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Failure of extra-embryonic progenitor maintenance in the absence of dosage compensation

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SUMMARY

Proper regulation of X-linked gene expression, termed dosage compensation, is required for the normal development of mammalian embryos. Through the process of X chromosome inactivation (XCI), somatic cells of mammalian females inactivate one of their two X chromosomes in order to balance X-linked gene dosage with their male counterparts. The process of XCI is dependent upon the long non-coding RNA *Xist*, which is expressed from and coats the inactivated X chromosome (Xi) in cis. During mouse embryogenesis, imprinted XCI inactivates the paternally inherited X chromosome (Xp) within the extra-embryonic lineages. Consequently, females harboring a paternally derived *Xist* mutation (X/X^{Xist-}) die owing to failure of imprinted XCI and, presumably, poor trophoblast development. Here, we investigate the consequence of two active X chromosomes in the extra-embryonic ectoderm (ExE) of X/X^{Xist-} female embryos. At embryonic day (E) 6.5, we find that the X/X^{Xist-} ExE lacks the transcriptional regulator CDX2, a factor required to maintain the ExE in a progenitor state. In addition, spongiotrophoblast progenitors are not maintained. Surprisingly, we observe evidence of an Xi in a subpopulation of X/X^{Xist-} ExE cells. We demonstrate further that trophectodermal stem cells derived from X/X^{Xist-} embryos completely reverse normal imprinted XCI patterns. Taken together, our data suggest that, much like in the cells of the epiblast, the initial imprint that establishes imprinted XCI is probably erased in ExE cells. Conversely, unlike the epiblast, in which XCI is not required for progenitor cell maintenance, we demonstrate that dosage compensation is indispensable for the maintenance of trophoblast progenitors.

KEY WORDS: X chromosome inactivation, Extra-embryonic ectoderm, CDX2, *Xist*, Mouse

INTRODUCTION

Unlike their male counterparts, mammalian female somatic cells must address a problem of X-linked gene dosage disequilibrium owing to the presence of two X chromosomes within the nuclei of each cell. In order to solve this problem, cells of female embryos undergo a process termed X chromosome inactivation (XCI), in which one of the two X chromosomes is rendered, for the most part, transcriptionally silent. During mouse embryogenesis, two forms of XCI are observed: imprinted and random. Imprinted XCI inactivates the paternally inherited X chromosome (Xp) and occurs in the extra-embryonic lineages. Random XCI occurs in the embryonic lineages in which either the Xp or the maternally inherited X chromosome (Xm) can be inactivated.

The transcriptionally silenced state of the Xi is characterized by the depletion of epigenetic modifications associated with transcriptional activation and the enrichment of those associated with transcriptional repression (reviewed by Chow and Heard, 2009). Both random and imprinted XCI are dependent upon the function of a long non-coding RNA, *Xist*, which is expressed from and coats the future inactivated X chromosome (Xi) in cis (Clemson et al., 1996). *Xist* is required to recruit the polycomb repressive complex 2 (PRC2) to the Xi (Plath et al., 2003; Zhao et al., 2008), which catalyzes the tri-methylation of lysine 27 of histone H3 (H3K27me3). In addition to H3K27me3, the Xi is enriched with other chromatin modifications associated with transcriptional repression, including, but not limited to, H4K20me1, macroH2A and methylation of CpG-rich domains (reviewed by Chow and Heard, 2009).

The inheritance of a non-functional copy of *Xist* on the Xp results in female-specific embryonic lethality (Marahrens et al., 1997). Such embryos typically die by embryonic day (E) 6.5 owing to lack of proper trophectodermal development, presumably due to failure of imprinted XCI in the extra-embryonic lineages, specifically the ExE. The consequence of failed imprinted XCI within the developing trophectoderm remains unexplored. As the development of the embryo proper is tightly associated with the proper development of the extra-embryonic tissues, it is increasingly important to understand the link between gene regulation and development of the extra-embryonic lineages.

Much of our knowledge regarding the generation of extra-embryonic tissues stems from studies in the mouse. All extra-embryonic progenitors are specified early in development and are largely derived from two cell populations: the trophectoderm and the primitive endoderm. During the blastocyst stages, the trophectoderm is morphologically distinguished as the outermost spherical monolayer of cells of the embryo. The inner cell mass (ICM) is a small population of cells clustered at one location attached to the inner surface of the trophectoderm.

The trophectoderm and ICM are not homogeneous cell populations. The trophectoderm consists of two populations of cells: the mural trophectoderm, which differentiates into primary giant cells, and the polar trophectoderm which gives rise to the extra-embryonic ectoderm (ExE). The ExE is a self-renewing progenitor population for the majority of cells within the placenta (reviewed by Roberts et al., 2004). Similarly, after the mid-blastocyst stage, the ICM can be further subdivided into two cell populations: the epiblast, which gives rise to the embryo proper, and the primitive endoderm, which gives rise to, among other tissues, extra-embryonic yolk sac.

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At the mid-blastocyst stage, the trophectoderm and epiblast are molecularly distinguished by the mutually exclusive expression of the transcriptional regulators *Cdx2* and *Oct4* (*Pou5f1* – Mouse Genome Informatics), respectively (reviewed by Ralston and Rossant, 2005). OCT4 is required for the maintenance of a pluripotent state within the epiblast and is thought to transcriptionally repress *Xist* in embryonic stem (ES) cells (Navarro et al., 2008), suggesting that XCI is not required for ES cell maintenance. Throughout early post-implantation development, *Cdx2* is required for the maintenance and multipotent state of the ExE (Strumpf et al., 2005). The requirement for XCI within the context of ExE maintenance is unknown, although it has been noted that stable dosage compensation might not be required in more differentiated trophectoderm cells (Ohhata et al., 2011).

Here, we use a paternally transmitted non-functional *Xist* allele ($X^{Xist1Lox}$) (Csankovszki et al., 1999) to investigate the consequence of failed imprinted XCI within the ExE of $X/X^{Xist1Lox}$ female embryos. Molecular analysis reveals that that the E6.5 ExE and ectoplacental cone of $X/X^{Xist1Lox}$ embryos mainly consists of differentiated giant cells and their progenitors, but does not maintain less differentiated spongiotrophoblast progenitors. Furthermore, we find that the E6.5 $X/X^{Xist1Lox}$ ExE lacks CDX2. Curiously, we observe evidence of an Xi in a few cells of the $X/X^{Xist1Lox}$ ExE. When grown in culture, $X/X^{Xist1Lox}$ embryo outgrowths retain CDX2 and harbor an Xi in many CDX2+ cells. Finally, by both cytological and allele-specific transcriptional analysis, trophectodermal stem (TS) cells derived from $X/X^{Xist1Lox}$ embryos harbor an inactive Xm.

Taken together, our data demonstrate a previously unappreciated capacity for the reversal of imprinted XCI in extra-embryonic progenitor cells, suggesting that, like epiblast cells, the ExE might no longer retain the initial imprint required for imprinted XCI. Furthermore, the poor trophectoderm development in $X/X^{Xist1Lox}$ embryos is due to a failed maintenance of trophectoderm precursors, probably through the premature differentiation and exhaustion of CDX2+ trophoblast progenitors. This latter finding demonstrates an absolute requirement for dosage compensation in order to maintain ExE progenitor cells during early mouse development.

