increases up to 10-fold. Thus, contrary to expectation, we show that the *Airn* ncRNA induces imprinted *Igf2r* expression not by silencing the paternal *Igf2r* promoter, but by creating an expression bias between the two parental alleles.

MATERIALS AND METHODS

ES cell culture and differentiation

ES cells were grown under standard conditions. CCE cells are feeder-independent; D3 cells were grown on irradiated 12.5 dpc mouse embryonic fibroblasts. Differentiation was induced by LIF withdrawal, feeder-cell depletion and 0.27 μM retinoic acid. Embryoid body differentiation was induced by hanging-drop culture for 5 days, followed by 9 days on gelatinised dishes.

Plasmid construction

The S12 targeting vector was constructed by ligation of a 5 kb *Bst*XI-*Eco*47III fragment (Mm.Build-37/Chr.17:12909688-12914937) into the *Eco*RV site, and diphtheria toxin fragment A (DTA) ligated into the *Sma*I site, of pBluescript KS. The C-to-T change that mutates the exon 12 *Pst*I site (bp:12912731) was generated with the QuikChange XL Site-Directed Mutagenesis Kit (Stratagene). The selection cassette Tk-Neo-loxP511 was introduced into the *Bsr*GI site in intron 11 (bp:12913929).

Allele-specific quantitative (Q) PCR

DNase I-treated RNA was assayed in duplicate or triplicate and normalised to cyclophilin A (peptidylprolyl isomerase A). Forward primers: MUTSEF (5′-CTGGCCTTCCCCTCCTGT-3′) detects the mutated allele, whereas WTSEF (5′-TGGCCTTCCCCTCCTGC-3′) detects the wild-type allele. Common reverse primer: GESER2 (5′-GCTATGACCTGTCTGTGTTG-GCT-3′). QPCR was performed using the Power SYBR Green PCR Mastermix (Applied Biosystems), with 9 mM $MgCl_2$ and 100 nM primers. Cycling: 2 minutes at 50°C, 10 minutes at 95°C, then 40 cycles of 15 seconds at 95°C and 1 minute at 64°C.

Southern blotting

Standard conditions were used. Probes: EEi:AJ249895:94104-99081, NEi:AJ249895:98070-99081, BEi:AJ249895:97090-99081, MEi:AJ249895:126086-127237, X12:Mm.Build-37/Chr.17:12916453-12917573, MSi:AJ249895:126087-127237, Oct4a:Mm.Build-37/Chr.17:35,643,244-35,644,955.

RNase protection and northern blots

Standard conditions were used. RPAIII Kit (Ambion) probes: AirF3b/AJ249895:100185-100446, Igf2rex34/NM_010515:4869-5002, Cycl/:Ambion7675. Northern probes: HX/NM_010515:1001-8877, Airp105/AJ249895:115522-116522, Oct4:(X52437:21-354).

Real-time QPCR

Real-time QPCR was performed as described (Seidl et al., 2006) (details available upon request) using the following primers and probes [5′ to 3′; F, forward primer; TM, Taqman probe (Applied Biosystems); R, reverse primer]:

Airn QPCR: F-GACCAGTTCCGCCCGTTT, TM-TACAAGTGAT-TATTAACTCCACGCCAGCCTCA, R-GCAAGACCACAAAATATTG-AAAAGAC;

Igf2rex48 QPCR: F-TCCTACAAGTACTCAAAGGTCAGCAA, TM-CCAAGACTAGGCAAGGACGGGCAAGA, R-GCGGTTGGTGGT-GATATGG;

Igf2rex4/5 QPCR: F-GACTACTGCAGCCTGCAAGAAA, TM-ACATATTTAAAGCTGATAAGGAGGTACCATGCTATGCA, R-AAT-CATGCTTCTGTAACTTGTCATCAA;

Cyclophilin A QPCR: F-AGGGTTCCTCCTTTCACAGAATT, TM-TCGTGGATCTGACGTGCCGCC, R-GTGCCATTATGGCGTGTAA-AGT;

Oct4 QPCR: F-ACCTTCAGGAGATATGCAAATCG, TM-AGACC-CTGGTGCAGGCCCGG, R-TTCTCAATGCTAGTTCGTTCGCTTT;

Fgf5 QPCR: F-AACTCCATGCAAGTGCCAAAT, TM-TACGGAT-GACTGTAAGTTCAGGGAGAGATTCCA, R-GGACGCATAGGTAT-TATAGCTGTTTTC;

Gata4 QPCR: F-CGCTGTGGCGTCGTAATG, TM-AGCCTGTATG-TAATGCCTGCGGCCTC, R-GGAACCCCATGGAGCTTCAT;

Slc22a2 QPCR: F-GGAAATCGGTGCCAGTCTC, TM-CTTCA-GAGCCTGACGGCAGATGAGGA, R-AAGGGTTCAATTTCATGCC-AGT; and

Slc22a3 QPCR: F-GAAATGCACGCTCATCCTTATG, TM-TTGCTTGGTTCACGAGCGCCGT, R-CAGGCGCATGACAAGTC-CTT.

