An *ex Vivo* Model for Imprinting: Mutually Exclusive Binding of Cdx2 and Oct4 as a Switch for Imprinted and Random X-Inactivation

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ABSTRACT In the early mammalian embryo, X chromosome inactivation (XCI) achieves dosage parity between males and females for X-linked genes. During mouse development, imprinted paternal XCI is observed first and switches to random XCI in the epiblast but not placental lineages. The mechanism by which this epigenetic switch occurs is currently unknown. Here, we establish an *ex vivo* model for imprinting and identify a novel *trans*-acting regulatory factor for imprinted XCI. Using an induced trophoblast stem cell (ITS) model, we show that embryonic stem (ES) cells transdifferentiated into trophoblasts retain partial memory of the XCI imprint. Cdx2, a stem cell factor that determines commitment to the extraembryonic lineage, directly binds *Xist* and activates expression of *Xist* RNA in extraembryonic cells. Cdx2 competes with Oct4, a stem cell factor that determines commitment to the embryonic lineage, for overlapping binding sites within *Xist*. We propose that mutually exclusive binding between Cdx2 and Oct4 in *Xist* underlies the switch between imprinted and random XCI in the early mouse embryo.

X chromosome inactivation (XCI) is an essential developmental program that achieves gene dosage parity in mammals between the XX female and the XY male (Wutz and Gribnau 2007; Payer and Lee 2008; Starmer and Magnuson 2009). XCI is epigenetically regulated and is tightly linked to changes in pluripotency and cell fate decisions in the early mouse embryo (Monk and Harper 1979). Between embryonic days 0.5 and 5.5 (E0.5–E5.5), two forms of XCI occur sequentially in the mouse. “Imprinted XCI” is a germline-determined process during which silencing occurs exclusively on the paternal X chromosome (X^P) (Takagi and Sasaki 1975; Takagi 2003). Evidence of imprinted XCI is observed by the two-cell stage, where repetitive elements on X^P are transcriptionally suppressed relative to those on the maternal X (X^M) (Huynh and Lee 2003; Namekawa *et al.* 2010). Silencing progressively encompasses genic elements on X^P during the next several divisions until the early mouse blastocyst stage (Okamoto *et al.* 2005; Kalantry *et al.* 2009; Namekawa *et al.* 2010).

In the later mouse blastocyst, embryonic (epiblast) and extraembryonic lineages (trophectoderm, primitive endoderm) become evident for the first time and it is during this time that evidence of “random XCI” is first detected. Whereas the extraembryonic cells retain imprinted XCI, the embryonic lineage reactivates X^P at E4.5 (Mak *et al.* 2004; Okamoto *et al.* 2004) and undergoes a second round of XCI (Harper *et al.* 1982; Tan *et al.* 1993), this time in a “random” way such that X^P and X^M have an equal chance of becoming the inactive X (Xi). Random XCI is essential for *in vivo* differentiation of the epiblast to the three germ lineages (ectoderm, mesoderm, and endoderm) and for the *ex vivo* differentiation of epiblast-derived embryonic stem (ES) cells. Recent work in stem cell engineering shows that mouse XCI is also intimately linked to the reprogramming process *ex vivo* in the derivation of mouse induced pluripotent stem (iPS) cells (Maherali *et al.* 2007).

How and why the early mouse embryo switches from imprinted to random XCI present two intriguing questions. Still unknown are specific factors that dictate the decision to switch XCI pathways. Also unclear is whether the switch from imprinted to random XCI necessitates erasure of the original germline imprint, which would then allow a zygotic counting/choice mechanism to initiate random XCI. An

alternative is that the zygotic counting/choice mechanism merely overwrites a parental imprint that is never erased in the epiblast. The switch in XCI pathways during early development is especially puzzling, given that the two forms are regulated by overlapping sets of factors, many based in the X-inactivation center (*Xic*).

The *Xic* includes a number of crucial long noncoding RNAs (lncRNAs). *Xist* RNA is a 17-kb transcript that is expressed only in female cells, coats the Xi in *cis*, and initiates global silencing on that chromosome (Brockdorff *et al.* 1992; Brown *et al.* 1992; Marahrens *et al.* 1997; Wutz and Jaenisch 2000). In the early embryo, *Xist* RNA is paternally expressed, required for imprinted XCI (Marahrens *et al.* 1997; Kalantry *et al.* 2009), and necessary for genic but not repetitive element silencing (Namekawa *et al.* 2010). Murine *Xist* is positively regulated by *Jpx* lncRNA (Tian *et al.* 2010) and negatively regulated by the antisense *Tsix* transcript (Lee and Lu 1999; Lee *et al.* 1999; Lee 2000; Luikenhuis *et al.* 2001; Sado *et al.* 2001; Stavropoulos *et al.* 2001). In cells that undergo random XCI (e.g., epiblast, ES cells), *Tsix* determines XCI choice and deleting *Tsix* results in skewed XCI to favor inactivation of the mutated X chromosome. In cells that undergo imprinted XCI (extraembryonic cells), *Tsix* suppresses expression of *Xist* on X^M , and deleting *Tsix* on X^M leads to ectopic X^M -inactivation and early loss of both XX and XY embryos. *Tsix* is in turn controlled by *Xite*, a positive regulator that sustains expression of *Tsix* on the future active X (Ogawa and Lee 2003).