MATERIALS AND METHODS

Mouse strains and genotyping

Animal care and research protocols were performed in accordance with The University of North Carolina at Chapel Hill's institutional guidelines, as approved by IACUC at The University of North Carolina at Chapel Hill. CD1 (Charles River Laboratories) and JF1 (Mager et al., 2003) were separately maintained through normal intercrossing of these two strains. $Xist^{tm2Jae}$ ($X^{Xist2Lox}$) (Csankovszki et al., 1999) were maintained on a 129/Sv background. $X^{Xist1Lox}$ mice were generated as previously described (Kalantry et al., 2009) and maintained on a 129/Sv background. The $X_{129}{}^{Xist2Lox;GFP}$ and $X_{129}{}^{Xist1Lox;GFP}$ lines were derived as previously described (Williams et al., 2011). The $Gt(ROSA)26Sor^{tm1Sor}$ (*R26R*) (Soriano, 1999), *Tg(Sox2-cre)1Amc* (*Sox2Cre*) (Hayashi et al., 2002) and *(TgCAG-GFP)D4Nagy* (X^{GFP}) (Hadjantonakis et al., 1998) transgenic lines are described previously. For staging of embryonic development, the morning of a detected vaginal plug was considered to be E0.5. To avoid potential contamination of maternal tissues of embryos fixed within the decidua (see below) or the disruption of blastocyst outgrowths, female embryos were identified by immunofluorescence for H3K27me3 (see below) within the epiblast or outgrowth, which identifies the territory of the Xi.

Cell and embryo culture

$X_{JF1}/X_{129}{}^{Xist2Lox;GFP}$ and $X_{JF1}/X_{129}{}^{Xist1Lox;GFP}$ TS cells were generated and maintained using standard methods for TS cell derivation (Himeno et al., 2008). TS medium (TSM) is mouse embryonic fibroblast (MEF) conditioned TS cell medium supplied with 1.5× FGF4 and heparin (F4H) (Himeno et al., 2008). In order to differentiate TS cells, F4H was removed from growth medium and cells were collected after 2, 4 and 6 days of F4H removal. To avoid feeder contamination in RT-PCR experiments, cells were grown in feeder-free conditions using MEF-conditioned medium with or without 1× F4H.

Attempts to generate $X_{JF1}/X_{129}{}^{Xist1Lox;GFP}$ TS cells from $X_{JF1}/X_{129}{}^{Xist2Lox;GFP}$ TS cells were performed by standard electroporation of a CAGGGS-*Cre* expression plasmid (AddGene) modified to include a PGK-*Neo* resistance cassette into $X_{JF1}/X_{129}{}^{Xist2Lox;GFP}$ TS cells. Neo-resistant $X_{JF1}/X_{129}{}^{Xist1Lox;GFP}$ TS cells were never obtained, whereas $X_{JF1}/X_{129}{}^{Xist2Lox;GFP}$ TS cells harboring partial *Cre* constructs were readily recovered. $X_{JF1}/X_{129}{}^{Xist2Lox;GFP}$ and $X_{JF1}/X_{129}{}^{Xist1Lox;GFP}$ TS cells were genotyped by PCR for the 2Lox or 1Lox *Xist* alleles as previously described (Csankovszki et al., 1999).

For blastocyst outgrowths, $X/X^{Xist2Lox}$ and $X/X^{Xist1Lox}$ blastocysts were flushed from the uteri of pregnant females into M2 media (Millipore) under embryo certified mineral oil (Sigma). Blastocysts were then washed through multiple pre-warmed droplets of either normal medium (α-MEM and 15% fetal bovine serum) or TSM (see above) under oil. Finally, blastocysts were distributed into gelatin-coated Lab-TekII (Thermo Scientific Nunc) 4-well chamber slides containing 500 μm of either normal medium or TSM per well. Embryos were cultured for three days in standard cell culture incubators at 5% carbon dioxide at atmospheric oxygen and nitrogen.

Histology

E6.5 $X/X^{Xist2Lox}$ or E6.5 $X/X^{Xist1Lox}$ embryos were obtained and fixed in 4% paraformaldehyde (Electron Microscopy Systems) in PBS for either 10 minutes at room temperature (RT) for immunofluorescence or overnight (O/N) at 4°C for in situ hybridization. To avoid mechanical disruption of the extra-embryonic tissues, embryos were fixed and sectioned while in the maternal decidua. After fixation, samples were washed three times in PBS at RT and placed in 30% sucrose in PBS O/N at 4°C. Samples were then mounted in OCT (Electron Microscopy Systems) and cryosectioned on a CM3050s cryostat (Leica Microsystems) at a thickness of 10 μm (immunofluorescence) or 16 μm (section in situ hybridization). Up to three serial sections were taken for each embryo on separate slides.

Immunofluorescence, in situ hybridization and fluorescence in situ hybridization

Immunofluorescence was carried out on cryosectioned tissue or blastocyst outgrowths as previously described (Mugford et al., 2008). Blastocyst outgrowths were fixed in chamber wells in an identical manner to E6.5 embryos prepared for immunofluorescence (see above). Antibodies used were as follows: rabbit anti-H3K27me3 (Cell Signaling, 1:100), mouse IgG3 anti-H3K27me3 (Abcam 1:100), mouse IgG2a anti-CDX2 (BioGenex, 1:200), mouse IgG1 anti-EED (Aire Otte, 1:200) (Hamer et al., 2002), mouse IgG2b anti-OCT3/4 (Santa Cruz, 1:100), rabbit anti-EOMES (Abcam, 1:500), rabbit anti-activeCASPASE3 (Cell Signaling, 1:200), rabbit anti-Ki67 (Abcam, 1:500) and chicken anti-GFP (AvesLabs, 1:200). All secondary antibodies, except for anti-chicken, were Alexa Fluor-conjugated (Invitrogen, 1:1000) appropriately matched to the primary antibody species and antibody isotype. The anti-GFP antibody was detected using Cy2-conjugated anti-chicken secondary antibodies (Jackson ImmunoResearch, 1:500). When multiple mouse antibodies were used, we observed no cross-reactivity among isotype-specific secondary antibodies.

RNA fluorescence in situ hybridization (FISH) on TS cells and section in situ hybridization on embryos were carried out as previously described (Kalantry et al., 2006; Mugford et al., 2009). At least eight E6.5 $X/X^{Xist1Lox}$ embryos were tested for each gene. The FISH *Xist* probe targeted against *Xist* exon 1 was generated by PCR using fosmid clone G135P63425C4 as a template (see supplementary material Table S1 for primer sequences). *Xist* exon 1 probes overlapped perfectly with full-length probes. Immunofluorescence followed FISH.

All images were taken on a Zeiss AxioImager M2 (Carl Zeiss) equipped with MRc and MRm cameras. Single plane, color images using Nomarski filters were taken for section in situ hybridization. For fluorescent images,

multiple grayscale images were taken through a given sample, such that the vertical distance between each image was half of the depth of field of the objective used. *z*-stacks were deconvolved using Axiovision software (Carl Zeiss) and projected into a single plane using a maximal projection. Grayscale images were pseudocolored and merged in Photoshop CS4 (Adobe Systems).

Allele-specific RT-PCR and qRT-PCR

For allele-specific RT-PCR and qPCR, total RNA was extracted from equal numbers of feeder-free $X_{JF1}/X_{129}^{Xist2Lox;GFP}$ and $X_{JF1}/X_{129}^{Xist1Lox;GFP}$ TS cells grown in the presence or absence of F4H using the normal Trizol protocol (Invitrogen). *JF1/JF1* and *129/129* RNA samples were isolated from placentas of E9.5 female embryos. For all assays except *Xist* allele-specific RT-PCR, the standard SuperScriptIII protocol (Invitrogen) was used with random hexamers to generate cDNA libraries from 1 μg of RNA of each sample. For *Xist*, a strand-specific primer was used to generate cDNA. Each result is representative of two independent $X_{JF1}/X_{129}^{Xist2Lox;GFP}$ and three independent $X_{JF1}/X_{129}^{Xist1Lox;GFP}$ TS cell lines.

