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Gata3 regulates trophoblast development downstream of Tead4 and in parallel to Cdx2

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SUMMARY

The mouse blastocyst and stem cells derived from its tissue lineages provide a unique genetic system for examining the establishment and loss of pluripotency. The transcription factor Cdx2 plays a central role by repressing pluripotency genes, such as *Oct4*, and promoting extraembryonic trophoblast fate at the blastocyst stage. However, genetic evidence has suggested that Cdx2 does not work alone in the trophoblast lineage. We have used bioinformatic and functional genomic strategies to identify the transcription factor Gata3 as a trophoblast factor. We show Gata3 to be capable of inducing trophoblast fate in embryonic stem cells and driving trophoblast differentiation in trophoblast stem cells. In addition, *Cdx2* is not required for Gata3-induced expression of a subset of trophoblast genes in embryonic stem cells. We show that Gata3 is coexpressed with Cdx2 in the blastocyst, but this does not depend on *Cdx2*. In the embryo, expression of Gata3, like that of Cdx2, depends on *Tead4*, and the expression of both factors becomes restricted to trophoblast by a mechanism that does not initially rely on *Oct4*. These observations suggest that Gata3 and Cdx2 can act in parallel pathways downstream of Tead4 to induce the expression of common and independent targets in the trophoblast lineage, whereas Oct4 is required for continued repression of trophoblast fate in the embryonic lineage.

KEY WORDS: Trophectoderm, Placenta, Implantation, Pluripotency, Lineage restriction, Embryogenesis, Mouse

INTRODUCTION

The first developmental decisions during mouse development lead to the establishment of the embryonic and extraembryonic tissue lineages. Stem cell lines have been isolated from these early lineages, including embryonic stem (ES) and trophoblast stem (TS) cells (Evans and Kaufman, 1981; Martin, 1981; Tanaka et al., 1998). Both stem cell types are self-renewing and capable of lineage-appropriate differentiation. For example, ES cells can differentiate into a wide range of fetal cell types, but fail to form trophoblast (Beddington and Robertson, 1989). Conversely, TS cells differentiate along the trophoblast/placenta lineage, and fail to form fetal cell types (Tanaka et al., 1998). To create a placenta, the trophoblast lineage must achieve several distinct goals simultaneously at the blastocyst stage. Trophoblast cells must override the pluripotency program of the embryonic lineage, they must establish the ability to self-renew, and they must maintain the ability to differentiate into mature trophoblast cell types. *Cdx2* and *Eomes* are required for trophoblast survival and maturation starting around the blastocyst stage (Russ et al., 2000; Strumpf et al., 2005). These genes are also important for TS cell establishment (Strumpf et al., 2005), suggesting roles in proliferation. However, not all cells of the trophectoderm are proliferative, as some trophectoderm cells visibly differentiate as early as implantation. This suggests that programs that promote proliferation and differentiation might coexist at the blastocyst stage.

Besides *Cdx2* and *Eomes*, genetic evidence suggests that other genes participate in trophoblast formation in the blastocyst. For example, loss of *Tead4*, which is required for expression of *Cdx2* in the trophectoderm (Yagi et al., 2007; Nishioka et al., 2008), leads to a more severe phenotype than loss of *Cdx2*. Thus, Tead4 must have multiple trophoblast targets acting at the blastocyst stage to regulate trophoblast development. Consistent with this proposal, constitutively active Tead4 is sufficient to induce trophoblast formation even in the absence of *Cdx2* in ES cells (Nishioka et al., 2009). Other factors capable of overriding the pluripotency pathway and promoting trophoblast fate must therefore exist.

To identify new factors involved in early lineage decisions in the mouse, we used a bioinformatic strategy to compare expression profiles of stem cells from the blastocyst. Transcripts encoding the transcription factor Gata3 were specifically enriched in TS cells and in the trophoblast lineage, consistent with recent reports (Home et al., 2009; Ray et al., 2009). Although Gata3 expression is restricted to the trophectoderm at the blastocyst stage, we found that this expression does not depend on *Cdx2*. Rather, expression of Gata3, like that of Cdx2, depends on *Tead4*. We show that *Oct4* (*Pou5f1*) is not initially involved in restricting expression of either Cdx2 or Gata3 to the trophectoderm, but Oct4 maintains repression of these genes in the epiblast. In ES cells, Gata3 is capable of overriding pluripotency and directing the expression of a multitude of *Cdx2*-independent trophoblast genes, whereas in TS cells Gata3 promotes differentiation.

MATERIALS AND METHODS

Bioinformatic analysis

For comparison of ES, TS and XEN cell expression profiles, MGU74v2A microarray.CEL files for ES (GSE3766), XEN (GSE2204) and TS (GSE3766) cells were downloaded from the GEO website

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(http://www.ncbi.nlm.nih.gov/projects/geo/) and processed using GCOS software (Affymetrix) with the 'statistical method' algorithm to generate signal intensities and absent/present calls. Log_2 ratios and fold-change calls for probe sets in TS and XEN cell samples versus ES cells were determined. Probe sets with more than a 2-fold difference in expression in TS cells ($P<0.003$) versus ES cells and with GO annotation as a transcription factor (GO term ID: 000635) were selected for hierarchical clustering. GO annotation was obtained from the Affymetrix annotation file for the gene chip. Hierarchical clustering was performed with Cluster 3.0 (de Hoon et al., 2004) using the 'uncentered correlation similarity' metric with average linkage, and resulting clusters were visualized with Java TreeView.

For comparison of Gata3 and Cdx2 activity in wild-type and *Cdx2* null cells, Affymetrix MOE430 2.0 microarrays were performed on four independent Gata3-expressing lines, two Cdx2-expressing lines, and three Gata3-expressing *Cdx2* null lines. Data for these and TS cells were batch processed using Expression Console software (Affymetrix) to normalize arrays. Probe intensities were calculated using the PLIER algorithm. To reduce sample complexity, redundant probes matching to a single gene were filtered for a representative probe that had the largest number of signals greater than three times the global median signal across all samples. Ratios of gene expression for *Gata3*-expressing and *Cdx2*-expressing cells were calculated relative to tamoxifen-treated R1 cells and were log_2 transformed. Ratios for *Gata3*-expressing *Cdx2* null cells were calculated relative to tamoxifen-treated *Cdx2* null cells. Expression ratios for TS cells and their differentiated samples were calculated relative to the median expression level of the probe set across all the TS samples. To facilitate mining of the data set, all expression and probe set annotation was placed into a custom database using MySQL and queried using command line scripts. Raw data are available at GEO with accession numbers GSE12985 and GSE12986.