In searching for pathway-specific factors, we reasoned that, because imprinted and random XCI are tied to trophectoderm and epiblast cell fates, regulatory factors are likely to be those involved in determining lineage commitment. For random XCI, two recent studies have implicated the pluripotency factor, Oct4 (Nichols *et al.* 1998), in the regulation of *Xite*, *Tsix*, and *Xist* (Navarro *et al.* 2008; Donohoe *et al.* 2009). By binding *Tsix* and *Xite*, Oct4 upregulates *Tsix* and *Xite* expression and controls the initiation of X chromosome counting and pairing in ES cells, an established *ex vivo* model to study random XCI. In addition, Oct4 sites can be found in the first intron of *Xist*, where Oct4 is thought to directly block expression of the linked *Xist* allele (Navarro *et al.* 2008). While Oct4 is a strong candidate for the initiation of random XCI, its expression alone cannot regulate the decision to undergo imprinted or random XCI. Indeed, Oct4 is expressed ubiquitously in 2-, 4-, 8-, and 16-cell stages (Palmieri *et al.* 1994; Nichols *et al.* 1998) at a time when imprinted, but not random, XCI is observed.

During these stages, *Cdx2*—a protein with well-defined roles in directing commitment to the trophectoderm lineage (Strumpf *et al.* 2005)—is known to be expressed. Genome-wide analysis has shown that *Cdx2* opposes the action of pluripotency factors by globally downregulating pluripotency factor target genes and thereby switching from embryonic to extraembryonic cell fates (Nishiyama *et al.* 2009). Moreover, ectopic expression of *Cdx2* in ES cells leads to transdifferentiation of ES to trophoblast stem (TS) cells (Niwa *et al.* 2005), and one significant report has utilized this system

to study imprinted XCI (Murakami *et al.* 2011). Given these observations, here we test whether *Cdx2* may be a regulator for the epigenetic decision to switch XCI pathways. We use the induced trophoblast stem cell (iT_{TS}) model to study imprinted XCI and show that mutually exclusive binding of *Cdx2* and Oct4 to *Xist* underlies the switch between imprinted and random XCI.

Materials and Methods

Allele-specific RT-PCR

Allele-specific RT-PCR was performed as described in Stavropoulos *et al.* (2001). Briefly, total RNA was isolated with Trizol (Invitrogen, Carlsbad, CA) from cells and 2 μ g was DNase with turbo DNase (Ambion) and reverse transcribed with random hexamer primers and SuperScriptIII (Invitrogen). The following primer and restriction digest combinations were performed to amplify polymorphic *Mus castaneus* and *M. musculus* fragments:

Xist: NS33 (5'-CAGAGTAGCGAGGACTTGAAGAG-3') and NS66 (5'-GCTGGTTCGTCTATCTTGTGGG-3'), *SrfI* digestion. *Mecp2*: NS43 (5'-ATGGTAGCTGGGATGTTAGGG-3') and NS44 (5'-GAGCGAAAAGCTTTTCCCTGG-3'), *DdeI* digestion. PGK: KHP72 (5'-CGTGATGAGGGTGGACTTCAAC-3') and KHP73 (5'-TAGTTTGGACAGTGAGGCTCGG-3'), *MseI* digestion

These fragments were fractionated on an agarose gel, Southern blotted, and probed with the ³²P-end-labeled probe *Xist*: NS67 (5'-CCAGAGTCTGATGTAACGGAGG-3'), *Mecp2*: NS65 (5'-CCACTACAACCTTCAGCCCACCAATTGAAGGCAAGCATGAG-3') PGK: KHP106 (5'-TGAACCTCAAATCTCTGCTGGGC-3'). Relative allelic amounts were quantitated by phosphorimage, using the software ImageQuant.

Immunofluorescence analysis

Colonies grown in ES media, TS media, or TS media + 1 μ g/ml of 4-hydroxy tamoxifen (Tx; Sigma, St. Louis) were fixed using 4% paraformaldehyde and rinsed with 20 mM Tris-HCl, 0.15 M NaCl, and 0.05% Tween-20. Cells were permeabilized in 1X phosphate buffered saline with 0.05% Triton X (blocking buffer) and blocked with 4% goat serum at room temperature. Monoclonal antibodies against mouse anti-Oct3/4 monoclonal Ab (C-10; Santa Cruz, 1:500 dilution) and mouse anti-*Cdx2* monoclonal Ab (*Cdx2*-88; Biogenex, 1:500 dilution) rabbit anti-NANOG (ab21603; Abcam) were incubated for 1 hr at room temperature. Cells were rinsed twice with blocking buffer and hybridized with the Cy3-conjugated goat anti-mouse and FITC-conjugated goat anti-rabbit secondary antibody (1:1000 dilution; Chemicon) for 1 hr. Cells were rinsed twice in blocking buffer and visualized.

Cell culture and generation of transgenic ES lines

Male J1 (40XY) and female 16.7 (40XX) ES cell lines and the TS line (TS3.5) have been described in Huynh and Lee

(2003). For iTS generation, 40 µg of *Sa*II linearized *pCAG-Cdx2ER-IP* (Niwa *et al.* 2005) was electroporated into EL16.7, J1, 3F1 (Δ Tsix female line), and CG7 (Δ Tsix male line) (Lee and Lu 1999) at 800 V and 3 µF in a 0.4-cm cuvette, using Gene Pulser (Bio-Rad, Hercules, CA) followed by selection with 1 µg/ml of puromycin for 8 days. Clones in which *pCAG-Cdx2ER-IP* were randomly integrated into genomic DNA were selected by the ability to undergo differentiation to TE by addition of 1 µg/ml of Tx (Sigma) in the standard trophoblast stem cell media (Tanaka *et al.* 1998), resulting in establishment of ELCDX, J1CDX, and Δ TsixCDX cells. For allele-specific RT-PCR analysis and RNA/DNA fluorescence *in situ* hybridization (FISH) analysis, cells were induced with 1 µg of 4-hydroxy tamoxifen and grown in standard TS media for 4 or 8 days as indicated. For chromatin immunoprecipitation (ChIP) analysis, cells were grown in ES cell media plus 1 µg of 4-hydroxy tamoxifen.