For qPCR, equal amounts of cDNA were used with transcript-specific primer pairs. SsoFast EvaGreen Supermix (BioRad) was used for all qPCR reactions and performed on a CFX96 Real Time System (BioRad) attached to a C1000 Thermal Cycler (BioRad). All samples were normalized to *Gapdh* and displayed relative to the undifferentiated state of the specific cell line. Error bars represent standard deviation of the mean among three technical replicates. Each result is representative of two independent $X_{JF1}/X_{129}^{Xist2Lox;GFP}$ and two independent $X_{JF1}/X_{129}^{Xist1Lox;GFP}$ TS cell lines.

All relevant information regarding primer sequences, restriction enzymes, and references for primers (if applicable) are available in supplementary material Table S1.

RESULTS

The $X/X^{Xist1Lox}$ ExE is deficient for spongiotrophoblast precursors

In order to generate *Xist* mutant and wild-type embryos, we used the $Xist^{tm2Jae}$ mouse strain (wild type, referred to as $X^{Xist2Lox}$) (Csankovszki et al., 1999), in which the promoter and first three exons of *Xist* are flanked by *loxP* sites, allowing for their removal via Cre-mediated excision. Males harboring the $X^{Xist2Lox}$ or the $X^{Xist1Lox}$ (mutant, see Materials and methods for generation) alleles were mated to wild-type CD1 females to generate $X/X^{Xist2Lox}$ and $X/X^{Xist1Lox}$ embryos, respectively. At E6.5 and prior, mutant and wild-type embryos were recovered in expected Mendelian ratios. As noted previously (Marahrens et al., 1997), E6.5 $X/X^{Xist1Lox}$ embryos display variable phenotypes beginning at E6.5, and are not readily recovered at normal Mendelian frequencies after E6.5. Regardless of phenotypic severity, $X/X^{Xist1Lox}$ embryos exhibit defects in extra-embryonic development, namely in the region normally occupied by the ExE and ectoplacental cone (Marahrens et al., 1997). As the ExE and its derivative harbor the precursors to the majority of the trophoblast lineages (reviewed by Roberts et al., 2004), we sought to identify whether failed dosage compensation led to defects in the initial specification or maintenance of trophoblast precursors in developing $X/X^{Xist1Lox}$ embryos.

The transcriptional regulator *Hand1* promotes the differentiation of spongiotrophoblasts into endoreduplicated giant cells (Hughes et al., 2004). Conversely, the transcriptional regulator *Ascl2* (*Mash2*) represses *Hand1*, and maintains spongiotrophoblast cells in a less differentiated state (Guillemot et al., 1994). Section in situ hybridization at E6.5 revealed that *Hand1* and *Ascl2* were both present in $X/X^{Xist2Lox}$ ExE (Fig. 1A,B, respectively). *Hand1*-expressing cells were detected in E6.5 $X/X^{Xist1Lox}$ embryos, though the size of the *Hand1* expression domain was markedly smaller in E6.5 $X/X^{Xist1Lox}$ embryos (Fig. 1C). We did not detect *Ascl2* transcripts $X/X^{Xist1Lox}$ ExE (Fig. 1D).

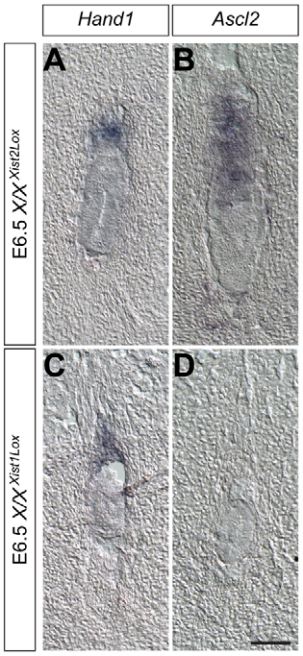

Fig. 1. Spongiotrophoblast progenitors are not maintained in E6.5 $X/X^{Xist1Lox}$ embryos. (**A-D**) Section in situ hybridization in E6.5 $X/X^{Xist2Lox}$ (A,B) and E6.5 $X/X^{Xist1Lox}$ (C,D) embryos for *Hand1* (A,C) (giant cell precursors) and *Ascl2* (B,D) (spongiotrophoblast precursors). Scale bar: 100 μm.

Section in situ hybridization for markers of more differentiated trophoblast cell types (Simmons et al., 2008), such as *Cebpb* and *Gcm1* (labyrinthine layer), *Prl3d1* (or placental lactogen 1, giant cells), and *Tpbpa* (spongiotrophoblast) was also performed. *Cebpb* and *Gcm1* were not detected in either E6.5 $X/X^{Xist2Lox}$ (supplementary material Fig. S1A,B, respectively) or $X/X^{Xist1Lox}$ (supplementary material Fig. S1E,F, respectively) embryos. Conversely, *Prl3d1*-expressing cells were found in both E6.5 $X/X^{Xist2Lox}$ (supplementary material Fig. S1C) and $X/X^{Xist1Lox}$ (supplementary material Fig. S1G) embryos. Finally, a small number of *Tpbpa*-expressing cells were identified at the posterior pole of E6.5 $X/X^{Xist2Lox}$ embryos (supplementary material Fig. S1D). The same staining pattern was not observed in E6.5 $X/X^{Xist1Lox}$ embryos, although a weak signal was observed at the posterior pole of mutant embryos (supplementary material Fig. S1H). These results held true across the spectrum of phenotypes observed in E6.5 $X/X^{Xist1Lox}$ embryos, suggesting that a failure of imprinted XCI compromises the ExE such that it cannot properly maintain precursors of the spongiotrophoblast lineage.

The $X/X^{Xist1Lox}$ ExE is not maintained as a progenitor population

We next investigated whether the diminished number of ExE cells in $X/X^{Xist1Lox}$ embryos was due to a diminished capacity for proliferation or a higher rate of cell death. We performed immunofluorescence for Ki67 (MKI67 – Mouse Genome Informatics), a protein present in actively dividing cells (Starborg et al., 1996). Compared with E6.5 $X/X^{Xist2Lox}$ embryos, we did not observe a defect in the presence of Ki67 in either the embryonic or the extra-embryonic E6.5 $X/X^{Xist1Lox}$ cellular compartments (supplementary material Fig. S2A,B,E,F). Active caspase 3 is found in cells undergoing apoptosis (Kuida et al., 1996). By

immunofluorescence, E6.5 *X/X*^{Xist2Lox} and *X/X*^{Xist1Lox} embryos were indistinguishable with regard to the number (zero to two cells per section) and distribution of active caspase 3+ cells (supplementary material Fig. S2C,D,G,H). Taken together, these data suggest that the diminished size of the *X/X*^{Xist1Lox} ExE was not due to either inhibited cellular proliferation or to excessive apoptosis within the extra-embryonic compartment.

Cdx2 is a transcriptional regulator required to maintain the ExE in a progenitor state and is not present in the embryo proper (Strumpf et al., 2005). Conversely, the transcriptional regulator *Pou5f1* (*Oct4*) is required for the pluripotent state of the epiblast and is not found in the ExE (Nichols et al., 1998). In addition, the transcriptional regulator eomesodermin (*Eomes*) is found in trophoblast progenitors, as well as in the developing mesoderm of the embryo proper, and in the visceral endoderm (Russ et al., 2000). Finally, H3K27me3 is enriched on the Xi at higher levels than any other region of the genome such that immunofluorescence for this modification can be used to visually identify the territory of the Xi in both the epiblast and ExE (Plath et al., 2003).

Using immunofluorescence for the above proteins, we investigated whether an aberrant distribution of these antigens in E6.5 *X/X*^{Xist1Lox} embryos gave further clues regarding the loss of the ExE. As expected, OCT4 and CDX2 were present in mutually exclusive populations of the developing E6.5 *X/X*^{Xist2Lox} embryos (Fig. 2A,B,D,E,L-O), whereas EOMES was present in the OCT4+ primitive streak, the CDX2+ ExE and the OCT4– CDX2– visceral endoderm (Fig. 2C-E).