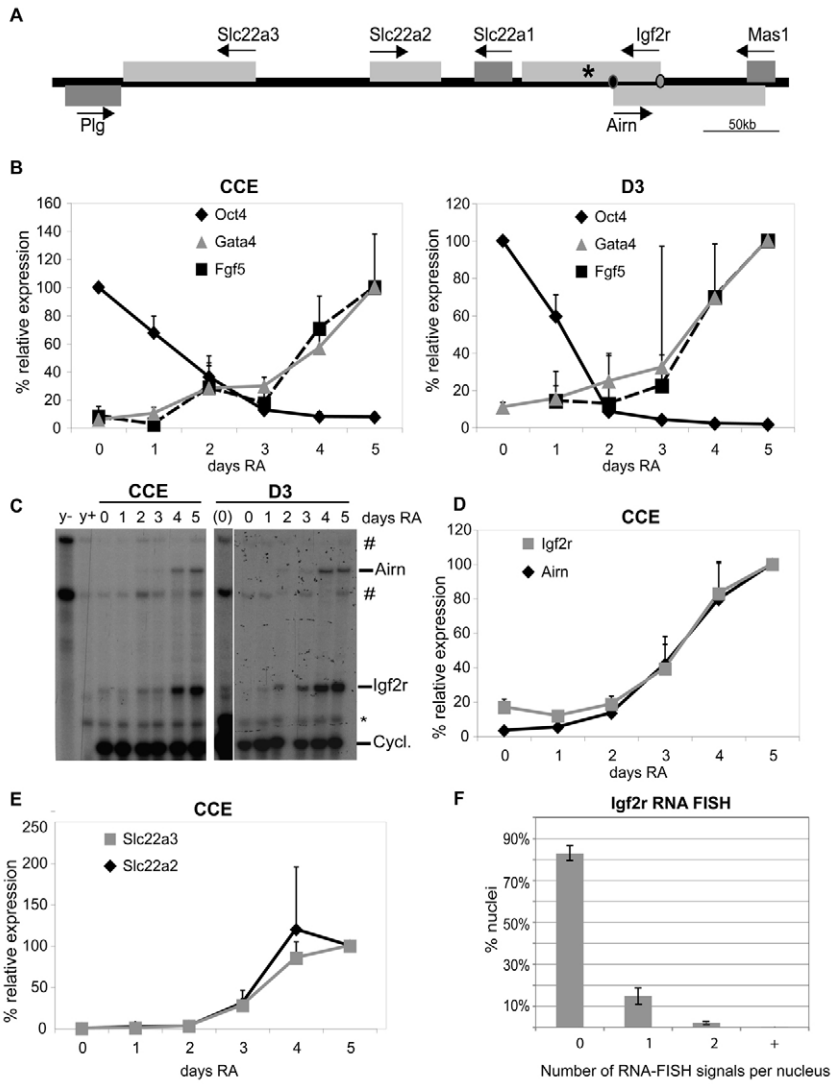

Fig. 1. Imprinted gene expression in differentiating ES cells. (**A**) The imprinted *Igf2r* cluster on mouse chromosome 17 spans 490 kb from *Plg* to *Mas1* and contains four imprinted (light grey boxes) and three non-imprinted (dark grey boxes) genes. Arrows indicate transcription orientation. Note the antisense *Airn* promoter in *Igf2r* intron 2 (black oval) and the 108 kb *Airn* ncRNA that overlaps the *Igf2r* promoter (grey oval) and the 3′ part of *Mas1*. The asterisk marks the exon 12 single nucleotide polymorphism (SNP), as described in Fig. 2. (**B**) Expression of *Oct4* (pluripotent cell marker; diamond), *Gata4* (endoderm marker; triangle) and *Fgf5* (embryonic ectoderm marker; square) as assessed by QPCR during retinoic acic (RA)-induced differentiation of CCE (left) and D3 (right) ES cells. The mean and s.d. of three independent replicates are shown. The day-5 value was set to 100, except for *Oct4* where day 0 was set to 100. (**C**) RNase protection assay (RPA) showing parallel upregulation of *Igf2r* (probe Igf2rex34 protects 133 bp) and *Airn* (probe AirF3b protects 261 bp) expression during RA-induced differentiation of CCE (left) and D3 (right) ES cells. Cycl., cyclophilin A loading control (protects 105 bp); #, undigested probe; *, non-specific band. 0, undifferentiated (day 0) ES cells; (0), longer exposure of the day-0 track; 1-5, days RA treatment; Y–, minus ribonuclease; Y+, plus ribonuclease. (**D**) Expression kinetics, as determined by QPCR, of *Igf2r* (square) and *Airn* (diamond) during RA-induced differentiation of CCE ES cells. (**E**) Expression kinetics, as determined by QPCR, of *Slc22a2* (diamond) and *Slc22a3* (square) during RA-induced differentiation of CCE ES cells. (**F**) Intronic RNA FISH for *Igf2r* detects nascent transcription in 17% of day-5 differentiated CCE cells, of which 88% show a single-spot signal indicating imprinted expression.

Native chromatin immunoprecipitation (ChIP)

ChIP was performed as described (Regha et al., 2007) using the antibodies and primers listed therein.

RNA fluorescence in situ hybridisation (FISH)

RNA FISH was performed as described (PROTO6, http://www.epigenome-noe.net). The single-stranded RNA FISH probe (2658 bp PCR product generated using AIFP1F 5′-GCTGGTCCTTACCTTGTGGA-3′ and AIFP1R 5′-GCAAGACCACATCACACACC-3′ from *Igf2r* intron 1) was transcribed with T7 RNA polymerase and digoxygenin labelled by reverse transcription. The RNA FISH signal was detected by sheep anti-digoxygenin antibody (Roche) and amplified by rabbit anti-sheep-FITC + goat anti-rabbit-FITC (Calbiochem). Fluorescent spots were independently counted twice.

RESULTS

Characterisation of an in vitro ES cell imprinting system

To study imprinted expression at the *Igf2r* cluster we used two inbred 129/Sv ES cell lines: the feeder-independent but germline-incompetent CCE line (Keller et al., 1993) and the feeder-dependent and germline-competent D3 line (Doetschman et al., 1985). ES cells were differentiated by exposure to retinoic acid (RA) for 5 days or by embryoid body (EB) formation for 14 days. Three marker genes were assayed by QPCR to follow differentiation: *Oct4* (*Pou5f1*) (an undifferentiated stem cell marker), *Gata4* (an endoderm marker) and *Fgf5* (a gastrulation marker). Fig. 1B shows RA-treated CCE and D3 cells; for the same analysis after EB formation, see Fig. S1A in the supplementary material. Both CCE and D3 cells showed a sharp decline in *Oct4* after 2 days of RA differentiation; the decline was more marked in D3. The *Oct4* decline was slower during EB formation than RA treatment and remained at 10-35% of the level found in undifferentiated cells after 14 days differentiation, indicating the continued presence of undifferentiated cells (see Fig. S1A in the supplementary material). After RA treatment or EB formation, expression of *Gata4* and *Fgf5* increased slowly up to 3 days and then sharply up to 5 days with RA differentiation, but *Fgf5* then decreased in EB populations (see Fig. S1A in the supplementary material). These expression patterns show that RA produces more-homogenously differentiated cells than does EB formation and identifies 2-3 days of RA treatment as the window when differentiation markers are induced.

The onset of *Airn* expression coincides with upregulation of *Igf2r* expression

We next determined the expression of genes from the imprinted *Igf2r* cluster. Based on previous data showing that *Igf2r* is biallelically expressed in undifferentiated ES cells and that *Airn* ncRNA

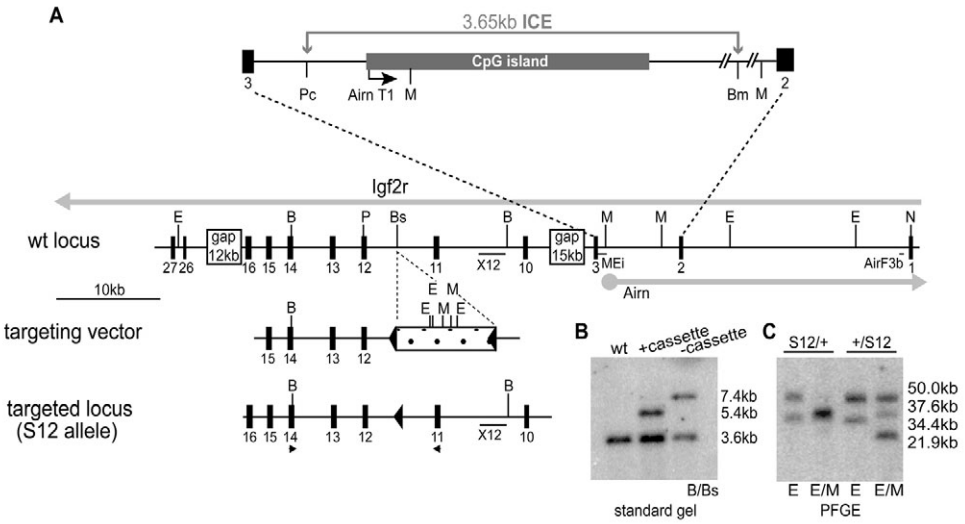

Fig. 2. Generation of the S12 allele carrying a SNP in exon 12 of *Igf2r*. (**A**) Mouse wild-type (wt) *Igf2r* locus, showing exons 1-27 (black boxes) and (above) an enlargement of intron 2 that contains the genetically defined 3.65 kb ICE and the CpG island lying immediately downstream of the *Airn* transcription start (arrow). Below is shown the targeting vector and targeted locus. The targeting vector contains the introduced SNP in exon 12 (that mutates a *Pst*I site) and a 3.8 kb *Tk-Neo* selection cassette (stippled box) flanked by loxP511 sites (triangles) inserted into the *Bsr*GI site in *Igf2r* intron 11. Arrowheads indicate PCR primers used for cDNA analysis. MEi/X12, Southern blot probes; B, *Bgl*II; Bm, *Bam*HI; Bs, *Bsr*GI; E, *Eco*RV; M, *Mlu*I; Pc, *Pac*I; P, *Pst*I; AirF3b, RPA probe as used in Fig. 1C. (**B**) Southern blot showing homologous recombination and removal of the selection cassette after Cre recombination (*Bgl*II+*Bsr*GI digest plus probe X12). (**C**) Parental origin of targeted allele analysed by pulsed-field gel electrophoresis (PFGE). ES cell DNA containing the selection cassette was digested with *Eco*RV or *Eco*RV+*Mlu*I and hybridised with probe MEi. In this assay, *Eco*RV generates a 50 kb wild-type and a 34.4 kb targeted allele (the selection cassette carries additional *Eco*RV sites), and a diagnostic 21.9 kb band is generated from a paternally targeted allele but not from a maternally targeted allele after digestion with the methyl-sensitive *Mlu*I enzyme. The blot shows that a *Eco*RV+*Mlu*I (E/M) digest of maternally targeted (S12/+) cells generates a 34.4 kb maternal targeted band (*Mlu*I does not cut the maternally methylated ICE) and a 37 kb wild-type paternal band (*Mlu*I cuts the unmethylated paternal ICE); however, these bands are not separated on the gel shown. An *Eco*RV+*Mlu*I digest of paternally targeted (+/S12) cells generates a 50 kb wild-type maternal band (*Mlu*I does not cut) and a 21.9 kb targeted paternal band (*Mlu*I cuts); the 37 kb band results from feeder contamination. Note that the *Mlu*I site outside the ICE is methylated on both parental alleles and does not participate in the assay.