RNA/DNA FISH

FISH experiments were carried out as previously described (Lee *et al.* 1999). *Xist* was detected by Sx9 probe labeled by a nick translation kit (Roche), using FITC-dUTP (Stratagene, La Jolla, CA). A telomeric BAC probe (RP23-461E16) and a probe detecting the wild-type X (pCC3) in *Tsix^{ΔCpG/+}* cells was labeled by nick translation (Roche), using Cy3-dUTP (Amersham, Piscataway, NJ).

Allele-specific ChIP

ChIP analyses were carried out as described in Lee *et al.* (2006) and the results were averaged for three independent biological replicates. ChIP antibodies included anti-Oct4 (8628; Santa Cruz), anti-CDX2 (Cdx2-88; BioGenex), and normal rabbit IgG (2729; Cell Signaling). qPCR was performed using an iCycler iQ real-time PCR detection system (Bio-Rad) by amplifying with primer pairs listed as described in Donohoe *et al.* (2009): *Xist* intron1 (p65, ATGTTTCCTTTTGAAGCAGTTACTTGTAC; p66, CATTGTCTGGCTCTCTAGGTGATAATAC), *Xite* (p77, CAAGGTTGGGAA CAAGGTATATCAGG; p78, GGACAAGGGACAGAAGTGCTTA TTTTAC), and *Lmb2* negative control (p89, ACTCCCATAAT TTATCCTCTGATGTC; p90, GACAGGCTAAGTAAAGGATGTC TATTTT). PCR was performed using Amplitaq Gold (Applied Biosystems, Foster City, CA) with the following primer/restriction enzyme combinations (Stavropoulos *et al.* 2001): *Xist* intron1, *XinAS-F*, ACCTGCTGCGAAGGAATTTA, and *XinAS-R*, TGCCAGCACTTTTCAACAGA followed by *Bsa*AI, which digests the *musculus* allele (63 bp and 129 bp) but not the *castaneus* allele (192 bp); *Xite*, NGP50, AGATAGGG TTTCTCTGTGTAG, and NGP51, GACTAGTTTCTGTTTCGTG CAG followed by *Aci*I digest, which does not digest the *musculus* allele (229 bp) and digests the *castaneus* allele (168 bp and 61 bp). PCR cycle number used was three cycles past the threshold cycle (*Ct*) from qPCR analysis. These fragments were fractionated on an agarose gel, Southern blotted, and probed with the ³²P-end-labeled probe *Xist*-intron

1 (*XINAS-F*), and *Xite* (NGP50). Relative allelic amounts were quantitated by phosphorimage, using the software ImageQuant.

RT-PCR analysis

Two micrograms of RNA were isolated with Trizol (Invitrogen) and DNA was removed by incubation with turbo DNase (Ambion) for 30 minutes. cDNA was synthesized by reverse transcription with random hexamer primers and SuperScriptIII (Invitrogen). Quantitative PCR was performed using an iCycler iQ real-time PCR detection system (Bio-Rad) by amplifying with the following primer pairs: *Xist* (NS33, CAGAGTAGCGAGGACTTGAAGAG; NS66, GCTGGT TCGTCTATCTTGTGGG), *Oct4* (*octF*, CAGAAGGAGCTAGAA CAGTTTGCC; *octR*, AGATGGTGGTCTGGCTGAACA), *Nanog* (*nanogF*, ACCTGAGCTATAAGCAGGTTAAGAC; *nanogR*, GTG CTGAGCCCTTCTGAATCAGAC), *Beta-actin* (*actin1*, agccatgta cgtagccatcc; *actin2*, ctctcagctgtggtgtgaa), and *Cdx2* as described in Niwa *et al.* (2005).

Electrophoretic mobility shift assay

Reactions were incubated 20 min on ice and contained either 120 ng of recombinant *Cdx2* protein (Novus Biologicals) or 140 ng of bacterially expressed rOct4 purified via a nickel column [Oct4 cDNA from J1 cells was cloned into pGEX4T1; GST-Oct4 was then purified on a glutathione resin and correct folding was verified by an electrophoretic mobility shift assay (EMSA) using an FGF4 enhancer oligo] and 0.2 pmol ³²P-labeled oligonucleotides in 10 mM Tris (pH 7.5), 50 mM KCl, 2.5 mM MgCl₂, 0.5 mM dithiothreitol, 0.5 µg poly(dIdC), and 4% glycerol. Cold competitors were added at 100× molar excess. The oligonucleotides were as follows: *Xite-F*, CCAGGTCTGCATTGATATGTAAAA TAAGCACTTCTGTC, and *Xite-R*, GACAGAAGTGCTTATTT TACATATCAATGCAGACCTGG; *Cdx2* mutated site, *Xite-CdxF*, CCAGGTCTGCATTGATATGGGGAATAAGCACTTCTGTC, and *Xite-CdxR*, GACAGAAGTGCTTATTTCCCATATCAATGCA GACCTGG; *Xist* intron 1 *XINF*, AATAGTTCTAGATGTATTAT CACCTAGAG, and *XINR*, CTCTAGGTGATAATACATCTAGAAC TATT; *Xist* intron 1 *Cdx2* site mutated, *XIN-CdxF*, AATAGTTC TAGATGTGGGGTCACCTAGAG, and *XIN-CdxR*, CTCTAGGT GACCCACATCTAGAACTAT; and *Xist* intron 1-Oct4 and *Cdx* sites mutated, *O-C-XIN-F*, AATAGTTCTAGCCCTGGGGTCACC TAGAG, and *O-C-XIN-R*, CTCTAGGTGACCCAGGGCTAGAA CTAAT. For antibody neutralization, anti-CDX2 (Cdx2-88; BioGenex) or normal rabbit IgG (2729; Cell Signaling) was co-incubated with the protein complex for 20 min on ice before an additional 20-min incubation on ice of labeled oligonucleotide. For competitive/cobinding experiments, equal molar ratios (16 pmol each) of recombinant protein were first incubated in the above binding buffer for 20 min on ice, and then the complexes were incubated for 20 min on ice with ds-labeled oligos. Protein-DNA complexes were resolved on a 4% PAGE gel run at 300 V for 1.5 hr at 4° under native conditions.