Phenotypic defects notwithstanding, the distributions of EOMES and OCT4 within E6.5 *X/X*^{Xist1Lox} embryos were similar to those of E6.5 *X/X*^{Xist2Lox} embryos (Fig. 2F,H-J). Although we detected OCT4– EOMES+ cells in a spatial location consistent with ExE, we did not observe CDX2+ cells in E6.5 *X/X*^{Xist1Lox} embryos (Fig. 2G,I,J,Q,S,T). Conversely, we note the existence of a properly formed EOMES+ OCT4– visceral endoderm lining the epiblast of E6.5 *X/X*^{Xist1Lox} embryos (Fig. 2F,H-J), suggesting that not all extra-embryonic cells are affected by the lack of imprinted XCI. To ensure that lack of a CDX2+ ExE was not due to improper specification of the ExE, we immunostained E5.5 *X/X*^{Xist1Lox} embryos for CDX2 and observed CDX2+ cells in E5.5 *X/X*^{Xist1Lox} and E5.5 *X/X*^{Xist2Lox} embryos (supplementary material Fig. S3). We do note, however, that the overall size of E5.5 *X/X*^{Xist1Lox} embryos is slightly smaller than that of their E5.5 *X/X*^{Xist2Lox} counterparts and that numbers of CDX2+ cells appeared to be diminished in E5.5 *X/X*^{Xist1Lox} embryos (supplementary material Fig. S3).

Serial sections through the same embryos displayed in Fig. 2A-J were immunostained for H3K27me3, CDX2 and OCT4 (Fig. 2K-T) or H3K27me3, CDX2 and EOMES (supplementary material Fig. S4A-J). CDX2+ cells were not found throughout the E6.5 *X/X*^{Xist1Lox} embryo. Notably, an Xi was readily observed in cells of the CDX2+, EOMES+ ExE and the OCT4+ EOMES+ epiblast of E6.5 *X/X*^{Xist2Lox} embryos (H3K27me3 punctate dots in Fig. 2K,N,O and supplementary material Fig. S4A,D,E). Similarly, within E6.5 *X/X*^{Xist1Lox} embryos, an Xi was observed in cells of the OCT4+ EOMES+ epiblast (Fig. 2P,S,T; supplementary material Fig. S4F,I,J). We did not detect evidence of an Xi, at least by H3K27me3 immunostaining, in OCT4– EOMES+ visceral endoderm lining the epiblast in either E6.5 *X/X*^{Xist2Lox} or E6.5 *X/X*^{Xist1Lox} embryos (Fig. 2K-T; supplementary material Fig. S4). As expected, we did not detect foci of H3K27me3 in the majority of the EOMES+ OCT4– ExE of E6.5 *X/X*^{Xist1Lox} embryos (Fig. 2S,T; supplementary material Fig. S4I,J), suggesting that imprinted XCI probably did not occur in the absence of paternally supplied *Xist*.

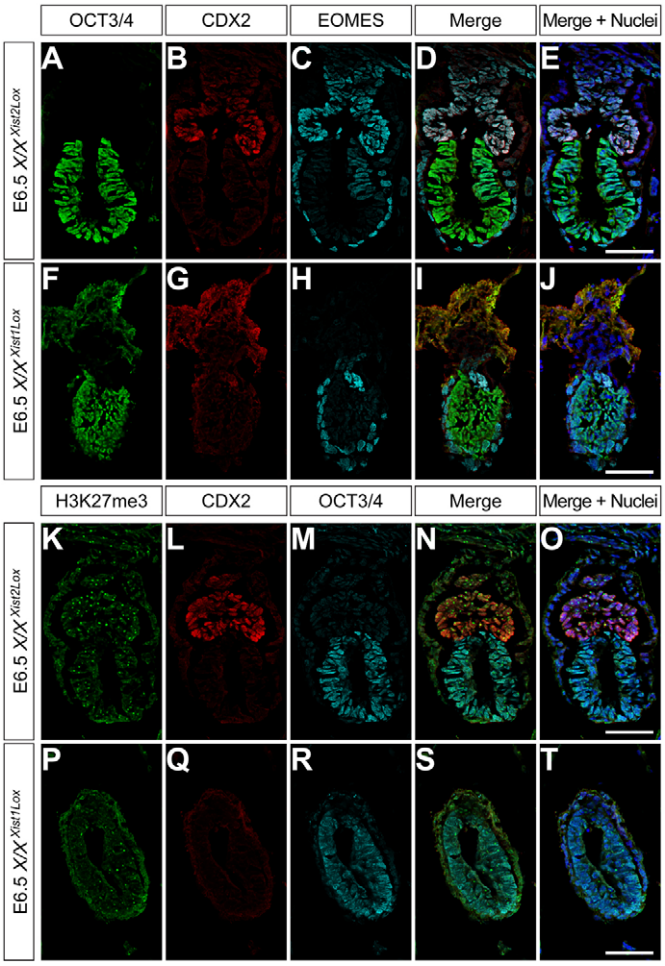

Fig. 2. E6.5 *X/X*^{Xist1Lox} embryos lack a CDX2+ ExE. (**A-T**) Immunofluorescence in serial sections of E6.5 *X/X*^{Xist2Lox} (A-E,K-O) and E6.5 *X/X*^{Xist1Lox} (F-J,P-T) embryos for Oct 3/4 (A,F,M,R), CDX2 (B,G,L,Q), EOMES (C,H) and H3K27me3 (K,P). Merged images without (D,I,N,S) and with (E,J,O,T) DAPI-stained nuclei are shown. Scale bars: 50 μm.

Taken together, our data suggest that in the absence of imprinted XCI, cells of the ExE are specified correctly, proliferate normally and are not apoptotic. However, failed dosage compensation leads to lack of ExE maintenance due to the absence of CDX2 function. We suggest that the ExE of E6.5 *X/X*^{Xist1Lox} embryos is rapidly exhausted by premature cell differentiation into syncitiotrophoblast progenitors.

An Xi is observed in a limited number of *X/X*^{Xist1Lox} ExE cells

As mentioned previously, the phenotype of E6.5 *X/X*^{Xist1Lox} embryos is variable and the majority of E6.5 *X/X*^{Xist1Lox} embryos did not harbor an Xi in cells of their ExE (Fig. 2; supplementary material Fig. S4). This latter observation applied to those E6.5 *X/X*^{Xist1Lox} embryos displaying the most severe (not shown) and moderate (Fig. 2; supplementary material Fig. S4) morphological phenotypes. However, we noticed a striking difference within the ExE of E6.5 *X/X*^{Xist1Lox} embryos displaying the mildest phenotypes (4/21 E6.5 *X/X*^{Xist1Lox} embryos). Using immunofluorescence for H3K27me3, OCT4 and EOMES in mildly affected E6.5 *X/X*^{Xist1Lox} embryos, we discovered a limited number of OCT4– EOMES+