Cell culture

The ES cell lines R1 and dKO23-5 (Niwa et al., 2005) were maintained on gelatin using standard culture conditions. To create stably transformed lines, $1\text{-}3\times10^7$ ES cells were electroporated with 20-30 μg plasmid (pCAG-hCdx2ERT2-ires-puro^r, or pCAG-hGata3ERT2-ires-puro^r), and were then seeded on two 10-cm gelatinized plates. Cells were fed with medium to select for expression of the plasmid (ES medium + 1.2 μg/ml puromycin) and individual colonies expanded. To induce transgene activity, cells were treated with induction medium [TS medium + 1 μg/ml tamoxifen (Sigma)]. For microarray analysis, 2×10^5 cells were seeded on gelatinized 35-mm wells in ES medium, and then switched to TS cell medium with tamoxifen the following day, and fed daily for 6 days. To derive TS-like cell lines, cells were treated with tamoxifen on mouse embryonic feeder layers for 6 days in TS cell medium, and then passaged onto fresh feeders in TS cell medium.

TS cell lines included TS3.5 and TS6.5, which were isolated from blastocyst and E6.5 embryos, respectively, and were maintained or differentiated as described (Tanaka et al., 1998), and TS_{WT}, which was isolated from ICR blastocysts. For overexpression of Gata3, TS_{WT} cells were electroporated with Gata3ER, followed by selection for plasmid expression (with 1.2 μg/ml puromycin in TS cell medium) and tamoxifen treatment (1 μg/ml) for 5 days.

Gene expression analysis

RNA was harvested from plated cells using Trizol (Invitrogen). For real-time PCR analysis, cDNA was synthesized using the Quantitect Kit (Qiagen). Real-time PCR analyses were performed using SYBR Green and a LightCycler 480 (Roche). All reactions (12 μl) were performed in triplicate, with 100-200 ng cDNA and 300 nM primers (shown 5′ to 3′) per reaction: *Hprt1*, AAACAATGCAAACTTTGCTTTCC and GGTCCTTTTCACCAGCAAGCT; *Gata3*, GGGTTCGGATGTAAGTCGAG and CCACAGTGGGGTAGAGGTTG; *Cdx2*, AGACAAATACCGGGTGGTGTA and CCAGCTCACTTTTCCTCCTGA; *Prl2c2*, AGCCCCATGAGATGCAATAC and CATCCAAAATCATGGCTCCT; *Bmp4*, AGGAGGAGGAGGAAGAGCAG and ACTGGTCCCTGGGATGTTCT; *Pdgfra*, ACGTTCAAGACCAGCGAGTT and CGATCGTTTCTCCTGCCTTA; *Ascl2*, TTTTCGAGGACGCAATAAGC and CACTGCTGCAGGACTCCCTA; *Eomes*, GTGACAGAGACGGTGTGGAGG and AGAGGAGGCCGTTGGTCTGTGG; *Elf5*, TGCCTTTGAGCATCAGACAG and TACTGGTCGCAGCAGAATTG; *Tead4*, ACGGAGGAAGGCAAGATGTA and CTGGAGACCTGCTTCCTTGT. A standard primer efficiency curve for each primer pair was generated using TS3.5 cDNA. Levels of *Hprt1* served as an internal reference for all reactions. Amplification of a single PCR product for each reaction was confirmed by melting curve analysis, and all primers spanned exon junctions. RNA samples used for microarray hybridization were collected using Trizol, and then further purified using the RNeasy Mini Kit (Qiagen). Samples were analyzed by Affymetrix mouse MOE 430 2.0 cDNA microarrays (The Center for Applied Genomics, Toronto, Canada).

For single-blastocyst qPCR, total RNA was extracted from individual blastocysts using the PicoPure RNA Isolation Kit (Arcturus Bioscience), and cDNA synthesized at 37°C for 2 hours using the high-capacity cDNA Archive Kit (Applied Biosystems). One eighth of each cDNA preparation was preamplified for 16 cycles (95°C for 15 seconds and 60°C for 4 minutes) using the TaqMan PreAmp Master Mix Kit (Applied Biosystems) and gene-specific primers. Products were then diluted 5-fold for PCR (Applied Biosystems) in 48.48 Dynamic Arrays on a BioMark System (Fluidigm). Threshold cycle (Ct) values were calculated using the system's software (BioMark Real-time PCR Analysis) and were normalized to *Actb* Ct values.

Immunofluorescence and in situ hybridization

Preimplantation embryos were harvested, stained and examined by confocal microscopy as described previously (Ralston and Rossant, 2008). Primary antibodies included mouse anti-Cdx2 (1:200, Biogenex CDX2-88), rabbit anti-Cdx2 (1:200) (Chawengsaksophak et al., 1997), mouse anti-Gata3 (1:20, Santa Cruz H-48) and mouse anti-Oct4 (1:10, Santa Cruz C-10). Secondary antibodies included Alexa488- or Alexa546-conjugated goat anti-mouse, rabbit or rat IgG (Molecular Probes) and Cy3-conjugated anti-mouse, rabbit or rat IgG (Jackson). Secondary antibody-only controls were performed in parallel (not shown). Whole-mount embryo in situ hybridization was performed as described (Yamanaka et al., 2007).

Mouse strains

Mouse strains used in this study included wild-type (ICR) mice and mice heterozygous for null alleles of *Cdx2* ($Cdx2^{tm1Fbe}$) (Chawengsaksophak et al., 1997), *Oct4* ($Pou5f1^{tm1Scho}$) (Kehler et al., 2004) and *Tead4* ($Tead4^{tm1Hssk}$) (Nishioka et al., 2008). All mice were treated in accordance with institutional guidelines. For genotyping, blastocysts were individually recovered following confocal microscopy and lysed using the Extract-N-Amp Tissue PCR Kit (Sigma) in a total of 10 μl per embryo, of which 2 μl was used for 10 μl PCR genotyping reactions, with 5 μl PCR Red mix and 0.5 μl each 10 μM primer (Kehler et al., 2004; Strumpf et al., 2005; Nishioka et al., 2008).