Figure 1 Generation of iTS cells via ES cells carrying an inducible Cdx2 transgene. (A) Southern blot analysis of ES clones carrying an inducible Cdx2 transgene, as detected by Cdx2-exon 1 probe. Four independent clones were tested. (B) Immunofluorescence analysis for Oct4, Nanog, and Cdx2. Tx, tamoxifen. (C) Quantitative RT-PCR analysis for Oct4, Nanog, and Cdx2 mRNA. Levels are normalized to β -actin levels, with ES level set to 1. Mean $\pm$ 1 SD is shown. $n = 3$ biological replicates. (D) iTS cells form TS-like colonies and giant cells (arrows) typically seen in the trophoblast lineage.

Results

An iTS model demonstrates partial retention of the XCI imprint in ES cells

Whereas the study of random XCI has benefited hugely from ES cells as an *ex vivo* model, our understanding of imprinted XCI has been hampered by the absence of one. Here we took advantage of Cdx2's known capacity to transdifferentiate ES to TS cells (Niwa *et al.* 2005) to develop an *ex vivo* model to study the transition between random and imprinted XCI. To generate iTS cells, we introduced a tamoxifen-inducible Cdx2 transgene into the hybrid female ES cell line, 16.7, which contains a paternally inherited X chromosome from *M. castaneus* and a maternally inherited X chromosome from *M. musculus*. Transgenic male ES cells were generated in parallel. Clones with low Cdx2 transgene copy numbers

were selected for further analysis (Figure 1A). Southern blot analysis with an exon 1 probe for Cdx2 indicated that only one to two copies were randomly integrated into the genome.

Prior to Tx induction of Cdx2, the ES cells grown in standard LIF-containing media expressed the pluripotency factors, Oct4 and Nanog, comparably to the parental cell lines (Figure 1, B and C). To induced transdifferentiation into iTS cells, we grew transgenic ES cells with tamoxifen in Fgf4- and heparin-containing media for 6 days and observed high levels of Cdx2 protein in the nucleus (Figure 1, B and C). In this inducible system, tamoxifen addition promotes translocation of Cdx2 into the nucleus and transcriptionally upregulates endogenous Cdx2 via a Cdx2 auto-feedback loop (Nishiyama *et al.* 2009). Immunofluorescence showed that the levels and localization patterns for Cdx2 were similar to

those observed in wild-type TS cells. At the same time, Oct4 and Nanog levels dramatically decreased. Furthermore, upon withdrawal of tamoxifen and growth for a further 7 days, the iTS cells differentiated into the multinucleated giant cells typically observed in differentiating TS cells, suggesting commitment to a placental lineage fate. These results are consistent with observations reported previously (Niwa *et al.* 2005) and were observed in both XX and XY backgrounds (Figure 1D).

We then asked whether the iTS cells would retain the random XCI pattern expected of differentiating ES cells or whether they would newly adopt the imprinted pathway ordinarily observed in TS cells. To address this question, we carried out SNP-based allele-specific RT-PCR for the X-linked genes, *Pgk1*, *Mecp2*, and *Xist* (Figure 2A). By inducing XCI through differentiation of wild-type female ES cells, we observed the expected allelic ratios of ~65:35 favoring inactivation of the *M. castaneus* chromosome. In differentiated iTS cells, we observed a similarly skewed inactivation of the *M. castaneus* chromosome. For both differentiated ES and iTS cells, the X-linked genes, *Pgk1* and *Mecp2*, have slightly skewed preferential expression from the *M. musculus* allele and *Xist* expression has slightly skewed preferential expression from the *M. castaneus* allele. The mild allelic skewing in ES cells is consistent with the strain-specific X-linked modifier, *Xce*, which favors genic expression from the X of *M. castaneus* origin (Cattanach and Isaacson 1967; Chadwick and Willard 2005). By contrast, in wild-type TS cells (Huynh and Lee 2004), expression of *Xist* was almost exclusively of paternal origin (*M. castaneus*), while *Pgk1* and *Mecp2* expression was of maternal origin (*M. musculus*), consistent with the occurrence of imprinted XCI in wild-type TS lines. Thus, iTS cells showed an allelic pattern that was more similar to that of the parental ES line.