expression is restricted to differentiated ES cells (Braidotti et al., 2004; Wang et al., 1994), we anticipated that *Igf2r* expression would decrease during ES cell differentiation, as *Airn* expression silences the paternal *Igf2r* promoter. Since QPCR only measures relative differences in gene expression, we first used RNase protection to assess non-amplified RNA levels. Fig. 1C shows that low-level *Igf2r* expression is detected in undifferentiated CCE and D3 ES cells (lane 0). However, instead of the anticipated decrease, we found that *Igf2r* was strongly upregulated during differentiation, with the most prominent increase between days 3 and 4 of RA treatment (Fig. 1C). The *Airn* ncRNA behaved as anticipated and was absent from undifferentiated cells (lane 0 is overexposed to show the absence of *Airn*), then sharply increased between days 3 and 4 of RA treatment. Notably, the levels of *Igf2r* and *Airn* increase during the same time window as when the differentiation markers change (Fig. 1B), and both also show a sharp increase in expression during days 3-4. The amount of stable *Igf2r* mRNA is greater than that of *Airn* ncRNA, in agreement with their differential stability (Seidl et al., 2006).

We then used QPCR to quantify expression of *Igf2r*, *Airn*, *Slc22a2* and *Slc22a3*. Fig. 1D and Fig. S1B in the supplementary material show that in CCE and D3 cells, *Igf2r* and *Airn* expression increases during ES differentiation with parallel kinetics: a sharp increase is first seen between days 2 and 3 for both genes. Relative to the day-0 undifferentiated state, *Igf2r* increased on average ~5-fold (from ~20 to 100) and *Airn* increased ~100-fold (from background to 100) by day 5 of RA treatment (Fig. 1D and see Fig. S1B, left panel, in the supplementary material) or by day 8 of EB differentiation (see Fig. S1C in the supplementary material). Parallel upregulation of a ncRNA with one mRNA is also seen in the *Kcnq1* and *Igf2* imprinted clusters during ES cell differentiation (see Fig. S2 in the supplementary material). Expression of *Slc22a2* and *Slc22a3* was undetectable by QPCR until days 2-3 of differentiation and then increased with similar kinetics to *Igf2r* and *Airn* (Fig. 1E and see Fig. S1B,D in the supplementary material). Although expression of both *Slc22a2* and *Slc22a3* is restricted to the trophoblast placenta (Zwart et al., 2001) and thus would be predicted to be absent from differentiated ES cells, the QPCR threshold cycle (Ct value) indicates that *Slc22a2* is expressed at low-to-background levels (Ct 34), whereas *Slc22a3* is expressed at clearly detectable levels in differentiated ES cells (Ct 27) (see Fig. S1E in the supplementary material).

Imprinted *Igf2r* expression in differentiated cells could result from transcriptional or post-transcriptional events. RNA FISH using intronic probes detects nascent transcription; however, probes that contain exons will also detect accumulated transcripts. In day-5 RA-treated ES cells, an intronic RNA FISH signal was detected in 17% of cells, and the majority (88%) of positive cells showed single-spot signals (Fig. 1F). Thus, imprinted expression of *Igf2r* arises from transcriptional differences between the two parental alleles.

Allele-specific assay of *Igf2r* expression during ES cell differentiation

Since *Igf2r* unexpectedly showed increased expression during ES cell differentiation, we used homologous recombination to generate an inbred ES cell line carrying a single nucleotide

polymorphism (SNP) to analyze allele-specific *Igf2r* expression (Fig. 2). The SNP in *Igf2r* exon 12 mutated a *Pst*I site but maintained the reading frame. To identify the parental origin of the targeted allele, we made use of the maternal-specific DNA methylation imprint on the ICE that results in a diagnostic 21.9 kb band from a paternally targeted allele but not from a maternally targeted allele, after digestion with *Eco*RV and the methyl-sensitive *Mlu*I. One maternally targeted cell line (S12/+) and one paternally targeted cell line (+/S12) are shown in Fig. 2C (note that the maternal allele is written on the left side throughout the text). We then used an allele-specific QPCR assay that distinguishes the exon 12 SNP to analyze a maternally targeted and a paternally targeted ES line for *Igf2r* expression. Fig. 3A shows the specificity of the allele-specific assay using plasmids containing cDNA with (S12) or without (WT) the SNP, indicating that the compatible QPCR assay reaches the detection threshold at least seven Ct cycles earlier than the incompatible assay. As shown in Fig. 3B, in both +/S12 and S12/+ cells, the ratio of maternal to paternal *Igf2r* expression increased from 1:1 to between 4:1 and 10:1 in differentiated cells. The relative increase in maternal *Igf2r* expression during ES differentiation varied between different experiments, but always coincided with the onset of *Airn* upregulation as shown in Fig. 1D.

Persistent paternal-specific *Igf2r* expression in differentiated ES cells

In allele-specific QPCR assays, the maternal to paternal *Igf2r* ratio did not exceed 10:1 in differentiated cells, even though the calculated specificity for the QPCR SNP assay was at least 50:1. To examine whether this might indicate incomplete silencing of the paternal *Igf2r* promoter in differentiated cells, we used D3 ES cells with a *lacZ*-polyA termination signal targeted into exon 1 that truncates the *Igf2r* mRNA (Wang et al., 1994). For northern blot analysis, we first used an *Igf2r*-specific probe downstream to exon 1 that only detects the wild-type parental allele (Fig. 3C). The +/*lacZ* cells showed strong upregulation of the wild-type maternal *Igf2r* allele during differentiation. However, the *lacZ*/+ cells also showed that wild-type paternal *Igf2r* expression is maintained at the same level throughout differentiation, and even increases slightly at day 5. Although this could, unexpectedly, indicate that the paternal allele is not silenced during differentiation, we noted that control double-knockout cells (*lacZ*/*lacZ*), which should lack all *Igf2r* expression, showed traces of *Igf2r*. This arises from contaminating wild-type feeder cells that persist at early time points despite feeder depletion. We therefore repeated the analysis using IPdel/Thp feeder cells, which completely lack *Igf2r* expression because the maternal allele carries an *Igf2r* promoter deletion and the paternal allele carries a 6 Mbp deletion of the whole imprinted cluster (Sleutels et al., 2003). Northern blots (Fig. 3D) showed that *Igf2r* is absent from *lacZ*/*lacZ* ES cells grown on IPdel/Thp feeder cells, and revealed approximately equal expression of the maternal and paternal *Igf2r* alleles in undifferentiated ES cells. *Igf2r* expression in these cells was quantified by QPCR, which showed that paternal *Igf2r* expression in *lacZ*/+ ES cells is indeed present throughout differentiation and at approximately constant levels until day 4, with a 2-fold increase at day 5 of RA treatment (Fig. 3E). By contrast, the maternal *Igf2r* allele (+/*lacZ*) was upregulated beginning at day 2 of RA treatment and increased sharply between days 4 and 5. Together, these results unexpectedly indicate that imprinted *Igf2r* expression occurs not by silencing the paternal allele, but by creating an expression bias between the two parental alleles.