RESULTS

Gata3 is enriched in trophoblast stem cells

To identify potential trophoblast-inducing factors, we compared microarray expression profiles of stem cells derived from the blastocyst lineages: ES, TS and extraembryonic endoderm stem (XEN) cells (Kunath et al., 2005) (see Materials and methods). Probe sets that were significantly increased in TS and XEN cells relative to ES cells ($P<0.003$) were retained, and analysis of corresponding gene ontology (GO) terms yielded 122 transcription factors specifically enriched in TS cells (see Fig. S1 in the supplementary material), as represented by 138 probe sets. Importantly, this list included genes known to be essential for early trophoblast development, such as *Cdx2* (Strumpf et al., 2005), *Eomes* (Russ et al., 2000; Strumpf et al., 2005) and *Tead4* (Yagi et al., 2007; Nishioka et al., 2008). We therefore reasoned that other transcription factors enriched in this list could likewise be important for trophoblast development.

We focused on the zinc-finger transcription factor Gata3, the expression of which was specifically highly enriched in TS cells. Consistent with the microarray data, quantitative RT-PCR (qPCR)

analysis of *Gata3* levels indicated a greater than 100-fold enrichment of *Gata3* in TS cells compared with ES cells (Fig. 1A). *Gata3* is known to be expressed in, and required for the function of, the trophoblast lineage at later stages of development of the placenta (Ma et al., 1997). However, its high level of expression in TS cells suggested a previously unrecognized role for Gata3 in regulating stem cells of the trophoblast lineage. Examination of differentiating TS cells revealed that *Gata3* levels increase during TS cell differentiation (Fig. 1B). These observations suggested that Gata3 might promote TS cell differentiation, consistent with its requirement in directing the formation of giant cells in the placenta (Ma et al., 1997).

Global comparison of Gata3 and Cdx2 trophoblast-inducing activity

ES cells are normally restricted in developmental potential to embryonic fates, having lost or suppressed the ability to generate trophoblast cell types (Beddington and Robertson, 1989). Overexpression of key trophoblast factors has been shown to lead to an increase in trophoblast gene expression in ES cells within a 6-day time frame (Niwa et al., 2005; Lu et al., 2008; Ng et al., 2008; Nishioka et al., 2009; Nishiyama et al., 2009). Subsequent passage of these cells in TS cell medium can lead to the establishment of self-renewing TS-like cells in some cases (Niwa et al., 2005; Lu et al., 2008; Nishioka et al., 2009). We examined the ability of Gata3 to induce the formation of TS-like cells by overexpressing *Gata3ER*, which encodes a fusion between Gata3 and the ligand-binding domain of the estrogen receptor (ER). Gata3ER was activated by addition of tamoxifen and, under TS cell derivation conditions, TS-like colonies were detected among cultures within 6 days (Fig. 1C; 4/5 lines examined), but not in control ES cells grown under the same conditions (Fig. 1E). However, endoderm-like cells were also present in all Gata3 cultures (Fig. 1C′), and these were not present in ES cells overexpressing Cdx2 (Fig. 1D). Continued passage of *Cdx2*-overexpressing ES cells led to the establishment of TS-like cell lines (4/5 lines examined). TS-like colonies were continuously detected among Gata3-overexpressing cells. However, cultures were consistently heterogeneous, and the TS cell phenotype could not be enriched under the conditions examined (5/5 lines examined). Thus, although both genes appear capable of inducing trophoblast differentiation in ES cells, only Cdx2 produced stable TS cell lines when overexpressed in ES cells.

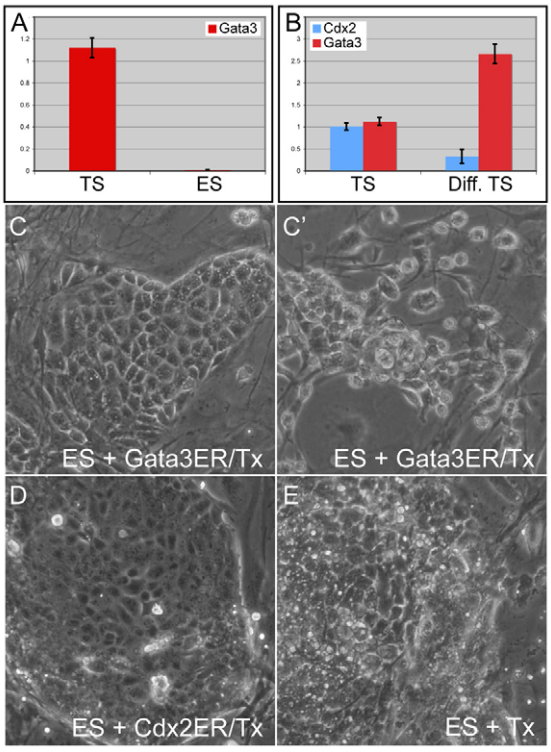

Fig. 1. *Gata3* is sufficient to induce trophoblast morphology in mouse ES cells. (**A**) qPCR analysis of *Gata3* levels in embryonic stem (ES) and trophoblast stem (TS) cells. For this and all subsequent qPCR analyses, expression levels have been normalized to those in TS cells. Error bars, variation in technical replicates. (**B**) qPCR analysis of *Gata3* levels in self-renewing and TS cells differentiated for 6 days. (**C**) TS-like morphology resulting from overexpression of Gata3 in ES cells for 6 days and subsequent passage on feeders. (**C′**) Endoderm-like morphology present among ES cells overexpressing Gata3. (**D**) TS-like cells derived from ES cells overexpressing Cdx2. (**E**) Control ES cells treated in parallel. Tx, tamoxifen.

We next compared the ability of Gata3 and Cdx2 to induce trophoblast at the gene expression level, comparing global gene expression profiles of ES cells overexpressing either gene. To restrict our analysis to trophoblast-specific genes, we began by defining a set of ~1800 core trophoblast genes, using TS cells as a reference (Fig. 2A; see Table S1 in the supplementary material; see Materials and methods). We then used this set to filter data sets from *Cdx2*-expressing and *Gata3*-expressing ES cell lines. This led to lists of genes induced by Gata3 (449/1794 core trophoblast genes) or Cdx2 (326/1794 core trophoblast genes) (see Table S2 in the supplementary material). Gata3 was therefore capable of inducing more trophoblast genes than Cdx2.

To examine qualitative similarities and differences in trophoblast genes induced by the overexpression of these two genes, we examined the overlap between the two lists. This revealed trophoblast genes induced by *Gata3* (225 genes) or *Cdx2* (102 genes) alone, as well as common genes induced by either factor (224 genes) (Fig. 2B; see Table S2 in the supplementary material). Thus, although around half of the trophoblast genes induced by *Gata3* were also induced by Cdx2, each factor also induced the expression of a unique set of trophoblast genes. These differences were validated by qPCR for a subset of the genes (see Fig. S2 in the supplementary material). This analysis suggested that Gata3 expression is induced by ectopic *Cdx2*, although Gata3 targets were not in turn detected in the *Cdx2*-overexpression array. This disparity could be due to differences in the levels of overexpressed Gata3 in these two conditions. Alternatively, Cdx2 might directly or indirectly repress the expression of a subset of Gata3 target genes. This possibility is further addressed below.