Although these observations seemed to indicate that iTS cells undergo random XCI, as recently observed (Murakami *et al.* 2011), allele-specific RT-PCR examines population-wide expression patterns and could potentially mask differences at the single-cell level. We therefore performed single-cell analysis by FISH to determine the allelic origin of *Xist* RNA. We used a genetically marked cell line carrying the *Tsix*^{ΔCpG} mutation on X^M, the *M. musculus* X chromosome (Lee and Lu 1999). *Tsix*^{ΔCpG} carries a promoter deletion of *Tsix* and, because *Tsix* regulates the choice step of random X chromosome inactivation, the chromosome deficient for *Tsix* is inactivated exclusively in ES cells and also in all somatic cells of the mouse, thereby enabling us to achieve a homogeneous population that would normally inactivate X^M during ES cell differentiation. However, when the deletion is maternally inherited in mice, placental tissues inactivate X^M as well as X^P, resulting in loss of cells carrying two Xi (Lee 2000; Sado *et al.* 2001).

We asked whether transdifferentiation of Cdx2-transgenic *Tsix*^{ΔCpG/+} female ES cells would give rise to iTS cells that retained the ES program in keeping with its original identity or adopted the imprinted program of its newly acquired TS

identity. If the transdifferentiated *Tsix*^{ΔCpG/+} cells upregulated *Xist* exclusively on the maternal *Tsix*^{ΔCpG} allele, they would recapitulate the pattern expected of the embryo proper (also ES cells). If *Xist* were instead expressed from the wild-type X^P allele, the transdifferentiated cells would recapitulate the pattern expected of imprinted XCI in the trophectoderm. Multiple independent iTS clones were examined and each demonstrated similar results. Interestingly, serial RNA-DNA FISH showed that 18% of iTS cells expressed *Xist* RNA from the wild-type *M. castaneus* chromosome (Figure 2B). This indicated unexpected inactivation of wild-type X^P in spite of the strong *Tsix* mutation on X^M. This contrasted sharply with normal ES differentiation, in which essentially 100% of cells inactivated the *Tsix*-disabled X^M. Notably, without TX induction of Cdx2, transgenic cells almost exclusively inactivated the mutated X (X^M), as expected for ES cells. Furthermore, 7% of cells displayed two foci of *Xist* expression, indicating inactivation of both X^M and X^P in the same cell, similar to what is observed in placental tissues of *Tsix*-mutant mice (Lee 2000; Sado *et al.* 2001) but is almost never observed in normal ES differentiation (Lee and Lu 1999) and in wild-type ES female cells when induced for Cdx2 expression. This provided further evidence for memory retention, as in spite of strong pressure against inactivation of two X's, the iTS cells could not suppress *Xist* from the previously imprinted X^P. All female lines were confirmed for diploidy and X chromosome number (XX) by DNA FISH. Thus, transdifferentiation into iTS cells induced imprinted XCI in a subset of cells, superseding the *Tsix* deletion and its strong effect on XCI choice. The relatively small percentages explain why allele-specific RT-PCR measured only a small shift in allelic ratios from ES to iTS cells and indicate that effects at the single-cell level were indeed masked by population-wide measurements (Figure 2A). This imprint retention was observed in multiple independent clonal lines. Taken together, these results argue that, although random XCI can be observed in iTS cells as reported in a recent publication (Murakami *et al.* 2011), the germline imprint to inactivate X^P may not be fully erased during X^P reactivation in the epiblast in our experimental system.

To investigate further, we performed single-cell analysis in male ES and iTS cells carrying the *Tsix*^{ΔCpG} mutation. In male ES cells, the mutation ordinarily has no effect on *Xist* expression during cell differentiation into embryoid bodies (EB) and also has no effect on the epiblast lineage or male soma in the mouse (Lee and Lu 1999). However, in placental tissues, maternal inheritance of the mutation results in ectopic *Xist* expression and inactivation of the single male X (Lee 2000). We asked whether transdifferentiation of ES into iTS cells altered the epigenetic program on X. When Cdx2 was induced for 4 days and the ES cells were transdifferentiated into iTS cells, 8% of the male cells consistently upregulated *Xist* (Figure 2, C–E), similar to that seen in placental tissues of mutant male embryos (Lee 2000; Sado *et al.* 2001) and almost never observed in normal

Figure 2 Evidence for partial memory of the XCI imprint in ES cells. (A) Allele-specific RT-PCR analysis for *Pgk1*, *Mecp2*, and *Xist*. d0, day 0; d10, day 10 of EB differentiation. % mus, percentage of RNA from the *M. musculus* allele. (B) RNA/DNA FISH of *Tsix^{ΔCpG}/+* female ITS clones differentiated for 4 days with tamoxifen (Cdx2). Arrow indicates wild-type allele. (C) RNA/DNA FISH of male ITS cells in a wild-type background after 4 days of Tx induction of Cdx2. (D) RNA/DNA FISH of *Tsix^{ΔCpG}/Y* male ITS clones after 4 days of Tx induction of Cdx2. (E) Xist expression patterns observed in *Tsix^{ΔCpG}/+* female and male Cdx2-transgenic clones differentiated in the absence or presence of tamoxifen (ES and ITS, respectively) for 8 days. X^{WT}, wild-type X chromosome; X^Δ, *Tsix*-mutant X chromosome. Solid black circle, Xist RNA cloud. Red oval indicates the wild type X in females; blue oval indicates mutated X in males. n, sample size.