Airn expression correlates with de novo DNA methylation of the paternal *Igf2r* promoter

The above result shows that the paternal *Igf2r* allele maintains the same expression level during ES cell differentiation. Since the paternal *Igf2r* promoter in 13.5 dpc embryos displays partial DNA methylation (Stoger et al., 1993), we tested whether methylation is also acquired in differentiated ES cells. Fig. 4 shows that in undifferentiated ES cells, the *Igf2r* promoter is unmethylated on both parental alleles, in agreement with the above data showing bi-parental *Igf2r* expression (Fig. 4A). In differentiated cells, a faint band indicative of a methylated *Igf2r* promoter was observed after day 3 of RA treatment, the period when *Igf2r* expression starts to be upregulated and *Airn* is induced. This methylated *Igf2r* band remained faint in RA-treated cells, but at day 14 of EB differentiation the intensity of the methylated and unmethylated *Igf2r* bands was similar (quantified in Fig. 4B), indicating complete methylation of the paternal allele (see the NIH3T3 lane, which shows the equal methylated and unmethylated signals). The *Airn* promoter is continuously modified by a maternal-specific DNA methylation imprint from the oocyte stage onwards, which silences the *Airn* ncRNA (Seidl et al., 2006). We confirmed that this methylation imprint is present and stably maintained during ES cell differentiation (Fig. 4A). As the *Oct4* gene has been shown to gain DNA methylation during ES cell differentiation (Hattori et al., 2004), we tested whether the kinetics of methylation gain were similar to those observed for the paternal *Igf2r* promoter. Fig. 4C (top panel) shows that differentiating CCE ES cells gained low-level *Oct4* methylation by day 2 and that this increased markedly during days 3 to 5, attaining similar levels to those seen in tail DNA. *Igf2r* methylation has similar kinetics, with a faint methylated band visible by day 3 (dashed line). However, in contrast to *Oct4*, *Igf2r* methylation was still partial by day 5 by comparison with tail DNA, which contains a fully methylated paternal allele.

Changes in histone modifications accompany the onset of *Igf2r* imprinted expression

We have previously shown in 13.5 dpc mouse embryonic fibroblasts (MEFs) (which show a maternal to paternal *Igf2r* expression ratio of 80:1, see Fig. S3 in the supplementary material) that the silent paternal *Igf2r* promoter and the silent maternal *Airn* promoter are each modified by a repressive 'heterochromatin' peak composed of H3K9me3/H4K20me3/HP1β, but are devoid of repressive H3K27me3 marks (Regha et al., 2007). In MEFs, the expressed maternal *Igf2r* and paternal *Airn* promoters lack repressive marks and are modified only by active histone marks (H3K4me2/3 and H3K9Ac). We therefore used chromatin immunoprecipitation (ChIP) of two active (H3K4me2, H3K9Ac) and two repressive (H3K27me3, H3K9me3) histone marks to test whether these modifications arise during ES cell differentiation. Fig. 5B depicts the expression of *Igf2r* and *Airn* in undifferentiated and differentiated ES cells, as described above. Fig. 5C-H shows ChIP analysis of the *Igf2r* promoter region using 12 primer pairs (the arrow indicates the transcription start site and direction). In undifferentiated ES cells (left panel), the *Igf2r* promoter is enriched for H3K27me3 on both sides of the transcription start site (Fig. 5D); however, this modification is lost in differentiated ES cells (right panel). H3K9Ac shows the inverse pattern, with enrichment in differentiated ES cells, mainly downstream of the transcription start (Fig. 5E). H3K4me2 is present downstream of the *Igf2r* transcription start in undifferentiated ES cells, but moves further

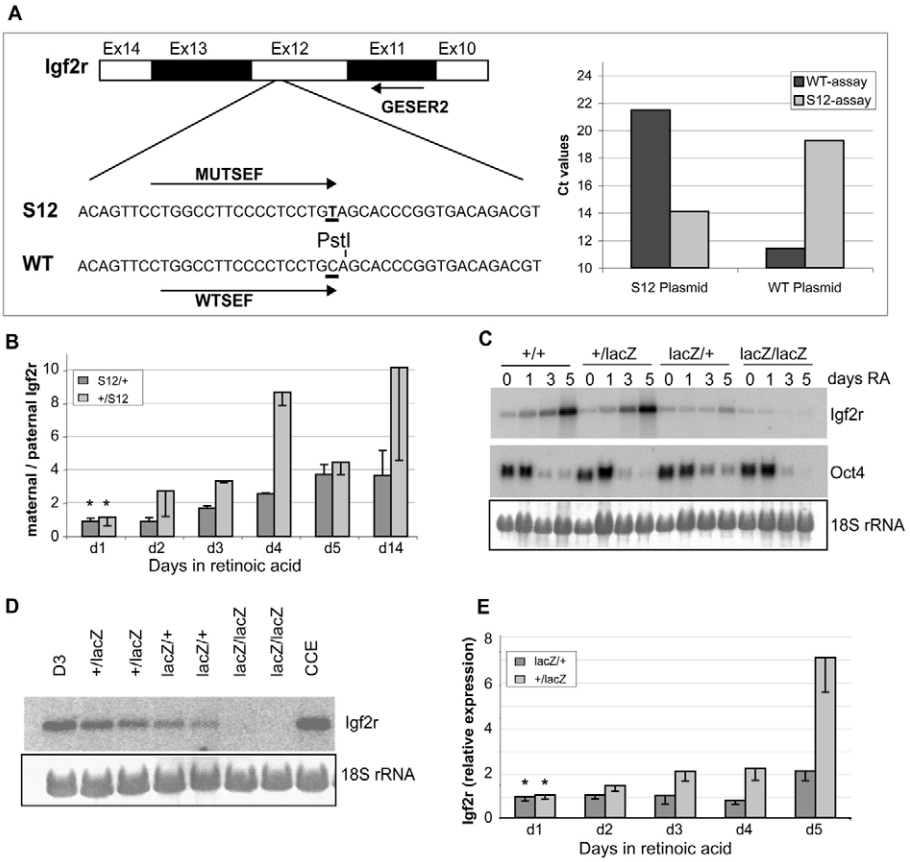

Fig. 3. Imprinted expression of *Igf2r* arises from a parental-specific expression bias. (**A**) Allele-specific QPCR distinguishes the mouse *Igf2r* wild-type (WT) and exon 12 SNP (S12) alleles. In the S12 allele, a C included in a *Pst*I restriction site in *Igf2r* exon 12 is replaced by a T. Primer MUTSEF (ending on T, S12-assay) or WTSEF (ending on C, WT-assay) in combination with a common reverse primer (GESER2) in exon 11 distinguish the alleles. QPCR with the common reverse primer and MUTSEF or WTSEF on equal amounts of plasmid containing *Igf2r* cDNA with (S12) or without (WT) the SNP shows that the compatible QPCR assay reaches the detection threshold at least seven Ct cycles earlier than the incompatible assay (compare black bar with grey bar for each plasmid). This indicates a high specificity of the assay (~50:1). (**B**) Allele-specific QPCR showing the ratio of maternal to paternal *Igf2r* expression during RA-induced differentiation of maternally targeted (S12/+, dark grey bars) and paternally targeted (+/S12, light grey bars) ES cells. Mean values and s.d. of three replicates are shown. Since *Igf2r* is biallelically expressed in undifferentiated ES cells, the middle value of the three replicates on day (d) 1 is set to 1 (*). The maternal to paternal *Igf2r* expression ratio increases during differentiation in both S12/+ and +/S12 ES cells, showing that expression of the maternal allele is greater than that of the paternal allele. (**C**) *Igf2r* expression in feeder-depleted differentiating D3 ES cells with disruption of full-length *Igf2r* by *lacZ*-polyA insertion into exon 1 on the maternal (*lacZ*/+) or paternal (+/*lacZ*) or on both (*lacZ*/*lacZ*) alleles, analysed by northern blot using a downstream *Igf2r* probe (HX). *Igf2r* expression is upregulated from the wild-type maternal allele (+/*lacZ*) and is expressed at similar levels throughout differentiation from the wild-type paternal allele (*lacZ*/+). *Igf2r* expression in *lacZ*/*lacZ* ES cells indicates expression from irradiated feeder MEFs that contaminate earlier time points, despite feeder depletion. *Oct4*, control for ES cell differentiation; 18S rRNA, loading control. (**D**) Northern blot confirming biallelic *Igf2r* expression in undifferentiated +/*lacZ* and *lacZ*/+ D3 ES cells and the absence of *Igf2r* expression in *lacZ*/*lacZ* D3 ES cells (details as C). Wild-type D3 and CCE undifferentiated ES cells are shown for comparison. All ES cells were grown on mutant MEF feeders that completely lack *Igf2r* as they have a targeted deletion of the maternal *Igf2r* promoter and a Thp (hairpin-tail) deletion on the paternal chromosome that includes the whole *Igf2r* imprinted cluster. (**E**) QPCR analysis (assay Igf2rex48) of maternal and paternal wild-type *Igf2r* alleles in +/*lacZ* (light grey bars) and *lacZ*/+ (dark grey bars) differentiating ES cells grown as in D on mutant feeders. Bars indicate mean values with s.d. of three replicates. The middle value of the three replicates on day 1 was set to 1 (*). Expression of the wild-type maternal *Igf2r* allele increases during ES cell differentiation from day 2 of RA treatment onward, whereas expression of the wild-type paternal allele is constant during early time points (days 1-4) and increases slightly by day 5.