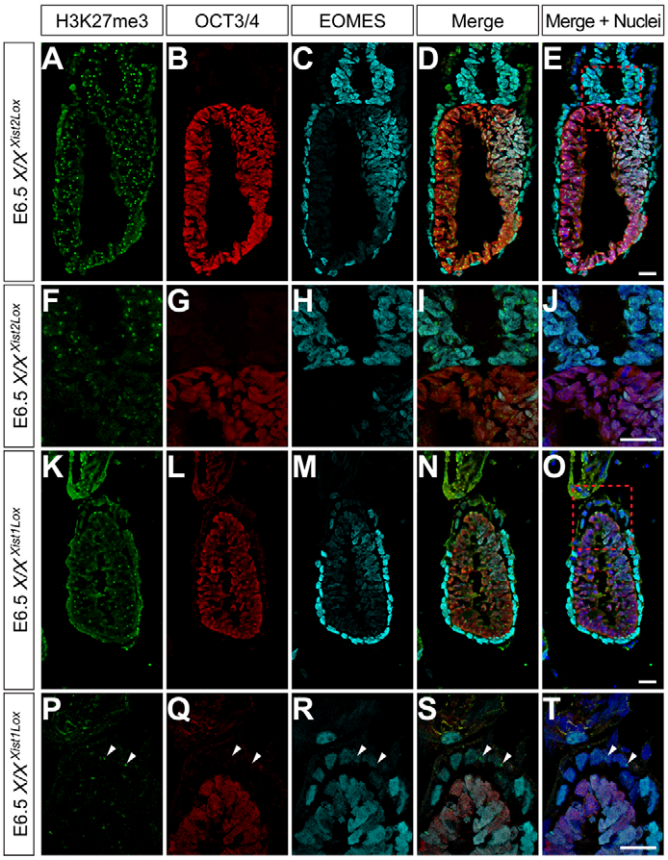

Fig. 3. A small number of E6.5 *X/X*^{Xist1Lox} ExE cells harbor a putative Xi. (**A-T**) Immunofluorescence in E6.5 *X/X*^{Xist2Lox} (A-J) and E6.5 *X/X*^{Xist1Lox} (K-T) embryos for H3K27me3 (A,F,K,P), Oct3/4 (B,G,L,Q) and EOMES (C,H,M,R). Merged images without (D,I,N,S) and with (E,J,O,T) DAPI-stained nuclei are shown. High magnification views of the red dashed boxes in E and O are represented in F-J and P-T, respectively. White arrowheads (P-T) indicate a focal enrichment of H3K27me3 in E6.5 *X/X*^{Xist1Lox} EOMES+ cells. Scale bars: 20 μm.

ExE cells (between three and five cells per embryo) that harbored a focal point of H3K27me3 (Fig. 3K-T, arrowheads in P-T). This H3K27me3 pattern was reminiscent of the Xi territory observed in CDX2+ EOMES+ ExE cells of E6.5 *X/X*^{Xist2Lox} embryos (Fig. 3A-J). In all cases in which we observed a focal enrichment of H3K27me3 (putative Xi) within the nuclei of E6.5 *X/X*^{Xist1Lox} ExE cells, CDX2 was not present. Furthermore, E6.5 *X/X*^{Xist1Lox} ExE cells harboring a putative Xi were located adjacent to OCT4+ epiblast cells. We did not observe a putative Xi in cells of the E6.5 *X/X*^{Xist1Lox} EOMES– ectoplacental cone.

Given that the epiblast is a source of growth factors required for the maintenance of the ExE (namely FGF4) (Goldin and Papaioannou, 2003) and that *X/X*^{Xist1Lox} ExE cells harboring a putative Xi were located adjacent to the epiblast, we reasoned that promoting ExE development in *X/X*^{Xist1Lox} cells might increase the frequency of finding a putative Xi. To this end, we cultured *X/X*^{Xist2Lox} and *X/X*^{Xist1Lox} blastocysts for three days in two different media: one composed of α-MEM and 15% FBS (normal medium) and the other a formulation normally used to derive trophectoderm stem (TS) cells (TSM, see Materials and methods).

Immunofluorescence for CDX2, OCT4 and H3K27me3 was carried out on blastocyst outgrowths. Regardless of the medium used, we observed the presence of CDX2+ cells and OCT4+ cells in the outgrowths of both *X/X*^{Xist2Lox} (Fig. 4A,B,D,E) and *X/X*^{Xist1Lox} embryos (Fig. 4F,G,I,J). As expected, the presence of OCT4 was mutually exclusive of CDX2. H3K27me3 immunofluorescence demonstrated the presence of an Xi in both OCT4+ and CDX2+ cells of *X/X*^{Xist2Lox} blastocyst outgrowths (Fig. 4A-E), as well as in OCT4+ cells of *X/X*^{Xist1Lox} blastocyst outgrowths (Fig. 4F,H-J).

CDX2 was not present in all *X/X*^{Xist1Lox} embryos (9/36 *X/X*^{Xist1Lox} outgrowths contained CDX2+ cells). In fact, culturing in the presence of TSM did not have an effect on the number of outgrowths containing CDX2+ cells, although when present, the population CDX2+ cells in embryos cultured in TSM was expanded compared with those cultured in normal medium. Interestingly, a putative Xi was observed in many *X/X*^{Xist1Lox} CDX2+ cells (8/9 *X/X*^{Xist1Lox} outgrowths with CDX2+ cells, 8/36 total *X/X*^{Xist1Lox} outgrowths) (Fig. 4G-J, H3K27me3 punctate dots). In contrast to their *X/X*^{Xist2Lox} counterparts, we note that giant cells present in *X/X*^{Xist1Lox} blastocyst cultures lack evidence of an Xi (supplementary material Fig. S5).

Taken together, our data suggest that in the absence of imprinted XCI, the ExE is not sustained as a progenitor population, though it is not necessarily incapable of responding to cues required for its proliferation and survival. Furthermore, our observations of a focal enrichment of H3K27me3 in both E6.5 *X/X*^{Xist1Lox} ExE cells and in CDX2+ cells of *X/X*^{Xist1Lox} blastocyst outgrowths suggest that the ExE either has a capacity to override imprinted XCI or that, much like the epiblast, the imprint underlying imprinted XCI is no longer maintained in the E6.5 ExE.

X/X^{Xist1Lox} trophectodermal stem cells harbor an inactive maternal X chromosome

To test whether the putative Xi observed in the ExE of *X/X*^{Xist1Lox} embryos and in *X/X*^{Xist1Lox} embryo outgrowths is indeed an inactive maternally derived X chromosome (Xm), we derived female TS cell lines from both *X/X*^{Xist2Lox} and *X/X*^{Xist1Lox} blastocysts using standard derivation methods (Himeno et al., 2008). Female TS cells maintain imprinted XCI and therefore normally harbor an inactive Xp (Kalantry et al., 2006). If the ExE has the ability to override imprinted XCI, this strategy should allow us to observe this switch on a large scale.

In order to assess accurately the transcriptional activity of the X chromosomes, we employed two different strategies. The *X*^{Xist2Lox} and *X*^{Xist1Lox} chromosomes are of 129/SvJae origin (*Mus musculus domesticus*). We intercrossed the *Xist* alleles with mice harboring an X-linked *Gfp* coding sequence under the control of the CAGGS promoter [*(TgCAG-GFP)D4Nagy*, termed *X*^{GFP}] (Hadjantonakis et al., 1998), also of 129/Sv origin, in order to generate $X_{129}^{Xist2Lox;GFP}$ or $X_{129}^{Xist1Lox;GFP}$ alleles. Previous studies have shown that *X*^{GFP} is subject to imprinted XCI in TS cells (Kalantry et al., 2006).

Males harboring either $X_{129}^{Xist2Lox;GFP}$ or $X_{129}^{Xist1Lox;GFP}$ were intercrossed with wild-type JF1 (*Mus musculus molossinus*) females. Therefore, in $X_{JF1}/X_{129}^{Xist2Lox;GFP}$ or $X_{JF1}/X_{129}^{Xist1Lox;GFP}$ TS cells, the global activity of the Xp and, indirectly, imprinted XCI, was monitored via the presence or absence of GFP. In addition, substrain-specific single nucleotide polymorphism (SNPs) located within coding regions of transcribed X-linked genes was leveraged via RT-PCR to assess the transcriptional activity of the Xm or Xp in $X_{JF1}/X_{129}^{Xist2Lox;GFP}$ or $X_{JF1}/X_{129}^{Xist1Lox;GFP}$ TS cells.