These observations predict that Gata3 and Cdx2 will have both shared and distinct roles during trophoblast development. Among the genes induced by either factor, an examination of phenotypes for those that have been knocked out (Fig. 2C) revealed defects in multiple trophoblast subtypes and at multiple developmental stages (see Table S3 in the supplementary material). No single trophoblast phenotype was predominant in any of the lists, suggesting that Cdx2 and Gata3 targets are likely to play diverse, and possibly overlapping, roles in trophoblast development.

This analysis, which was designed to focus on the trophoblast roles of these genes, excluded genes that were not included among the core trophoblast gene list. We noted 347 ‘non-trophoblast’ genes induced by *Gata3* and 72 induced by *Cdx2* (see Table S4A,B in the supplementary material). Interestingly, among the genes induced by

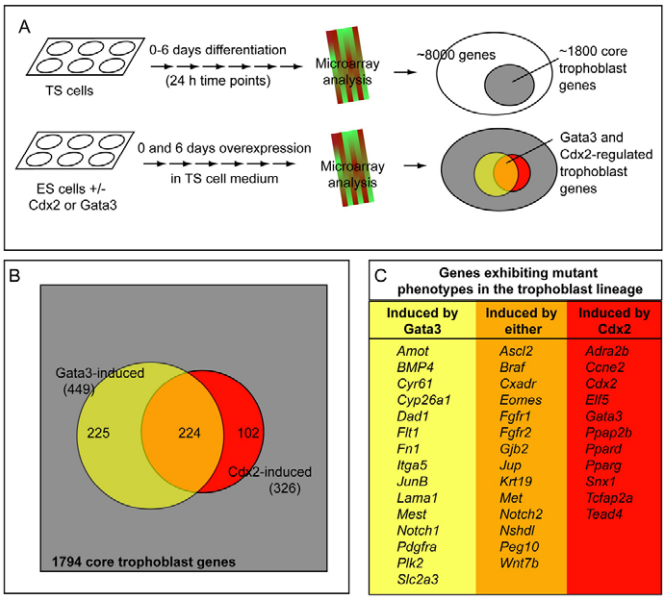

Fig. 2. Gata3 is sufficient to induce trophoblast gene expression in ES cells. (**A**) Data mining strategy for examining trophoblast gene expression in ES cells overexpressing *Cdx2* or *Gata3*. TS cells were differentiated for 6 days, and samples harvested daily during this period to generate a 6-day differentiation profile. Around 1800/8000 genes (core trophoblast genes, gray) exhibited a 2-fold or greater change in absolute expression level over the course of the experiment and were retained as likely to be important for trophoblast development. Changes in the expression of core trophoblast genes were then examined in ES cells overexpressing *Gata3* or *Cdx2* for 0 and 6 days. Genes exhibiting a greater than 2-fold increase in expression level in at least one of the cell lines examined were retained. (**B**) Venn diagram showing overlap between the lists of core trophoblast genes (gray) exhibiting a 2-fold or greater enrichment in lists from *Gata3*-expressing (yellow) or *Cdx2*-expressing (red) cells. The number of genes within each subset is indicated. (**C**) Subset of genes represented in B with Mouse Genome Informatics (MGI)-archived mutant phenotypes that affect the trophoblast lineage.

Gata3 were many known endodermal genes, including *Foxa2*, *Sox17* and *Sox7*. Thus, both Cdx2 and Gata3 are capable of inducing non-trophoblast targets in ES cells, consistent with the diverse developmental roles played by these genes and the plasticity of ES cells to respond to inductive cues.

Gata3 exhibits both *Cdx2*-dependent and -independent induction of trophoblast gene expression

In ES cells, downregulation of *Oct4* leads to upregulation of *Cdx2* and the adoption of trophoblast fate (Niwa et al., 2000), raising the possibility that *Gata3* overexpression could induce trophoblast gene expression by simply altering Oct4/Cdx2 levels. To address this possibility, we examined trophoblast gene expression following *Gata3* overexpression in the dKO23-5 ES cell line that is *Cdx2* null and expresses *Oct4* constitutively (Niwa et al., 2005). Gata3 overexpression in dKO23-5 cells led to changes in cell morphology, and TS cell lines could not be established in this genetic background, as expected (5/5 lines examined). A microarray comparison of differences in the induction of trophoblast genes following overexpression of *Gata3* in wild-type and dKO23-5 ES cells revealed that *Gata3* was still able to induce a large number of core trophoblast genes (284/1794, compared with 449/1794 in wild-type cells). *Gata3* is therefore sufficient to induce trophoblast gene expression in a *Cdx2*-independent manner.

However, the expression of many trophoblast genes was lost in this genetic background. Examining the intersection between the lists of core trophoblast genes induced by *Gata3* in either wild-type or dKO23-5 cells (Fig. 3A) revealed that the expression of 172/449 Gata3 targets was unchanged, whereas 277/449 targets were no longer induced by Gata3 in dKO23-5 cells. Therefore, the expression of some Gata3 targets relied on the Oct4/Cdx2 pathway, whereas the expression of others, such as *Eomes* and *Ascl2*, did not (Fig. 3B). The genes that were dependent on *Cdx2*, however, did not necessarily overlap with those induced by Cdx2, suggesting differences in the necessity and sufficiency of *Cdx2* for trophoblast gene expression.

This analysis also identified 112 genes that were induced by Gata3 in dKO23-5 and not wild-type ES cells (Fig. 3A; see Table S5 in the supplementary material). This suggests that Cdx2 might repress the Gata3-mediated induction of some trophoblast genes. Taken together, these observations suggest that Gata3 can act via Cdx2, and in parallel to Cdx2, to induce trophoblast gene expression. In addition, these observations suggest that Gata3 might play a unique role in regulating trophoblast development independently of Cdx2.