ES differentiation (Lee and Lu 1999). Ectopic expression also did not occur in *Cdx2*-transgenic *Tsix^{ΔCpG}/Y* ES cells without tamoxifen induction of *Cdx2* (Figure 2E). Multiple independent clonal male lines demonstrated the same behavior. Collectively, these findings argue that ES cells retain at least partial memory of the germline imprint, which can be unmasked when ES cells are transdifferentiated into ITS

cells by *Cdx2*. Memory retention is independent of sex and clonal derivation.

Cdx2 binds the *Xite* and *Xist* loci

We next investigated *Cdx2*'s mechanism of action and asked whether *Cdx2*'s effect on XCI could be mediated through the *Xic*. We bioinformatically searched for potential *Cdx2*

Figure 3 Cdx2 and Oct4 bind to *Xist* and *Xite* in vivo. (A) Map of Oct4- and Cdx2-binding motifs within *Xist* and *Xite*. The Cdx2 consensus and binding motifs within *Xist* intron 1, *Xite*, and *HoxC8* (a known Cdx2 target gene) are shown. (B) qChIP analysis of Oct4 and Cdx2 binding at designated sites in the indicated cell lines. ES, Cdx2-transgenic ES clones without Tx induction of Cdx2. iTS, Cdx2-transgenic clones transdifferentiated to TS cells by Tx induction of Cdx2. TS, trophoblast stem cells. Mean $\pm$ SEM of three independent biological replicates is shown. The statistical significance of each result is calculated compared to the control IgG ChIP (background), using a paired, one-tailed Student's *t*-test. $*P < 0.05$. (C) qRT-PCR for Oct4 RNA in the indicated cell lines. Levels are normalized to β -actin levels. Mean $\pm$ 1 SD is shown.

binding sites, using the known Cdx2 consensus motif, and identified several sites within the *Xic* that were notable for their proximity to Oct4-binding sites (Figure 3A). Specifically, within *Xite* and the first intron of *Xist*, Cdx2 motifs colocalize with Oct4 motifs. To determine whether Cdx2 occupies these sites *in vivo*, we performed ChIP and observed statistically significant reciprocal binding patterns for Cdx2 and Oct4 that are dependent on cell type ($P < 0.05$; Figure 3B). Oct4 associated with *Xist* intron 1 and *Xite* in both male and female ES cells, consistent with previous findings (Navarro *et al.* 2008; Donohoe *et al.* 2009; Nishiyama *et al.* 2009) (Figure 3B).

Induction of Cdx2 for 48 hr and transdifferentiation into iTS cells resulted in loss of binding of Oct4 to both sites in male and female iTS cells. ChIP for Cdx2 showed the opposite pattern. Cdx2 binding was not seen in ES cells, as expected since ES cells do not ordinarily express Cdx2. Forced expression of Cdx2 and transdifferentiation into iTS cells led to *de novo* binding of Cdx2 protein to *Xist* intron 1 in

iTS cells (Figure 3B). Binding to this locus was also observed in wild-type TS cells. Although Cdx2 was detected at *Xite* in wild-type TS cells, it did not associate significantly with *Xite* in iTS cells at 48 hr. In subsequent analyses, we therefore focused primarily on *Xist* intron 1. Quantitative RT-PCR showed that, during transdifferentiation to iTS cells, the loss of Oct4 binding occurred in advance of Oct4 depletion at 48 hr after Cdx2 induction (Figure 3C). Combined, these experiments demonstrate that the Cdx2 pathway intersects with XCI through the *Xic* and raise the possibility that Cdx2 binding to *Xist* intron 1 may be a key determinant of imprinted XCI.

Cdx2 competes with Oct4 for *Xist* intron 1 binding and activates the paternal *Xist* allele

The fact that Oct4 binding to intron 1 occurred before a large increase in Oct4 levels (Figure 3C) hinted that expression of Cdx2 may displace Oct4 from the *Xic*. The possibility that Cdx2 and Oct4 may compete for binding to *Xic*

Figure 4 Cdx2 and Oct4 bind directly to *Xist* intron 1 and *Xite* *in vitro*. (A) Gel shift of *Xist* intron 1 (Int1), using recombinant Cdx2 (rCdx2). Arrow, protein–DNA shift. Comp, cold competitor added at 100-fold molar excess; Mut, mutation. (B) Gel shift of the *Xite* motif using rCdx2. (C) Gel shift of *Xist* intron 1 and *Xite*, using recombinant Oct4 (rOct4). (D) Gel shift of *Xist* intron 1 motif using rOct4 and rCdx2. Two times (2x) and three times (3x), molar excess of rOct4 are shown. mCdx, mutated Cdx2-binding site; mOct-mCdx, mutated Cdx2-Oct4-binding sites. (E) qRT-PCR for *Xist* RNA, with levels normalized to β -actin levels. Mean $\pm$ SD is shown. ES, Cdx2-transgenic ES clones without TX induction of Cdx2. ITS, Cdx2-transgenic clones transdifferentiated to TS cells by TX induction of Cdx2 for 1 (d1) or 2 (d2) days. (F) Allele-specific gChIP analysis for Cdx2 and Oct4 binding from indicated samples.

was intriguing, considering that Cdx2 has been shown to interact with Oct4 (Niwa *et al.* 2005) and also regulate the same target genes (Nishiyama *et al.* 2009). Furthermore, Cdx2 sites at *Xite* and *Xist* intron 1 overlap Oct4 sites (Figure 3A). To explore this idea, we first asked whether Cdx2 directly binds the *Xite* and *Xist* motifs by performing EMSAs. Using recombinant Cdx2 protein (rCdx2), we tested its ability to shift a ³²P-labeled *Xist* intron1 probe (Figure 4A). Indeed, Cdx2 protein shifted the intronic probe, and this

interaction was significantly competed away by excess cold wild-type but not mutant oligos. Addition of α -Cdx2 antibodies neutralized and/or supershifted the protein–DNA interaction, whereas addition of normal IgG did not. Cdx2 also strongly shifted the *Xite* probe and this interaction was competed away by excess cold wild-type but not mutant oligos (Figure 4B). Together, these results demonstrated a direct and specific interaction of Cdx2 protein with both *Xite* and intron 1 of *Xist*.