upstream in differentiated cells (Fig. 5G). H3K9me3 is mostly absent from *Igf2r* in undifferentiated ES cells, but is enriched in differentiated ES cells, mainly downstream of the transcription start site (Fig. 5H).

Fig. 5I-N shows the same modifications analysed over the *Airn* promoter region. Although *Airn* is only expressed in differentiated ES cells (Fig. 1C), both H3K9Ac and H3K4me2 show the same dynamic changes as seen at the *Igf2r* promoter. H3K9me3 differs, as it is already present on the *Airn* promoter region in undifferentiated ES cells and the pattern is largely unchanged in differentiated ES cells. Similarly, H3K27me3 is lost from the *Airn* promoter region in differentiated ES cells.

We then examined the same samples by QPCR. Fig. 5O demonstrates that H3K27me3 is present only in undifferentiated ES cells that lack imprinted *Igf2r*/*Airn* expression and is absent (i.e. signals are similar to mock ChIP levels, arrows) in differentiated ES

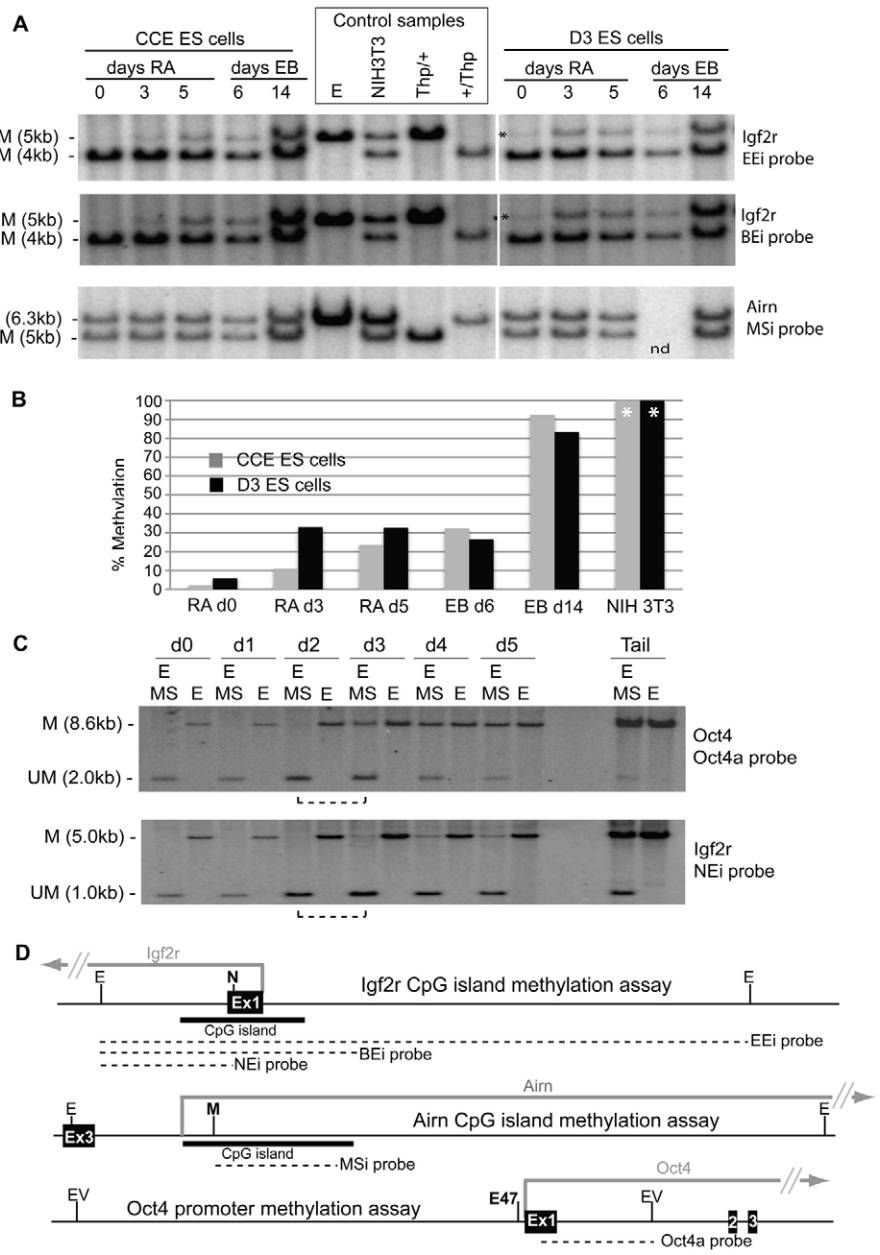

Fig. 4. Gain of de novo methylation of the paternal *Igf2r* promoter. (**A**) DNA methylation of mouse *Igf2r* and *Airn* promoters during RA or embryoid body (EB) differentiation of CCE and D3 ES cells. Upper and middle panels show gain of methylation on the *Igf2r* promoter on a *Not*I site close to the transcription start. The lower panel shows stable methylation of the *Airn* promoter on a *Mlu*I site in the same DNA samples. Thp/+ and +/Thp uniparental deletion cells show that the *Igf2r* promoter is paternally methylated (Thp/+), whereas the *Airn* promoter is maternally methylated (+/Thp) in tail DNA. NIH3T3 diploid cells show a 50:50 ratio of methylated:unmethylated signal. *Igf2r* promoter: *Eco*RI+*Not*I digest with probes EEi (upper panel) and BEi (middle panel), both of which are contained in the *Eco*RI fragment. *Airn* promoter: *Eco*RI+*Mlu*I digest with probe MSi. M, methylated allele; UM, unmethylated allele; E, *Eco*RI; *, feeder cell contamination; nd, not done. (**B**) ImageJ quantification of *Igf2r* promoter methylation during RA or EB differentiation of CCE and D3 cells (probe EEi). Bars indicate percentage methylation on the paternal *Igf2r* promoter as compared with the fully methylated paternal *Igf2r* promoter in NIH3T3 cells. Methylation on the paternal *Igf2r* promoter in NIH3T3 cells was set to 100 (*). (**C**) DNA methylation kinetics at the *Oct4* promoter (*Eco*RV + methyl-sensitive *Eco*47III) and the *Igf2r* promoter (*Eco*RI + methyl-sensitive *Not*I) during RA-induced differentiation of CCE cells assayed in the same DNA sample. Both promoters gain DNA methylation between days 2 and 3 of RA treatment (dashed line), but the *Oct4* promoter gains substantially more methylation than the *Igf2r* CpG island promoter. (**D**) Details of the Southern blot methylation analyses in A and C (to scale).

cells that have imprinted expression. Thus, differentiated ES cells lack a correlation between H3K27me3 and imprinted *Igf2r/Airn* expression, as previously shown for 13.5 dpc MEFs (Regha et al., 2007).