Gata3 is expressed in the trophoblast lineage in vivo

The findings that *Gata3* is enriched in TS cells and is sufficient to induce trophoblast gene expression in ES cells suggested that Gata3 might be expressed in the trophoblast during lineage establishment in vivo. We examined the expression of Gata3 during trophoblast development at preimplantation stages (Fig. 4A-E). Gata3 protein was detectable within the nuclei of the trophectoderm at the blastocyst stage, where it colocalized with Cdx2 (Fig. 4D) (n=10). In fact, Gata3 colocalized with Cdx2 at earlier preimplantation stages as well (Fig. 4A-C) (n=31 embryos, 8- to 32-cell stages). Prior to becoming restricted to outside cells of the nascent trophectoderm, Cdx2 is expressed in an unpatterned, mosaic manner beginning around the late 8-cell stage (Dietrich and Hiiragi, 2007; Ralston and Rossant, 2008). Gata3 colocalized with Cdx2 in nuclei on a cell-by-cell basis (723/730 cells) in embryos examined at the 8- to 32-cell stages (31 embryos). Among embryos in which Gata3 and Cdx2 expression did not perfectly correlate (5/31 embryos), Gata3-positive/Cdx2-negative and Cdx2-positive/Gata3-negative nuclei were detected at equivalent frequency (four and three nuclei, respectively). Thus, Gata3 is coexpressed with Cdx2 from the earliest developmental stages.

Since TS cells have also been derived from post-implantation embryos, around the time of gastrulation (Tanaka et al., 1998; Uy et al., 2002), we next examined *Gata3* expression around gastrula stages by in situ hybridization. *Gata3* expression was detected throughout the trophoblast lineage from embryonic day (E) 6.5 to 8.5 (Fig. 4E) (n=12). This expression was consistent with previous reports (George et al., 1994) and included the extraembryonic ectoderm (EXE)/chorion and ectoplacental cone (EPC). However, we noted that higher levels of *Gata3* were detected in the EPC than in the EXE. By contrast, *Cdx2* levels appeared higher in the EXE than in the EPC (Fig. 4F). These differences were confirmed by qPCR, following microdissection of these regions (Fig. 4G). Since the EPC is thought to be more differentiated than the EXE, these observations suggest that *Gata3* levels increase during trophoblast differentiation.

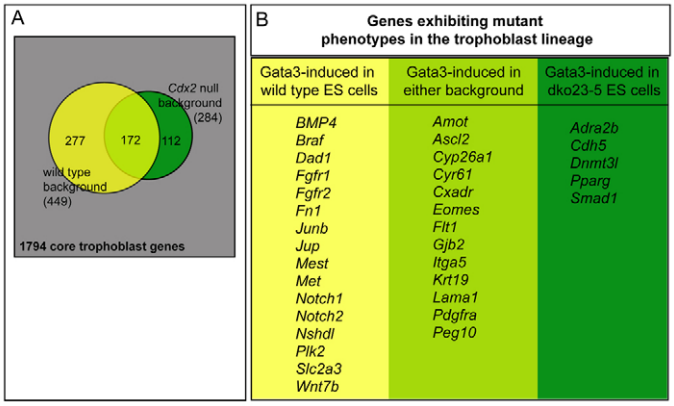

Fig. 3. Gata3 induces trophoblast though *Cdx2*-dependent and -independent mechanisms. (**A**) Venn diagram showing overlap between lists of core trophoblast genes (gray) upregulated by Gata3 in wild-type (yellow) or *Cdx2* null (green) ES cells, with the number of genes indicated. (**B**) Subset of genes represented in A with MGI-archived mutant phenotypes that affect the trophoblast lineage.

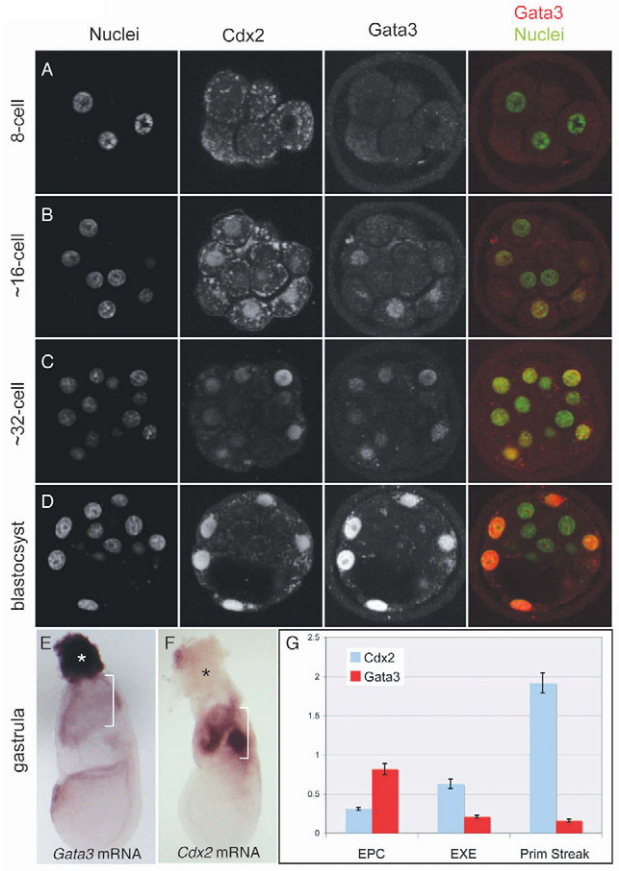

Fig. 4. Gata3 is expressed in the trophoblast lineage. (**A-D**) Representative confocal sections of preimplantation mouse embryos (stages indicated) showing simultaneous localization of Cdx2 and Gata3. Merged images show Gata3 and nuclei, emphasizing the localization of Gata3 in nuclei in outside cells of the embryo (yellow), as previously shown for Cdx2. Since the expression levels of Cdx2 and Gata3 appear to increase steadily during preimplantation stages, confocal settings were changed between embryos so as to optimize the signal-to-noise ratio for each developmental stage examined. Note that neither Cdx2 nor Gata3 is detectable in early 8-cell embryos (shown), but they become detectable during the 8- to 16-cell transition. Background fluorescence from the zona pellucida (zp) can be detected in some channels. The apparent cytoplasmic staining detectable in the Cdx2 channel is likely to be background as it is still present in *Cdx2* mutants stained with this polyclonal antibody (not shown). (**E**) Representative image of *Gata3* whole-mount in situ hybridization at ~E7.5. Note expression in the extraembryonic ectoderm (EXE) (bracket) and apparently higher levels of expression in the ectoplacental cone (EPC) (asterisk). (**F**) Representative image of *Cdx2* in situ hybridization at ~E7.5, with expression in EXE bracketed. (**G**) Quantification of *Cdx2* and *Gata3* levels in EXE, EPC and primitive streak regions from a pool of ten E7.5 embryos. Results are representative of experiments performed in triplicate.