Several lines of $X_{JF1}/X_{129}^{Xist2Lox;GFP}$ and $X_{JF1}/X_{129}^{Xist1Lox;GFP}$ TS cells were derived from separate crosses of male and female mice. The overall cell morphology, colony organization and growth rates of $X_{JF1}/X_{129}^{Xist2Lox;GFP}$ and $X_{JF1}/X_{129}^{Xist1Lox;GFP}$ TS cells were comparable. A differentiation time course was performed on both

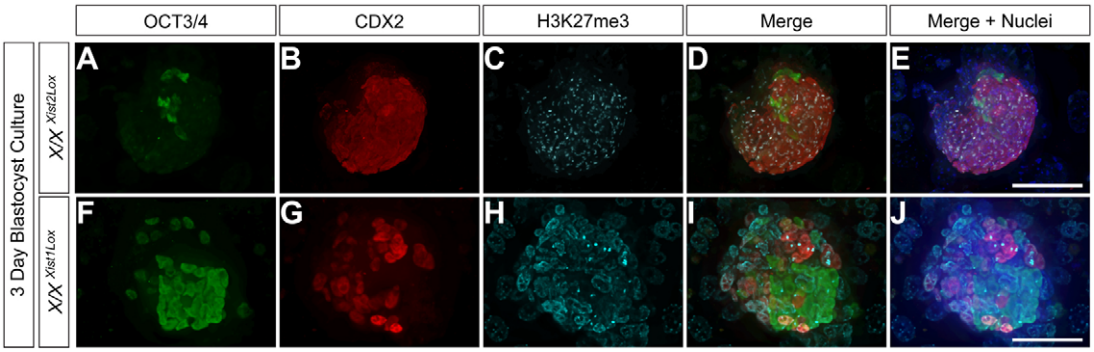

Fig. 4. CDX2+ cells harboring a putative Xi are observed in *X/X^{Xist1Lox}* blastocyst outgrowths. (**A-J**) Immunofluorescence in *X/X^{Xist2Lox}* (A-E) and *X/X^{Xist1Lox}* (F-J) blastocyst outgrowths for Oct3/4 (A,F), CDX2 (B,G) and H3K27me3 (C,H). Merged images without (D,I) and with (E,J) DAPI-stained nuclei are shown. Scale bars: 50 μm.

$X_{JF1}/X_{129}^{Xist2Lox;GFP}$ and $X_{JF1}/X_{129}^{Xist1Lox;GFP}$ TS cells by the removal of FGF4 and heparin (see Materials and methods). As differentiation occurred, we noted that both $X_{JF1}/X_{129}^{Xist2Lox;GFP}$ and $X_{JF1}/X_{129}^{Xist1Lox;GFP}$ TS cells inactivated genes required for a TS cell progenitor state (*Cdx2* and *Eomes*), transiently activated genes required for syncitiotriophoblast and spongiotrophoblast differentiation (*Gcm1* and *Ascl2*, respectively), and activated genes found in differentiated spongiotrophoblast and giant cells (*Tpbpa* and *Prl3d(1-3)*, respectively) (supplementary material Fig. S6).

We next assayed cell lines for the presence of GFP, CDX2, EED, H3K27me3 and *Xist* (Fig. 5). CDX2 was present in both $X_{JF1}/X_{129}^{Xist2Lox;GFP}$ (Fig. 5B,D,E) and $X_{JF1}/X_{129}^{Xist1Lox;GFP}$ TS cells (Fig. 5G,I,J). As expected, GFP was present in $X_{JF1}/X_{129}^{Xist1Lox;GFP}$ TS cells (Fig. 5F,I,J), but not in $X_{JF1}/X_{129}^{Xist2Lox;GFP}$ (Fig. 5A,D,E) TS cells, suggesting that the Xp is active in $X_{JF1}/X_{129}^{Xist1Lox;GFP}$ TS cells, whereas imprinted XCI was properly maintained in $X_{JF1}/X_{129}^{Xist2Lox;GFP}$ TS cells. EED is a member of the polycomb repressive complex 2 (PRC2) and is also enriched on the Xi in TS cells (Kalantry et al., 2006). A focal enrichment of EED was present in both $X_{JF1}/X_{129}^{Xist2Lox;GFP}$ (Fig. 5C-E) and $X_{JF1}/X_{129}^{Xist1Lox;GFP}$ TS cells (Fig. 5H-J), suggesting the presence of an Xi.

In order to assess whether maternally supplied *Xist* is active and functional in $X_{JF1}/X_{129}^{Xist1Lox;GFP}$ TS cells, we performed RNA fluorescent in situ hybridization (FISH) for *Xist* transcripts along with immunofluorescence for EED and H3K27me3. If *Xist* is functional, signals for *Xist*, EED and H3K27me3 should overlap, as *Xist* is thought to recruit PRC2 to the Xi (Zhao et al., 2008). Importantly, our *Xist* RNA FISH probe is directed against *Xist* exon 1, a region deleted in the paternally supplied $X^{Xist1lox}$ allele. Therefore, any *Xist* RNA FISH signal detected in $X_{JF1}/X_{129}^{Xist1Lox;GFP}$ TS cells must be supplied by the maternal copy of *Xist*. Indeed, we observe *Xist* RNA FISH signals overlapping with EED and H3K27me3 in $X_{JF1}/X_{129}^{Xist1Lox;GFP}$ TS cells (Fig. 5P-T), a pattern indistinguishable from that of $X_{JF1}/X_{129}^{Xist2Lox;GFP}$ TS cells (Fig. 5K-O).

Although a contribution of the earliest ICM to the ExE has not been documented, we wanted to ensure that $X_{JF1}/X_{129}^{Xist1Lox;GFP}$ TS cells were not derived from transdifferentiated early embryonic cells that had undergone normal erasure of imprinted XCI and subsequent skewed random XCI. To test this, we generated TS lines from an intercross of a WT female harboring the *Gt(ROSA)26Sor^{tm1Sor}* (*R26R*) reporter (Soriano, 1999) with a $X^{Xist1lox;GFP}$ male harboring the *Tg(Sox2-cre)1Amc* (*Sox2Cre*) transgene (Hayashi et al., 2002). *Sox2Cre* is active in the embryonic ICM, but not in extra-embryonic compartments. Therefore, if *X/X$^{Xist1lox;GFP}$;R26R/+;Sox2Cre^{+}* TS lines were of embryonic origin, they should express *lacZ* and have β-galactosidase activity. Using X-gal, a substrate for β-galactosidase, *X/X$^{Xist1lox:GFP}$;R26R/+;Sox2Cre^{+}* TS cells were indistinguishable from TS cells derived from male siblings (supplementary material Fig. S7), suggesting that TS cell lines derived from X/X^{Xist1lox} embryos are of ExE origin.