DISCUSSION

We show here that in vitro differentiating ES cells constitute a reliable model in which to study the onset of *Igf2r/Airn* imprinted expression. Although it is thought that ES cells are vulnerable to epigenetic instability (Pannetier and Feil, 2007), we show using two independent lines that ES cells not only maintain a gametic DNA methylation imprint, but also mimic the onset of imprinted *Igf2r* expression previously only observed in vivo. Differentiating ES cells are a model for: (1) the switch from biallelic to imprinted *Igf2r* expression that occurs between 4.5 and 6.5 dpc of development (Lerchner and Barlow, 1997; Szabo and Mann, 1995); (2) the activation of the *Airn* ncRNA, which is silent in the blastocyst inner cell mass and expressed in post-implantation embryos (Terranova et al., 2008; Zwart et al., 2001); (3) the gain of *Igf2r* promoter DNA methylation seen in post-implantation embryos (Stoger et al., 1993); and (4) the histone modification pattern present in 13.5 dpc MEF cells (Regha et al., 2007). Contrary to expectation, we find that the paternal *Igf2r* promoter is expressed at similar low levels in undifferentiated and differentiated ES cells, despite *Airn* ncRNA expression and gain of DNA methylation on the *Igf2r* promoter region. Together, these results demonstrate that *Airn* expression does not silence the paternal *Igf2r* promoter, but instead creates an expression bias between the two parental alleles.

Control of imprinted gene expression by DNA methylation

DNA methylation is a late event relative to imprinted expression: methylation is incomplete at the time when differentiated ES cells show maximum differences between maternal and paternal *Igf2r*

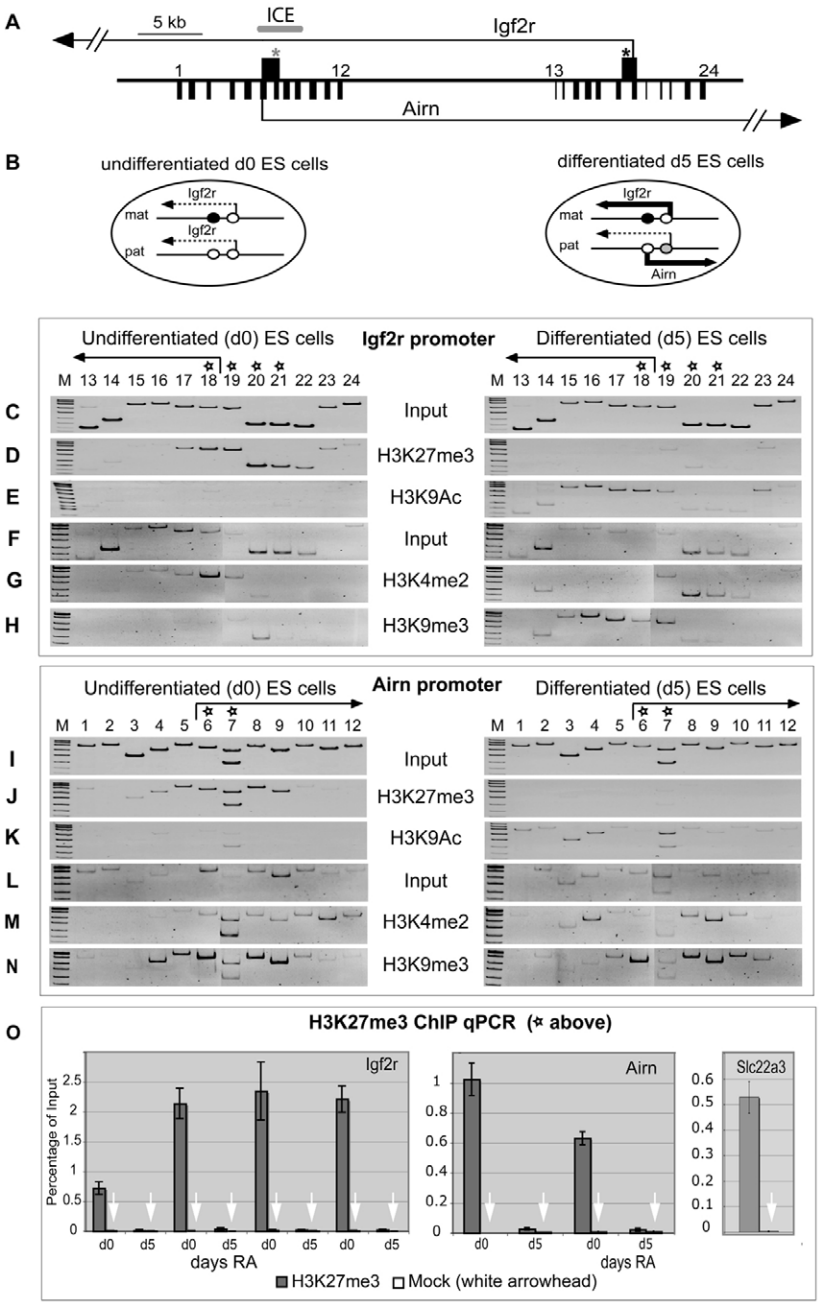

Fig. 5. Histone modification dynamics at *Igf2r* and *Airn* promoter regions upon ES cell differentiation. (**A**) Mouse *Igf2r* and *Airn* promoter regions. Arrows, direction of transcription; black boxes above line, CpG islands; black bars below line, regions analysed using 24 primer pairs spanning 12 kb around the *Airn* and *Igf2r* transcription start sites and spaced at ~1 kb intervals [from Regha et al., with permission (Regha et al., 2007)]; short grey bar, the 3.65 kb genetically defined ICE; grey and black asterisks, the *Mlu*I and *Not*I sites assayed in Fig. 4. (**B**) In undifferentiated ES cells, *Igf2r* is expressed biallelically and *Airn* is silent, the maternal *Airn* promoter carries a DNA methylation imprint and the *Igf2r* promoter is unmethylated. In differentiated ES cells, *Igf2r* is upregulated from the maternal allele and *Airn* is upregulated from the paternal allele, the paternal *Igf2r* promoter gains DNA methylation while the maternal *Airn* promoter methylation imprint is unchanged. Dotted arrow, low-level expression; thick arrow, high-level expression; black oval, methylated CpG island; white oval, unmethylated CpG island; grey oval, partially methylated CpG island. (**C-N**) Scanning ChIP-PCR analysis of *Igf2r* (C-H) and *Airn* (I-N) promoter regions for two repressive (H3K27me3, H3K9me3) and two active (H3K9Ac, H3K4me2) marks in undifferentiated (left panels) and differentiated (right panels) ES cells. Two to four replicates of each ChIP-PCR were performed and representative images are shown. M, size marker. (C) Input PCR for *Igf2r* promoter region for H3K27me3 and H3K9Ac ChIPs. (D,E) Scanning ChIP-PCR using H3K27me3 and H3K9Ac antibodies and PCR assays 13-24 (see A) to assay 12.7 kb around the *Igf2r* promoter. (F) Input PCR for *Igf2r* promoter region for H3K4me2 and H3K9me3 ChIPs. (G,H) Scanning ChIP-PCR using H3K4me2 and H3K9me3 antibodies. Details as D,E. (I) Input PCR for *Airn* promoter region for H3K27me3 and H3K9Ac ChIPs. (J,K) Scanning ChIP-PCR using H3K27me3 and H3K9Ac antibodies and primer pairs 1-12 (see A) to assay 11.6 kb around the *Airn* promoter. (L) Input PCR for *Airn* promoter region for H3K4me2 and H3K9me3 ChIPs. (M,N) Scanning ChIP-PCR using H3K4me2 and H3K9me3 antibodies. Details as J,K. (**O**) QPCR assays of H3K27me3 ChIP DNA on *Igf2r* (left) and *Airn* (middle) using primer pairs 18-21 for *Igf2r* and 6-7 for *Airn* (stars in C and I). Bars indicate the percentage ChIP/input (with s.d.). Arrows, mock antibody negative control showing background signals only. The *Slc22a3* CpG island (right) was used as a positive control for the day-5 H3K27me3 ChIP.