Notably, *Gata3* and *Cdx2* were also detected within the embryo proper around the gastrula stage and later, with *Gata3* in a restricted anterior region (Fig. 4E and data not shown), consistent with previous reports (Manaia et al., 2000), and *Cdx2* in posterior regions (Fig. 4F and data not shown) (Beck et al., 1995). This pattern is consistent with the proposal that both genes can also induce non-trophoblast targets in ES cells.

Gata3 is sufficient to induce differentiation of TS cells

Increasing levels of *Gata3* during trophoblast differentiation, both in TS cells and in the post-implantation embryo, suggested that Gata3 promotes differentiation. We therefore examined whether Gata3 is sufficient to induce differentiation in TS cells. We introduced the Gata3ER fusion construct into a TS cell line and examined changes in morphology and gene expression following treatment with tamoxifen for 5 days. As in previous experiments, cells expressing the Gata3ER fusion protein were selected by drug resistance. Control TS cells treated with tamoxifen maintained a generally undifferentiated state (Fig. 5A). However, *Gata3*-overexpressing TS cells appeared largely differentiated, with numerous giant cells present throughout the culture (Fig. 5B), despite the continued presence of TS cell medium. Plasmid-electroporated cells treated with lower doses of tamoxifen did not appear differentiated, arguing that the differentiation effect was Gata3 dependent. We therefore conclude that Gata3 overexpression is sufficient to induce differentiation of TS cells. This proposal was confirmed by an examination of TS cell and giant cell markers by qPCR (Fig. 5C).

Common mechanisms of regulation of Cdx2 and Gata3 in the blastocyst

Coexpression of Cdx2 and Gata3 at the blastocyst stage led us to investigate whether these genes are regulated by a common mechanism in vivo. The transcription factor Tead4 is required for Cdx2 expression prior to the blastocyst stage (Yagi et al., 2007; Nishioka et al., 2008). In *Tead4* mutants, Cdx2 is initially detected around the 16-cell stage (Nishioka et al., 2008), but this expression is lost and embryos die prior to blastocyst formation around the 32-cell stage (Yagi et al., 2007; Nishioka et al., 2008). We hypothesized that Tead4 could play a role in the regulation of Gata3 prior to blastocyst formation, and examined expression of Gata3 in *Tead4* mutants at E3.5. Nuclear levels of Gata3 were greatly reduced in *Tead4* mutants (n=5) compared with non-mutants (n=17) (Fig. 6A,B). Similar to Cdx2, however, low levels of Gata3 could be detected in the nuclei of some cells (not shown), consistent with Tead4 regulating the maintenance, rather than initiation, of *Gata3* expression.

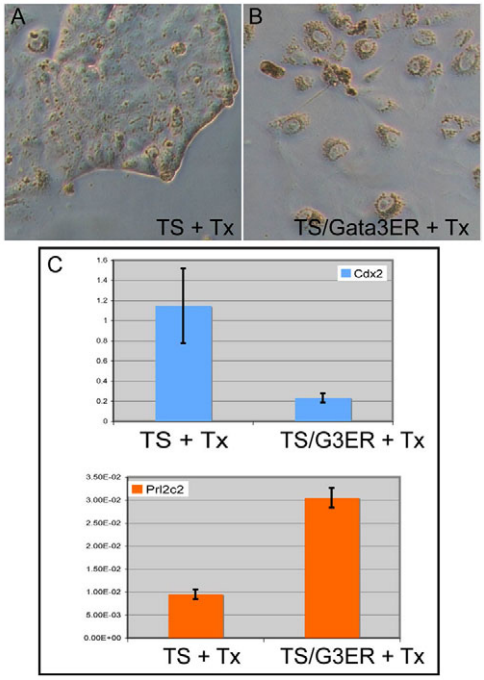

Fig. 5. Gata3 is sufficient to induce differentiation in TS cells. (**A**) Control TS cells treated with tamoxifen (Tx) in TS cell medium for 5 days. (**B**) Giant cells have formed after 5 days of overexpression of Gata3ER in TS cells in the presence of tamoxifen and TS cell medium. (**C**) qPCR analysis of stem cell (*Cdx2*) and giant cell (*Prl2c2*) markers in control and Gata3-expressing TS cells. Results are representative of experiments performed in duplicate.

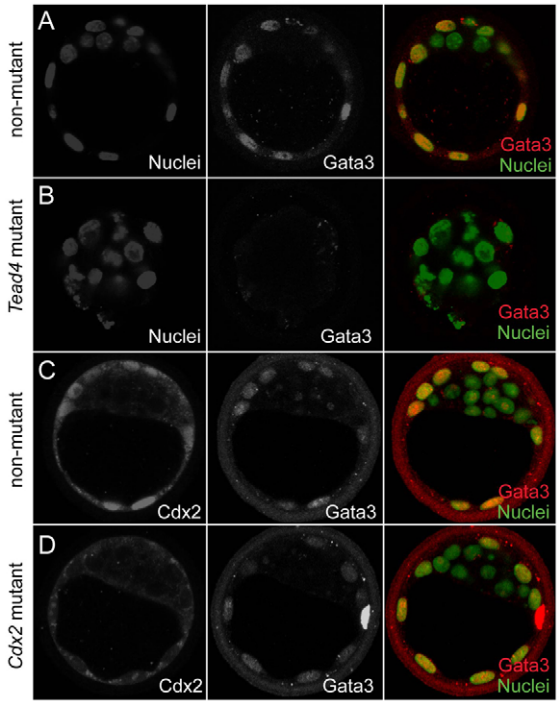

Fig. 6. Parallel regulation of Cdx2 and Gata3 during blastocyst formation. (**A**) Confocal section of a non-mutant blastocyst from a *Tead4* heterozygous intercross, showing Gata3 protein and nuclear stain. Merged red and green signals appear yellow. (**B**) Confocal section of a *Tead4* mutant at the same time point as in A, showing greatly reduced levels of Gata3 in outside cells of the embryo. *Tead4* mutants contain roughly the same number of cells as non-mutants at this stage. (**C**) Confocal section of non-mutant blastocyst from a *Cdx2* heterozygous intercross, following immunofluorescent staining to detect Cdx2 and Gata3 proteins and nuclear stain. Note that the zona pellucida (zp) and polar body (pb) can also be detected in some channels/planes. (**D**) Confocal section of *Cdx2* mutant blastocyst showing Cdx2 and Gata3 protein within the trophectoderm. For each mutation examined, images were collected within a single confocal session and with identical settings.