In order to test definitively whether the Xm is the Xi in $X_{JF1}/X_{129}^{Xist1Lox;GFP}$ TS cells, we performed allele-specific RT-PCR for *Ddx3x*, *Utx* (*Kdm6a* – Mouse Genome Informatics), *Ube1x* (*Uba1* – Mouse Genome Informatics), *Atrx*, *Xist*, *Atp7a*, *Rnf12* (*Rlim* – Mouse Genome Informatics), *Pgk1*, *Kdm5c*, *Rhox6* and *Pdha1* (Fig. 6). In all cases, the patterns of XCI were inversed in $X_{JF1}/X_{129}^{Xist1Lox;GFP}$ TS cells compared with $X_{JF1}/X_{129}^{Xist2Lox;GFP}$ TS cells, indicating a complete reversal of imprinted XCI in $X_{JF1}/X_{129}^{Xist1Lox;GFP}$ TS cells. Interestingly, genes known to escape XCI, such as *Kdm5c*, escaped XCI in $X_{JF1}/X_{129}^{Xist1Lox;GFP}$ TS cells in a reciprocal pattern to that of $X_{JF1}/X_{129}^{Xist2Lox;GFP}$ TS cells. Genes known to be imprinted in adult tissues, such as *Rhox6* (Maclean et al., 2011), followed the patterns of genes subject to imprinted XCI. It should be noted that when *Cre*-expressing constructs were introduced into $X_{JF1}/X_{129}^{Xist2Lox;GFP}$ TS cells, we were unable to recover $X_{JF1}/X_{129}^{Xist1Lox;GFP}$ TS cells, whereas the same *Cre* constructs introduced into *X/X^{Xist2Lox}* embryonic stem (ES) cells produced *X/X^{Xist1Lox}* ES colonies. Taken together, our data demonstrate a complete reversal of imprinted XCI in TS cells devoid of paternally derived *Xist* function.

DISCUSSION

In this study, we link the maintenance of the ExE, the progenitor population for the majority of the cells within the placenta, with X-linked gene dosage. We demonstrate that in the absence of proper gene dosage compensation (imprinted XCI), the ExE is exhausted owing to premature differentiation and is probably incapable of maintaining precursors of the spongiotrophoblast lineage. Furthermore, we demonstrate the ability of ExE cells to reverse imprinted XCI patterns, suggesting that the imprint responsible for imprinted XCI is no longer present in the ExE (see below).

CDX2+ cells are present in E5.5 *X/X^{Xist1Lox}* embryos, *X/X^{Xist1Lox}* embryo cultures and *X/X^{Xist1Lox}* TS cells, suggesting that overabundance of X-linked gene products does not prevent the specification of ExE or the response of the ExE to extrinsic factors

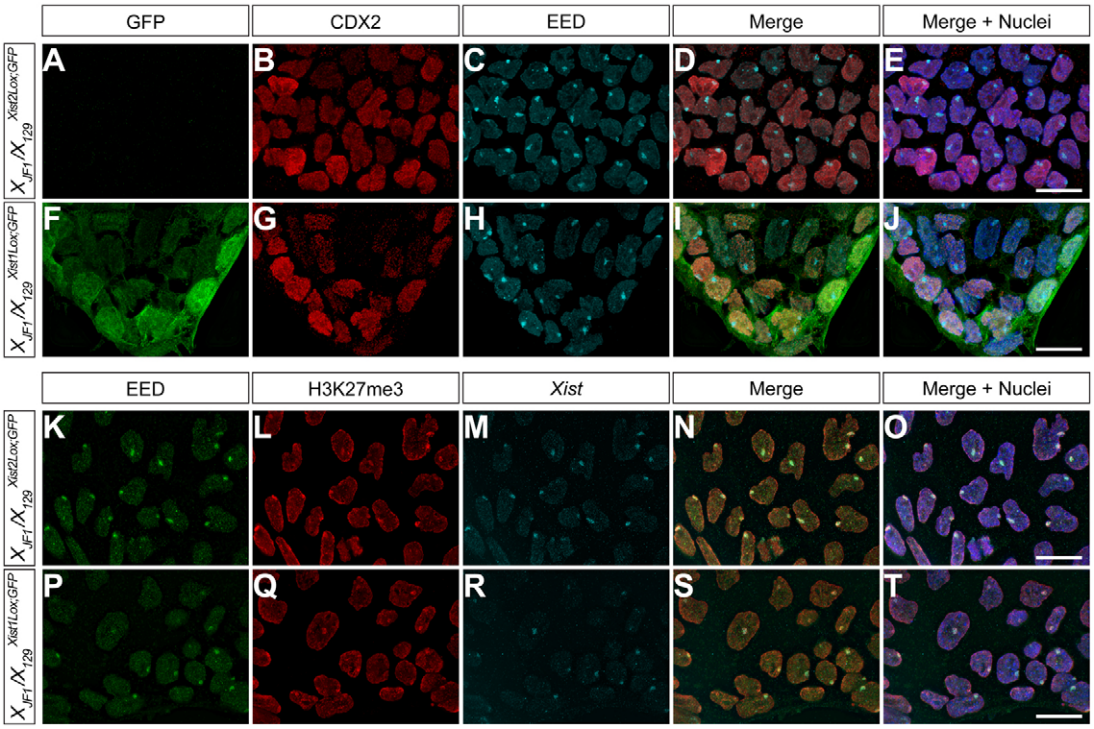

Fig. 5. *X/X*^{Xist1Lox} TS cells harbor a definitive Xi. (**A-T**) Immunofluorescence in $X_{JF1}/X_{129}^{Xist2Lox;GFP}$ (A-E,K-O) and $X_{JF1}/X_{129}^{Xist1Lox;GFP}$ (F-J,P-T) TS cells for GFP (A,F), CDX2 (B,G), EED (C,H,K,P) and H3K27me3 (L,Q). RNA Fluorescent in situ hybridization for *Xist* exon 1 is shown in M and R. Merged images without (D,I,N,S) and with (E,J,O,T) DAPI-stained nuclei are shown. Scale bars: 20 μm.

(such as FGF4) normally required for its maintenance. This is especially evident in *X/X*^{Xist1Lox} blastocyst outgrowths where we detected CDX2+ cells in which an Xi was not observed. We do not observe CDX2+ cells in E6.5 *X/X*^{Xist1Lox} embryos, we cannot convert *X/X*^{Xist2Lox} TS cells to *X/X*^{Xist1Lox} TS cells, and we have not observed *X/X*^{Xist2Lox} or *X/X*^{Xist1Lox} TS cells lacking an Xi. Taken together, these facts suggest that, in vivo, the CDX2+ ExE is highly sensitive to X-linked gene dosage and that the developmental problems observed are not due to specification, but rather maintenance of a CDX2+ progenitor pool.

It is curious that more differentiated extra-embryonic cells are able to survive despite the absence of an Xi. For instance, giant cells are present in both E6.5 *X/X*^{Xist1Lox} embryos and *X/X*^{Xist1Lox} cultured embryo outgrowths. Furthermore, the giant cells present in *X/X*^{Xist1Lox} embryo cultures do not show evidence of an Xi, even though their *X/X*^{Xist2Lox} counterparts do. This finding is similar to the findings of Ohhata et al. (Ohhata et al., 2011), which demonstrated that more differentiated trophectodermal cell types seem to no longer require stable XCI for their survival. Similarly, cells of the visceral endoderm lining the epiblast do not harbor an Xi, at least by H3K27me3 immunostaining, in either E6.5 *X/X*^{Xist2Lox} or E6.5 *X/X*^{Xist1Lox} embryos. A differential requirement for PRC2 function in XCI in the visceral endoderm has been noted previously (Kalantry et al., 2006), further suggesting a greater tolerance of elevated X-linked gene dosage in more differentiated extra-embryonic cells. However, it should be noted that differentiated extra-embryonic cells in E6.5 *X/X*^{Xist1Lox} embryos have not been extensively compared with those in E6.5 *X/X*^{Xist2Lox} embryos, so it is possible that although differentiated *X/X*^{Xist1Lox} extra-embryonic cells are present and appear normal, they might in fact be deficient in either gene expression and/or cellular function.

Recent work describing a hypomorphic *Xist* allele suggests that development of giant cells is impaired in the presence of failed imprinted XCI (Hoki et al., 2011), a finding inconsistent with our observations. Female embryos harboring a paternally inherited *Xist* hypomorphic allele survive to E13.5. Conversely, *X/X*^{Xist1Lox} embryos cannot be recovered at this stage. It is therefore possible that our results are specific to primary giant cells, and not those that arise later in development. If so, the combination of these two results suggests a difference in properties of primary versus secondary giant cells, at least in relationship to X-linked gene dosage. Alternatively, the differences between the *Xist* hypomorph and *Xist* null alleles, namely unstable XCI versus lack of XCI, might affect giant cell function and/or differentiation differently.