Although the direct binding of Oct4 protein to *Xite* was previously demonstrated (Donohoe *et al.* 2009), whether Oct4 can directly bind *Xist* intron 1 has not been addressed, as ChIP results leave open the possibility of either direct or indirect binding (Navarro *et al.* 2008). To address this point, we performed EMSA using purified recombinant Oct4 protein (rOct4). Consistent with previous findings (Donohoe *et al.* 2009), we observed a strong rOct4-*Xite* complex that is neutralized by addition of α -Oct4 antibodies (Figure 4C). rOct4 also shifted *Xist* intron 1, although to a lesser extent than *Xite*. In both cases, the shifted complex was neutralized by addition of α -Oct4 antibodies. These data demonstrate that binding of Oct4 to the *Xite* and *Xist* intron 1 sequences is direct.

Collectively, these findings indicate that Oct4 and Cdx2 bind overlapping sites at the *Xic* and make possible the idea that they may bind mutually exclusively. Under the same *in vitro* conditions, Cdx2 appeared to bind the intron 1 probe more strongly than Oct4 (Figure 4D). In support of this idea, mixing equimolar amounts of Cdx2 and Oct4 with 32 P-labeled *Xist* intron 1 resulted predominantly in formation of the Cdx2:DNA complex, suggesting that Cdx2 outcompetes Oct4 for binding when protein concentrations are equal. Increasing the amount of Oct4 protein led to increased formation of an Oct4:DNA complex. When the Cdx2-binding site was mutated (mCdx Int1), Cdx2 binding was dramatically decreased at the same time that Oct4 binding became more apparent. When both Cdx2 and Oct4 sites were mutated (mOct-mCdx Int1), binding to both sites was abolished. Our results support the notion that Oct4 and Cdx2 compete for binding to *Xist* intron 1. The more intense Cdx2 shifts relative to Oct4 shifts (Figure 4D) may suggest that Cdx2 has higher affinity than Oct4 for the intronic site, raising the intriguing possibility that Cdx2 expression in the early embryo may displace Oct4 from the *Xic* and trigger imprinted XCI.

Our data suggest that competition between Cdx2 and Oct4 for binding to *Xist* intron 1 may be a crucial factor in switching from random to imprinted XCI in iTS cells. We asked how Cdx2 binding to the *Xic* regulates *Xist* expression. By qRT-PCR, we observed that *Xist* expression increased 3- to 12-fold after 24–48 hr of Cdx2 induction (Figure 4E). This finding implies that Cdx2 may be an activator of *Xist*. To determine which allele binds Cdx2, we carried out allele-specific ChIP in wild-type TS cells carrying X^P of *M. castaneus* origin and X^M of *M. musculus* origin. Indeed, at *Xist* intron 1, Cdx2 preferentially bound X^P (Figure 4F), the chromosome expressing *Xist*. By contrast, Oct4 was observed to be biallelically bound to ES cells, consistent with Oct4's proposed repressive effect on *Xist* and with the presence of two active X (Xa) chromosomes in undifferentiated ES cells. We conclude that Cdx2 preferentially associates with X^P in extraembryonic cells, demonstrating imprinted X^P inactivation. We propose that Cdx2 is an activator of *Xist* and that its competitive binding with Oct4 in *Xist* intron 1 is a key determinant of the transition between imprinted and random XCI.

Discussion

Here we have established an *ex vivo* model to study imprinted XCI and identified a novel *trans*-acting factor that regulates imprinted XCI. Based on a Cdx2-iTS model, we show that mouse ES cells retain at least partial memory of the germline imprints that govern imprinted X^P silencing. In this iTS model, a subset of cells demonstrates ectopic *Xist* upregulation, with female cells displaying two *Xist* RNA foci and male cells displaying one. Thus, although the X^P has reactivated in ES cells, it still demonstrates a predisposition toward inactivation and this predisposition can be unmasked when the ES cells are transdifferentiated into trophoblast stem cells—a lineage that normally undergoes imprinted X^P silencing.

It should be noted that our findings largely agree with those of a recent report using a similar iTS model (Murakami *et al.* 2011), in that a majority of iTS cells do overcome the X^P imprint. Several experimental differences may explain why our iTS model retained the X^P imprint in a subset of cells. First, there may be strain-specific differences in imprint retention, as indeed our ES cells are a *M. musculus* $\times$ *castaneus* hybrid, whereas the ES cells of Murakami *et al.* are of CBA $\times$ MsM background. Second, the F₁(CBA $\times$ MsM) female cell line carried a puromycin-marked X^P and a neomycin-marked X^M and was maintained in the ES state under dual drug selection to prevent loss of the X chromosomes (there is a strong tendency for female mouse ES cells to become XO in culture). By requiring that X^P remains continuously active, the system might have disrupted potential asymmetry between X^M and X^P, thereby selecting for ES cells that have a weakened or lost imprint. Finally and perhaps most likely, our ES cells carried a deletion of *Tsix* on X^M, a key regulator of imprinted XCI that enables X^M to resist inactivation (Lee 2000; Sado *et al.* 2001). It is therefore possible that the retained X^P imprint may be revealed only when the maternal block to imprinted XCI is disabled. Importantly, this revelation occurs only when ES cells are transdifferentiated to iTS and is not evident when ES cells are normally differentiated into embryoid bodies.