Cdx2 expression is also lost in *Tead4* mutants (Yagi et al., 2007; Nishioka et al., 2008), suggesting that Cdx2 could be required for *Gata3* expression during preimplantation. We therefore examined the requirement for *Cdx2* in Gata3 expression in the trophectoderm by examining Gata3 expression in *Cdx2* null embryos at E3.5. By confocal analysis, Gata3 expression was unaffected by loss of *Cdx2* (Fig. 6C,D) (n=6) at the blastocyst stage. This was validated at the mRNA level by qPCR (see Fig. S3 in the supplementary material). We conclude that *Cdx2* is not required for the expression of Gata3 during trophectoderm formation, consistent with the similar timing of their expression at earlier stages. Rather, *Gata3* appears to be regulated by Tead4 in parallel to *Cdx2* during blastocyst formation.

Oct4 does not restrict trophectoderm gene expression during early blastocyst formation

We have shown that, like Cdx2, Gata3 is initially expressed throughout the preimplantation embryo, suggesting that both genes become patterned by a process of repression within inside cells during blastocyst formation. *Oct4* is required for repression of *Cdx2* in ES cells (Niwa et al., 2000), consistent with a possible role for *Oct4* in repressing trophectoderm fates in the embryonic lineage. However, whether *Oct4* is required for repression of *Cdx2* in the inner cell mass during blastocyst formation has not been examined. Likewise, the role of Oct4 in regulating *Gata3* expression during blastocyst formation remains unknown.

To examine the requirement for *Oct4* in repressing *Cdx2* and *Gata3* in vivo, we examined the expression of these markers in embryos lacking zygotic *Oct4* (Kehler et al., 2004). At the blastocyst stage (E3.5), Oct4 protein was undetectable in *Oct4* mutants (Fig. 7B) (n=3), whereas Oct4 was detected throughout the blastocyst at this stage in non-mutants (Fig. 7A). However, Cdx2 and Gata3 expression patterns were largely unaffected in *Oct4* mutants (n=4/5 and n=2/2, respectively) (Fig. 7C). Although weak Cdx2 expression was detected in the inner cell mass of one *Oct4* mutant embryo, Cdx2 expression levels in the trophectoderm of this mutant embryo were also weaker than in non-mutant littermates (not shown). This pattern is normally observed in early blastocysts (Ralston and Rossant, 2008), suggesting that *Oct4* mutants can exhibit a slight developmental delay relative to non-mutant littermates. Indeed, this proposal is consistent with the previous observation that the trophectoderm marker keratin 8 (Krt8, detected by TROMA1 antibody) is detected in the inner cell mass of some, but not all, *Oct4* null embryos (Nichols et al., 1998). Since Krt8 is also expressed in the inner cell mass of early blastocysts (Ralston and Rossant, 2008), a developmental delay of *Oct4* mutants could explain this phenotype. Nonetheless, the majority of *Oct4* mutants exhibited the normal trophoblast-restricted expression of Cdx2 at the blastocyst stage. These results therefore suggest that zygotic *Oct4*

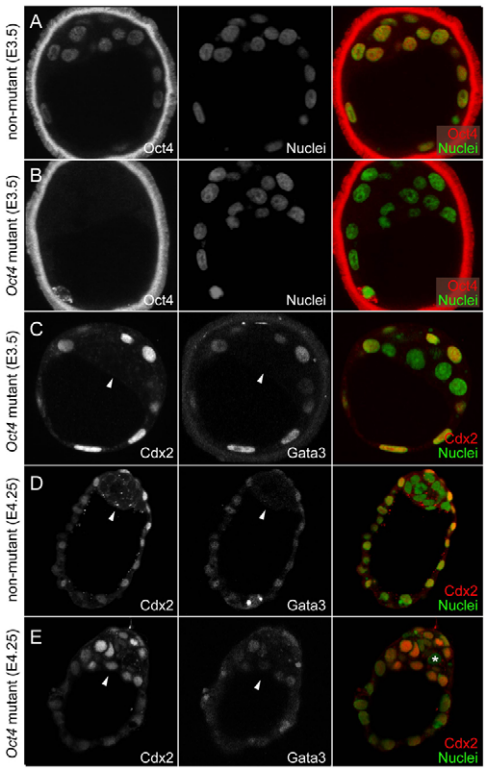

Fig. 7. Oct4 maintains, but does not initiate, repression of trophectoderm genes in the inner cell mass. (**A**) Confocal section showing Oct4 protein and nuclear stain in a non-mutant blastocyst resulting from intercrossing *Oct4* heterozygous mice. (**B**) Confocal section showing nuclear stain and lack of detectable Oct4 protein in *Oct4* mutant blastocyst. (**C**) Confocal sections showing Gata3 and Cdx2 protein in *Oct4* mutant blastocysts. Note the absence of detectable Gata3 or Cdx2 in cells of the inner cell mass (arrowheads). (**D**) Confocal sections showing Gata3 and Cdx2 protein and nuclei in a non-mutant implanting blastocyst from an *Oct4* heterozygous intercross. Note the lack of detectable nuclear Gata3 and Cdx2 in epiblast and primitive endoderm cells (arrowheads). (**E**) Confocal sections showing nuclear Gata3 and Cdx2 in cells occupying epiblast and primitive endoderm regions (arrowheads), and a cell that lacks expression of either (asterisk). For each litter examined, images were collected within a single confocal session and with identical settings.

is not required for initial repression of trophectoderm genes *Cdx2* or *Gata3*, indicating that other mechanisms lead to the restriction of both of these genes to the trophectoderm.

To examine whether *Oct4* is required for maintaining restricted expression of trophectoderm genes at later developmental stages, we attempted to examine the expression of Cdx2 and Gata3 in *Oct4* mutants after implantation, at ~E4.25. At this stage, *Oct4* mutants were extremely rare (1 mutant/27 non-mutant embryos), consistent with a requirement for *Oct4* for embryo survival. However, in a rare mutant recovered at this stage, Cdx2 and Gata3 were clearly upregulated in cells occupying epiblast and primitive endoderm territories (Fig. 7E). By contrast, both Cdx2 and Gata3 were always excluded from nuclei in epiblast and primitive endoderm populations in non-mutants (Fig. 7D) (n=26). Thus, *Oct4* is required for continued repression of *Cdx2* and *Gata3* in the late blastocyst, similar to its proposed role in repressing trophoblast fate in ES cells (Niwa et al., 2005). These observations indicate that the establishment of trophoblast and embryonic lineages proceeds by a mechanism that is distinct from the program that regulates this lineage restriction in established ES cells or the epiblast.