We suggest that an X-linked factor, or factors, forces the differentiation of extra-embryonic progenitor populations, namely the ExE, towards a more differentiated state. In addition, the ExE appears to lose the ability to properly maintain spongiotrophoblast progenitors. In both cases, these observations provide further evidence of a direct link between the regulation of dosage compensation with the maintenance of a progenitor pool. As expected Mendelian ratios of *X/X*$^{Xist-}$ embryos begin to decline after E6.5 (Marahrens et al., 1997), we are unable to assess reliably the presence of other cell types of the placenta, such as labyrinthine cells. Whether the tendency towards a differentiated state, presumably giant cells, is direct or indirect is unknown, however, the X chromosome contains ~800 known protein-coding genes, providing numerous candidates upon which further studies can be based. Pure speculation suggests an effect on the regulation of *CDX2*, the repression or antagonism of which would immediately release ExE cells from an undifferentiated state.

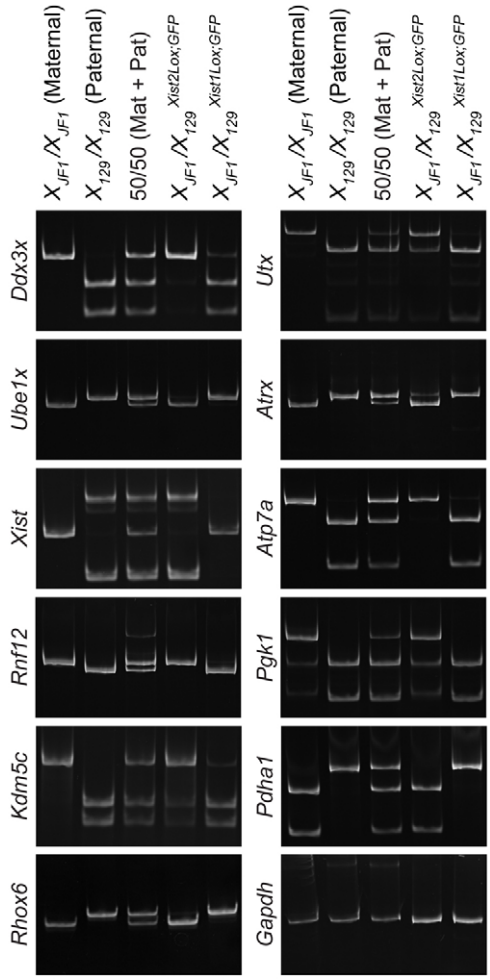

Fig. 6. $X/X^{Xist1Lox}$ TS completely reverse imprinted XCI. Allele-specific RT-PCR for the indicated transcripts from cDNA derived from X_{JF1}/X_{JF1} placental RNA, X_{129}/X_{129} placental RNA, a 50/50 mixture of maternal and paternal RNA, $X_{JF1}/X_{129}^{Xist2Lox;GFP}$ TS cell RNA, and $X_{JF1}/X_{129}^{Xist1Lox;GFP}$ TS cell RNA.

The imprint underlying imprinted XCI is unknown, though it is established during oocyte growth and maturation (Tada et al., 2000). It is also known that *Xist* can be activated at the blastocyst stage within gynogenetic embryos (Latham and Rambhatla, 1995), though this is likely to be due to the initiation of random XCI within the ICM of these embryos. Imprinted XCI is initiated throughout the embryo during early cleavage stages (Kalantry and Magnuson, 2006; Williams et al., 2011) and continues throughout development in the extra-embryonic lineages (reviewed by Lyon, 1994). Around mid-blastula stage, epiblast cells erase imprinted XCI and undergo random XCI (Williams et al., 2011). To date, the ability for extra-embryonic cells to completely erase imprinted XCI has not been documented.

We observe a complete reversal of imprinted XCI in TS cells derived from $X/X^{Xist1Lox}$ embryos. Additionally, at single-cell resolution, we observe evidence of an Xi, presumably the Xm, in both developing E6.5 $X/X^{Xist1Lox}$ embryos in vivo and $X/X^{Xist1Lox}$ blastocyst outgrowths cultured ex vivo. Most notably, imprinted XCI reversal occurs in CDX2+ cells in embryo cultures and in CDX2+ TS cells. As embryo cultures and TS cell derivation remove developing embryos from their normal environments, imprinted XCI reversal may be construed as an artifact of cell culture. However, our demonstration of a focal point of H3K27me3 (a putative Xi) within EOMES+ Oct3/4– E6.5 $X/X^{Xist1Lox}$ ExE cells in vivo suggests that imprinted XCI reversal happens, though on a limited basis, in vivo.

During mouse development, the Xp remains transcriptionally silent in extra-embryonic lineages, a phenomenon thought to be due to the retention of an imprint on the Xm. Our data provide evidence contrary to the prevailing model and suggest that, like the epiblast, the imprint no longer exists in the ExE. Recent work has demonstrated a link between *Xist* transcriptional repression and the pluripotency factors present in epiblast cells (reviewed by Deuve and Avner, 2011). Specifically, OCT4, NANOG and SOX2 are known to bind within the first exon of *Xist*, an event coincident with imprint reversal and *Xist* downregulation in the epiblast (Navarro et al., 2008). The pluripotency factors are not present in ExE cells, suggesting that the mechanism to downregulate *Xist* does not exist in the extra-embryonic lineages, precluding the possibility of ever observing random XCI in the ExE, even in the absence of an imprint. Furthermore, if the imprint remained, reversal of imprinted XCI should never be observed in embryo culture, nor in TS cells derived from $X/X^{Xist1Lox}$ embryos. We suggest that the X-linked expression patterns normally present in ExE might not be the consequence of sustained imprinted XCI, but rather maintenance of an XCI choice previously biased by imprinted XCI.

E6.5 $X/X^{Xist1Lox}$ ExE cells that have reversed imprinted XCI reside adjacent to the epiblast, a source of ExE survival factors. It is possible that these factors are required, either directly or indirectly, for reversal of imprinted XCI to take place, possibly by maintaining a partial progenitor state in $X/X^{Xist1Lox}$ ExE cells. The observation that the addition of FGF4 and heparin to culture media did not substantially increase the percentage of $X/X^{Xist1Lox}$ embryos in which imprinted XCI had been reversed suggests that other effects, possibly the same that lead to phenotypic variability, have added an additional layer of complexity to the infrequency of this phenomenon.

By placing $X/X^{Xist1Lox}$ ExE cells in an artificial environment (TS cell derivation or embryo culture), the sensitivity of ExE cells to X-linked gene dosage appeared to be partially suppressed, probably owing to undefined components in growth media (i.e. FBS). However, undergoing XCI clearly confers a survival advantage for ExE cells, as $X/X^{Xist1Lox}$ TS cells are not a heterogeneous mixture of cells which harbor an Xi and those that do not.

Our data demonstrate that, unlike early epiblast or ES cells, the ExE requires proper dosage compensation for its survival as a multipotent progenitor pool. We suggest that in the developing $X/X^{Xist1Lox}$ embryo, differentiation into a cell type more tolerant of an overabundance of X-linked gene activity offers a 'path of least resistance' to $X/X^{Xist1Lox}$ ExE. Although a few cells are able to reverse imprinted XCI patterns, the pre-mature differentiation leads to depletion of the progenitor status of the ExE, poor placental development and, ultimately, embryonic lethality.

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Competing interests statement
The authors declare no competing financial interests.

Supplementary material
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