expression. This is consistent with the ongoing reassessment of the biological role of DNA methylation, which indicates that it largely modifies promoters already downregulated by other means (Suzuki and Bird, 2008). The lack of a direct role for promoter methylation in inducing *Igf2r* imprinted expression is supported by experiments demonstrating that *Igf2r* is silenced, but *Airn* is upregulated, in embryos globally deficient in DNA methylation (Li et al., 1993; Seidl et al., 2006). Note that in this and other imprinted gene clusters, DNA methylation can be viewed as repressing a repressor (e.g. the *Airn* promoter) on one parental chromosome. The differential behaviour of the *Igf2r* and *Airn* promoters in response to global demethylation indicates an inequality between somatic DNA methylation imprints that coincide with the gain of imprinted expression on one parental chromosome, and gametic DNA methylation imprints that modify the ICE on the other parental chromosome. The former appear to be a consequence of imprinted expression, whereas the latter have been shown to directly repress ICE activity and thereby regulate imprinted gene expression (Barlow and Bartolomei, 2007). The molecular basis of the inequality between gametic-derived and somatic-derived DNA methylation imprints is not yet clear, but might reflect the ability of the ICE to recruit accessory proteins, such as the recently described KRAB zinc-finger ZFP57 protein (Li et al., 2008).

The role of histone modifications in regulating imprinted gene expression

Repressive histone modifications show dynamic changes on the *Igf2r/Airn* promoters during ES cell differentiation, but in a manner that does not correlate with the gain of imprinted expression. H3K27me3 showed strong enrichment in undifferentiated ES cells on

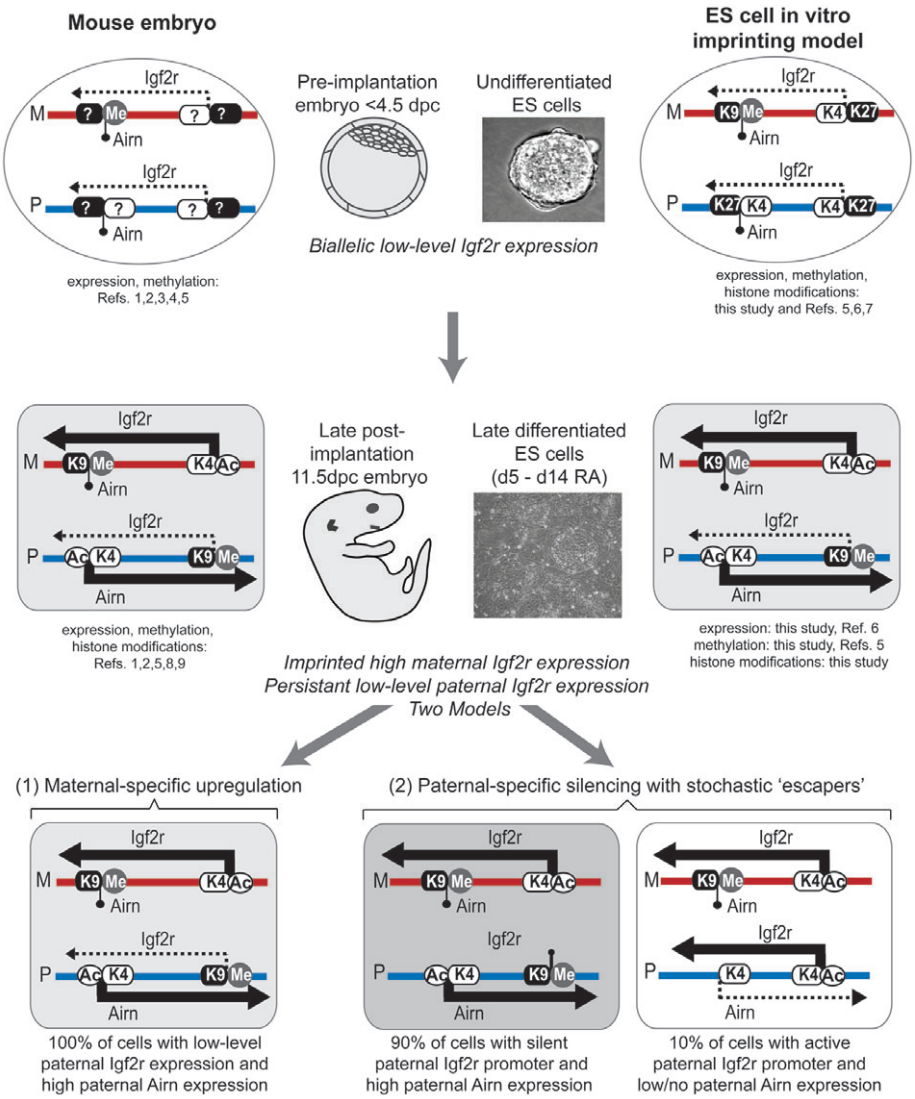

Fig. 6. Differentiating ES cells mimic the developmental gain of *Igf2r*/*Airn* imprinted expression. Changes in *Igf2r*/*Airn* expression (black lollipop, no expression; dotted arrow, low-level expression; solid arrow, medium- or high-level expression), DNA methylation (Me), repressive histone modifications (black; K9, H3K9me3; K27, H3K27me3), active histone modifications (white; K4, H3K4me2/me3; Ac, H3K9Ac), shown on the left-hand side for the mouse embryo and on the right-hand side for ES cells. In pre-implantation embryos, *Igf2r* is biallelically expressed and *Airn* is silent. The histone modification status is unknown, but DNA methylation is present on the maternal *Airn* CpG island and is absent from the *Igf2r* CpG island. These patterns are mimicked in undifferentiated ES cells that also show repressive H3K9me3 modifications on the maternal *Airn* CpG island and bivalent histone modifications comprising H3K27me3 and H3K4me3 on the *Igf2r* and *Airn* promoters, which is typical of all CpG islands in ES cells irrespective of expression status. In 11.5-13.5 dpc embryos, *Igf2r* shows imprinted maternal-specific expression, whereas *Airn* shows imprinted paternal-specific expression in all tissues except post-mitotic neurons (which lack *Airn* and express *Igf2r* biallelically). Persistent expression of the paternal *Igf2r* promoter is detected in some tissues of the post-implantation embryo. DNA methylation is maintained on the maternal *Airn* promoter and is also now present on the paternal *Igf2r* promoter (the latter is not fully methylated until after birth). Active histone marks (H3K4me2/3, H3K9Ac) are only found on the expressed paternal *Airn* and expressed maternal *Igf2r* CpG island promoters. Repressive histone marks (H3K9me3, H4K20me3) plus HP1 are only found on the silent maternal *Airn* and the silent paternal *Igf2r* promoters. Notably, both the expressed and silent *Airn* and *Igf2r* promoters and their gene bodies are free of H3K27me3. All these features, including the persistent low-level expression of the paternal *Igf2r* promoter and the loss of H3K27me3, are fully mimicked in differentiated ES cells. We propose two models, as discussed in the text, to explain the persistence of low-level *Igf2r* expression from the paternal allele: (1) maternal-specific upregulation and (2) paternal-specific silencing with stochastic 'escapers'. References: [1](Szabo and Mann, 1995), [2](Lerchner and Barlow, 1997), [3](Wang et al., 1994), [4](Terranova et al., 2008), [5](Stoger et al., 1993), [6](Braidotti et al., 2004), [7](Mikkelsen et al., 2007), [8](Sleutels et al., 2002), [9](Regha et al., 2007). M, maternal; P, paternal; ?, unknown status; d RA, days of RA-induced differentiation.

both the biallelically expressed *Igf2r* and the non-expressed *Airn* promoters. However, this modification was completely lost in differentiated cells that gain imprinted expression of both *Igf2r* and *Airn*. This indicates that H3K27me3 is not used to regulate imprinted *Igf2r/Airn* expression. This interpretation is supported by our previous studies of parental-specific histone modification in 13.5 dpc MEF cells, which also demonstrated a complete absence of H3K27me3 over the repressed alleles of *Igf2r* and *Airn* (Regha et al., 2007). The lack of a role for H3K27me3 in regulating imprinted expression of *Igf2r* is further supported by examination of 7.5 dpc embryos deficient

for *Eed*, a PRC2 component that catalyzes H3K27me3. *Eed* mutant embryos lose imprinted expression of 4/14 of the imprinted genes tested, but *Igf2r* imprinted expression was unaffected in embryonic and extra-embryonic tissue (Mager et al., 2003). Differentiated ES cells model embryonic differentiated cells, and their analysis does not exclude a role for H3K27me3 in regulating imprinted expression of the *Slc22a2* and *Slc22a3* genes in the trophoblast placenta. Although this has not been directly tested, a recent publication has described a physical association between the *Airn* ncRNA and a nuclear compartment marked by Polycomb proteins in the trophectoderm cells of the late blastocyst that give rise to the trophoblast cells of the placenta (Terranova et al., 2008).