DISCUSSION

Here, we have used a combination of bioinformatic and functional genomic approaches to address fundamental questions about the first lineage restriction in the mouse. Specifically, what other factors act downstream of *Tead4*, are these sufficient or necessary to induce trophoblast fate, and are trophoblast factors themselves regulated in the embryo through mechanisms similar to those used in ES cells? Through genetic analyses performed in stem cells and in the mouse embryo, we provide evidence that *Gata3* acts downstream of *Tead4* and in parallel to *Cdx2*. A fundamental challenge in the field of stem cell biology is the paucity of truly trophoblast-specific markers. To overcome this challenge, we used TS cells as a reference tissue to define a set of core trophoblast genes. This enabled a deeper molecular comparison of trophoblast phenotypes resulting from the overexpression of *Gata3* or *Cdx2*, and provides a reference for future studies of this type.

We have shown that *Gata3* is sufficient to induce trophoblast genes in ES cells, consistent with another study (Nishiyama et al., 2009). Our analysis, however, revealed differences between *Gata3* and *Cdx2*. First, although expression of *Gata3* can induce trophoblast differentiation in ES cells, stable TS cell lines could not be maintained, unlike the situation with Cdx2. Rather, Gata3 appears to act as a pro-differentiation factor in TS cells. Second, unlike *Cdx2*, *Gata3* is probably not required for the early lineage decision in the embryo. Whereas shRNA-mediated knockdown of *Gata3* leads to developmental delay during the morula-to-blastocyst transition (Home et al., 2009), *Gata3* null embryos survive until E10.5, whereupon they exhibit defects in the placenta and numerous fetal tissues (Ma et al., 1997). Thus, *Gata3* is both necessary and sufficient to promote trophoblast maturation, but is not sufficient to stabilize the stem cell state. Other studies have shown that other factors, including Eomes, Elf5 and activated Ras, can also destabilize the pluripotent state of ES cells and drive trophoblast differentiation (Niwa et al., 2005; Lu et al., 2008; Ng et al., 2008; Nishiyama et al., 2009). Together, these observations suggest that there are multiple pathways capable of overriding the pluripotency program to induce trophoblast fate in ES cells.

Another intriguing difference between *Cdx2* and *Gata3* lies in their expression patterns at later stages of trophoblast development. Whereas *Cdx2* and *Gata3* were coexpressed in the EXE, *Gata3* was expressed at much higher levels within the EPC around the time of gastrulation. These observations suggest where Cdx2/Gata3 targets might be expressed. For instance, common trophoblast targets would be expected to be expressed in the EXE. Consistent with this proposal, many genes involved in EXE development, such as *Fgfr2*, *Wnt7b* and *Bmp4* (Orr-Urtreger et al., 1993; Coucouvanis and Martin, 1999; Kemp et al., 2005), were induced by either *Cdx2* or *Gata3* overexpression in ES cells. In addition, EXE genes such as *Eomes* and *Ascl2* (Guillemot et al., 1994; Ciruna and Rossant, 1999; Russ et al., 2000) were induced by Gata3 even in the absence of *Cdx2*, suggesting that Gata3 can reinforce trophoblast fate through a *Cdx2*-independent mechanism. Intriguingly, *Gata3* was expressed at higher levels in the EPC than in the EXE, whereas *Cdx2* was not. This provides potential biological relevance for the set of trophoblast genes that were induced by *Gata3* only in the absence of *Cdx2*. Genes in this list included *Pparg* and *Dnmt3l*, loss of which lead to defects in trophoblast differentiation (Barak et al., 1999; Bourc'his et al., 2001). Thus, Gata3 may promote a program of trophoblast

differentiation in the EPC where *Cdx2* expression is low or lacking. When overexpressed in TS cells, Gata3 induced differentiation of the cells towards more differentiated cell fates, consistent with this role. In this way, Gata3 could play a dual role, either promoting stem cell (EXE) fates or differentiation (EPC/giant cell fates) depending on the presence of other factors such as Cdx2. This proposal is consistent with evidence that *Gata3* is required for self-renewal of TS cells (Home et al., 2009). Moreover, Gata2 has been proposed to promote self-renewal versus differentiation of hematopoietic progenitor cells in a level-dependent manner (Heyworth et al., 1999), arguing that Gata3 might play a similar role in the trophoblast lineage.

We have also examined whether trophoblast factors are regulated in the embryo through mechanisms similar to those used in ES cells. In ES cells, Oct4 normally represses trophoblast fate (Niwa et al., 2000). However, it has not been clear whether this relationship applies to the embryo. Since Cdx2 and Gata3 are initially expressed in both inside and outside cells, Oct4 could repress the expression of these factors in inside cells during blastocyst formation. However, we show that Oct4 is not involved in the repression of trophoblast fate in the embryo until around the time of implantation. Indeed, trophoblast cells can coexpress Cdx2 and Oct4 in a variety of contexts (Niwa et al., 2005; Strumpf et al., 2005; Lu et al., 2008; Ng et al., 2008), arguing that Oct4 cannot be providing the initial patterning information along the inside/outside axis of the embryo. Rather, it was recently shown that the absence of Hippo signaling promotes Cdx2 expression in outside cells during blastocyst formation (Nishioka et al., 2009). Thus, the maintenance of ES cell fate might reflect molecular interactions that are relevant to stages of development following the initial lineage decisions. This proposal could help to explain why *Cdx2* is not required for Gata3-mediated induction of *Eomes* in ES cells, even though *Cdx2* is initially required for expression of Eomes in the blastocyst (Ralston and Rossant, 2008). Studies conducted in ES cells may therefore be viewed as reflecting a lineage maintenance, rather than establishment, program.

Finally, our study suggests that culture conditions influence cell fate changes induced by transcription factor overexpression in ES cells. Cdx2, and not Gata3, was sufficient to induce the formation of TS-like cells from ES cells. However, the isolation of stable TS-like cells is only possible following continued passage in TS cell medium after an initial period of transient transcription factor overexpression, a process that takes at least 6 days. This is reminiscent of the process involved in reprogramming mature cell types to pluripotency (Takahashi and Yamanaka, 2006), and suggests that similar mechanisms might be involved in the generation of stable TS cell lines. Given that many non-trophoblast genes were induced by the overexpression of either *Cdx2* or *Gata3*, altering culture and selection conditions could therefore lead to an enrichment of different cell fate outcomes. For instance, the use of different cell culture medium, growth factors or small molecules might enable the enrichment of TS-like cells or endoderm from *Gata3*-expressing ES cells. Given that multiple pathways can override the pluripotent state, the manipulation of intrinsic and extrinsic factors could facilitate the selection of other lineage-specific stem cell types during this process.

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Competing interests statement
The authors declare no competing financial interests.

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