While not all of our iTS cells revert to imprinted XCI, we observe reproducible X^P skewing, strongly arguing for imprint memory. Our work is consistent with earlier studies implicating X^P memory in mouse ES cells that differentiate into visceral endoderm and mesoderm of the yolk sac, an inner cell mass (ICM) lineage that also demonstrates imprinted XCI (Tada *et al.* 1993). In our work, remnants of the X^P imprint can be observed over many ES passages, are independent of sex (both male and female ES cells demonstrate the same remnant), and are not clonally unique (multiple independent iTS clones showed similar behavior). This observed memory of an XCI imprint in ES cells supports the hypothesis that cell-type-specific factors regulate imprinted XCI in the early embryo and that the imprint can be truly erased only during gametogenesis, as is the case for autosomal imprinting (Edwards and Ferguson-Smith 2007;

Figure 5 Opposing stem cell commitment factors, Cdx2 and Oct4, regulate the switch between imprinted and random XCI in the early mouse embryo. See Discussion for details.

Wan and Bartolomei 2008; Barlow 2011). Several factors within any given ES/iTS culture could contribute to differences in reversion to imprinted XCI. For example, there could be variability at the single-cell level. Recent work suggests that a small transient population within mouse ES cultures lacks Oct4, Sox2, and Nanog expression and can contribute to either embryonic or extraembryonic tissues (Macfarlan *et al.* 2012). Such a subpopulation could be more permissive for imprinted XCI upon Cdx2 induction. Variable Cdx2 expression between cells could also contribute to variable recapitulation of imprinted XCI.

Using the iTS model, we identify Cdx2—a stem cell factor that defines commitment to the extraembryonic lineage—as a regulator of imprinted XCI. Cdx2 directly binds intron 1 of *Xist*. Its binding site overlaps with that for Oct4, the stem cell factor that defines commitment to the embryonic lineage and random XCI. Using both *in vitro* and *in vivo* techniques, we show that Cdx2 and Oct4 binding is mutually exclusive. Given that Cdx2 and Oct4 proteins interact with each other (Niwa *et al.* 2005), we propose that Cdx2 physically competes against Oct4 for binding in *Xist* intron 1 and that in Cdx2 expression in the early embryo effectively ensures that *Xist* is paternally expressed (Figure 5). We suggest that Cdx2 does not bind to X^M because *Tsix* RNA—a known *cis*-acting maternal factor responsible for protecting X^M from inactivation (Lee 2000; Sado *et al.* 2001)—is expressed from X^M in the early embryo. At the blastocyst stage, Cdx2 becomes sequestered to the trophectoderm, maintaining paternal *Xist* expression in this extraembryonic lineage. In the epiblast lineage, Cdx2 is repressed, and Nanog—newly expressed in the morula—collaborates with Oct4 to repress *Xist* expression from both alleles. Our work shows that epiblast-derived cells (ES cells) do not fully lose memory of the paternal imprint and most likely merely override the imprint on X^P to enable binding of Oct4-Nanog to the *Xist* promoter. Indeed, the imprint is at least partially unmasked when ES

cells are transdifferentiated into TS cells and Cdx2 can once again bind to the paternal *Xist* allele. Thus, expression and competition between two lineage-commitment factors, Cdx2 and Oct4, underlie the switch between imprinted and random XCI in the early mouse embryo.

Transcriptional networks of pluripotency transcription factors (pTFs) maintain expression of ES-specific genes and repression of cell-type-specific genes through a positive feedback loop. Previous work identified Cdx2 as a potent transcription factor that causes widespread downregulation of pTF-target genes by interfering with the binding of pTFs to the regulatory region of pTF-target genes on a genome-wide level (Nishiyama *et al.* 2009). In demonstrating Cdx2 inhibition of Oct4 binding to the *Xist*-intron 1 region, our results are consistent with this notion. Also consistent with our results, *Xist* was identified in a ChIPseq analysis as one of 337 Cdx2 targets in ES cells (Nishiyama *et al.* 2009).

Our data raise the possibility that Cdx2 may be involved in reading specific imprints. The nature of such imprints remains elusive. Genetic analysis suggests that DNA methylation maintains random XCI but plays a lesser role in imprinted XCI (Sado 2000, 2005; Takagi 2003; Sado *et al.* 2004). Strong evidence supports a maternally transmitted imprint, which acts to maintain an active X^M by preventing *Xist* upregulation (Goto and Takagi 2000; Lee 2000; Okamoto *et al.* 2000; Tada *et al.* 2000). This maternal imprint is established during oocyte growth, can be set onto maternally transmitted autosomal transgenes, and may lie within the *Tsix* gene. The presence of a specific paternal imprint remains under debate. Recent work suggests that preinactivated intergenic repeats on X^P may underlie paternal XCI in the early mouse embryo (Namekawa *et al.* 2006, 2010). If there are biparental contributions to imprinting, it is formally possible that a maternal imprint prevents Cdx2 from binding to *Xist* intron 1 (e.g., *Tsix* RNA) and/or a paternal imprint attracts Cdx2 binding to *Xist* intron 1. Our

work provides a basis for future investigation into the molecular underpinnings of imprinting and offers a tractable *ex vivo* system in which to do so.

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