In contrast to H3K27me3, the repressive H3K9me3 modification is only found on *Igf2r* when it shows imprinted expression in differentiated ES cells. Notably, this H3K9me3 mark is lost in MEFs that carry a 3 kb *Airn* ncRNA truncation, a shortened form that is unable to silence *Igf2r* (Regha et al., 2007). Despite the finding that H3K9me3 also modifies the *Airn* promoter irrespective of expression status (i.e. in undifferentiated and differentiated ES cells), it is possible that this mark plays a direct or accessory role in allele-specific repression in differentiated cells. H3K9me3 has been shown to specifically mark the maternal DNA-methylated *Airn* promoter in ES cells (Mikkelsen et al., 2007). We have also shown in 13.5 dpc MEF cells that H3K9me3 specifically marks the repressed paternal *Igf2r* and repressed maternal *Airn* promoters and is probably mediated by the ESET histone methyltransferase (Regha et al., 2007). The absence of viable ES cells lacking ESET (Dodge et al., 2004) precludes the significance of this H3K9me3 mark from being directly tested.

An in vitro ES system for studying epigenetic gene regulation

Genomic imprinting is a well-studied model of epigenetic gene regulation that has identified a role for DNA methylation in regulating cis-acting insulator elements and macro ncRNA CpG island promoters. We show here that in vitro ES cell differentiation can faithfully mimic key developmental steps as imprinted expression is established (see Fig. 6 for an in vivo and in vitro comparison). Maternal methylation of the ICE, which is the imprint for the *Igf2r* cluster and acquired in oocytes and lost in 12.5 dpc germ cells (Labosky et al., 1994; Stoger et al., 1993), cannot be studied in an ES cell model system that mimics events in embryonic somatic cells. However, the ICE gametic methylation imprint is stably maintained during early embryonic development and we show that it is similarly maintained during ES cell differentiation (Fig. 4). The switch from non-imprinted to imprinted expression is also modelled by an in vitro ES system. As we show here, undifferentiated ES cells express *Igf2r* biallelically in a similar manner to pre-implantation embryos (Lerchner and Barlow, 1997; Stoger et al., 1993; Szabo and Mann, 1995). The onset of imprinted *Igf2r* expression that takes place between 4.5 and 6.5 dpc in the embryo (Lerchner and Barlow, 1997) is also recapitulated by the gain of imprinted expression as ES cells differentiate (Fig. 3). Similarly, dynamic changes in histone modifications and gain of methylation on the *Igf2r* promoter that have been shown to be parental specific in MEF cells (Fournier et al., 2002; Regha et al., 2007) are recapitulated in differentiating ES cells (Fig. 5). One drawback to an ES model system is that it cannot be used to study genes that show placental trophoblast-specific imprinted expression, as ES cells are established from the blastocyst inner cell mass, which does not contribute to the trophoblast. However, alternative stem cells, derived from trophectoderm and primitive endoderm lineages, are available and these might prove useful models of placental trophoblast imprinted expression (Rossant, 2007). Thus, we show that ES cell differentiation offers a reliable model system in which to dissect some stages of genomic imprinting. In addition, analysis of the epigenetic regulation of imprinted genes can itself provide valuable information about existing epigenetic mechanisms related to the control of pluripotency in stem cell populations. For example, we have also shown that absence of the *Airn* and *H19* ncRNAs, as well as equal biallelic expression of *Igf2r*, are stringent markers of undifferentiated pluripotent ES cells.

Persistent expression of the 'silent' allele of an imprinted gene

Imprinted expression is generally interpreted as an epigenetic silencing event (Solter, 2006). However, we show that the paternal *Igf2r* allele maintains persistent low-level expression despite the gain of DNA methylation and repressive H3K9me3 histone modifications. Although we use an ES cell in vitro differentiation system, we consider that this accurately reflects imprinted expression because post-implantation mouse embryos similarly show persistent paternal expression in some tissues (Lerchner and Barlow, 1997). In addition, 13.5 dpc MEF cells with complete DNA methylation on the paternal promoter also show persistent paternal *Igf2r* expression that is readily detected in northern blots and by non-quantitative PCR (see Fig. S3 in the supplementary material). Several reports have also noted expression of the 'silent' allele of an imprinted gene, including *Igf2* (Sasaki et al., 1992) and genes in the *Kcnq1*, *Dlk1* and *Sgce-Peg10* imprinted clusters (da Rocha et al., 2007; Fitzpatrick et al., 2002; Ono et al., 2003). A recent review has suggested that the majority of imprinted genes might actually show preferential, rather than exclusive, parental-specific expression (Khatib, 2007).

Although the *Airn* ncRNA has traditionally been viewed as inducing paternal-specific silencing of *Igf2r*, our data allow an alternative epigenetic regulation model (Fig. 6). In model 1, we propose that *Airn* does not silence the paternal *Igf2r* promoter but instead acts to prevent its upregulation. We advocate this maternal-specific upregulation model because we observe that the maternal *Igf2r* allele, which contains the silent methylated *Airn* promoter, shows dramatic upregulation during ES cells differentiation. On the paternal chromosome, the unmethylated *Airn* promoter is upregulated and low-level paternal *Igf2r* expression is unchanged. The *Airn* ncRNA would act in this model to prevent the gain of activating epigenetic marks on either the paternal *Igf2r* promoter or its enhancers. It is interesting, in view of this model, that H3K9me3 has been shown to block active histone marks such as H3K9Ac and H3Ser10P (Rea et al., 2000). This maternal-specific upregulation model is supported by the parallel kinetics of *Igf2r* and *Airn* upregulation and our previous analysis of DNase I hypersensitive sites in this region, which concluded that the *Igf2r* and *Airn* promoters share the same cis-acting regulatory elements, albeit on different parental chromosomes (Pauler et al., 2005). In model 2, we propose the more classical viewpoint that *Airn* does silence the paternal *Igf2r* promoter but a small percentage of cells, in some unknown stochastic way, escape silencing and express high levels of *Igf2r* biallelically. Analysis of single-cell *Igf2r* transcription would distinguish between these two models. However, RNA FISH is currently insufficiently sensitive to detect either the low-level

Igf2r expression seen in undifferentiated ES cells (data not shown), or the full biallelic expression seen in differentiated ES cells that lack a functional *Airn* ncRNA (Stricker et al., 2008). Thus, we cannot directly distinguish between persistent low-level expression of the paternal *Igf2r* allele in all cells in the population as proposed by model 1 and a low percentage of cells in the population that express *Igf2r* biallelically as proposed by model 2. Both models, however, accommodate the view that repressive marks may follow the lack of activation and play accessory roles in regulating the paternal *Igf2r* promoter, as well as our recent proposal that *Airn* transcription, and not the *Airn* ncRNA itself, is important for its function (Pauler et al., 2007). The demonstration here that the gain of imprinted expression can be mimicked in vitro in differentiating ES cells provides a valuable tool with which to determine whether the *Airn* ncRNA acts by blocking the access of activating epigenetic marks, or by recruiting repressive epigenetic marks, to induce imprinted *Igf2r* expression.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/136/3/437/DC1

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