LETTERS

Generation of nuclear transfer-derived pluripotent ES cells from cloned *Cdx2*-deficient blastocysts

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The derivation of embryonic stem (ES) cells by nuclear transfer holds great promise for research and therapy but involves the destruction of cloned human blastocysts. Proof of principle experiments have shown that 'customized' ES cells derived by nuclear transfer (NT-ESCs) can be used to correct immunodeficiency in mice[1]. Importantly, the feasibility of the approach has been demonstrated recently in humans[2], bringing the clinical application of NT-ESCs within reach. Altered nuclear transfer (ANT) has been proposed as a variation of nuclear transfer because it would create abnormal nuclear transfer blastocysts that are inherently unable to implant into the uterus but would be capable of generating customized ES cells[3]. To assess the experimental validity of this concept we have used nuclear transfer to derive mouse blastocysts from donor fibroblasts that carried a short hairpin RNA construct targeting *Cdx2*. Cloned blastocysts were morphologically abnormal, lacked functional trophoblast and failed to implant into the uterus. However, they efficiently generated pluripotent embryonic stem cells when explanted into culture.

Survival of the normal embryo beyond implantation depends on the formation of the trophectoderm lineage, the extra-embryonic lineage that forms the fetal–maternal interface within the placenta. The second embryonic lineage that forms, the inner cell mass (ICM), gives rise to all subsequent lineages in the embryo proper, and it is the ICM that, upon explanting in culture, gives rise to ES cells. The 'altered nuclear transfer' (ANT) concept[3] is based on the premise that the inactivation of a gene crucial for trophectoderm development will eliminate the potential to form the fetal–maternal interface, but will spare the ICM lineage. By genetically altering a somatic donor cell before nuclear transfer, one could generate cloned blastocysts that have no potential to develop beyond the blastocyst stage because no placenta could be formed. However, such cloned blastocysts could generate NT-ESCs derived from the ICM.

In this study we have performed a proof-of-principle experiment in mice to test the validity of the ANT approach and chose *Cdx2* as a candidate gene, as this gene encodes the earliest-known trophectoderm-specific transcription factor that is activated in the 8-cell embryo and is essential for establishment and function of the trophectoderm lineage[4,5]. *Cdx2*-deficient blastocysts fail to maintain a blastocoel, lack epithelial integrity, dysregulate the ICM-specific transcription factors *Oct-4* and *Nanog*, and show increased cell death[5]. Importantly, *Cdx2*-deficient blastocysts are able to form an ICM and generate ES cells when explanted in tissue culture[4,5].

We selected for functional short hairpin (sh)RNAs against *Cdx2* as described in Supplementary Figs S1 and S2 (Supplementary Information). The experimental scheme, outlined in Fig. 1a, involved the introduction of a conditional *Cdx2* shRNA lentiviral vector (Fig. 1b) into primary tail-tip fibroblasts from neonatal F_1 mice (C57BL/6x129/SvJae). Green fluorescent protein (GFP)-positive $Cdx2^{2Lox}$ tail-tip fibroblasts were selected and used as donors for nuclear transfer. *Cdx2*-deficient blastocysts derived from the manipulated donor cells were tested for their potential to implant into the uterus and to generate pluripotent ES cells.

Of a total of 526 reconstructed oocytes, 350 formed pronuclei, of which 61 cleaved and developed into nuclear transfer morula/blastocysts. *Cdx2* knockdown nuclear transfer embryos showed no delay in developing to the early blastocyst stage compared to nuclear transfer embryos expressing a shRNA targeting *CD8*[6] (data not shown). Figure 2a shows that GFP-positive $Cdx2^{2Lox}$ nuclear transfer blastocysts did not express *Cdx2* as assessed by immunohistochemistry, in contrast to wild-type blastocysts (columns 1 and 2, Fig. 2a). Figure 2b shows that, when compared to control nuclear transfer blastocysts, $Cdx2^{2Lox}$ nuclear transfer blastocysts were morphologically abnormal and failed to maintain a blastocoel cavity during *in vitro* cultivation, similar to previous results with *Cdx2* knockout blastocysts[5]. Using semi-quantitative polymerase chain reaction with reverse transcription (RT–PCR), we confirmed the

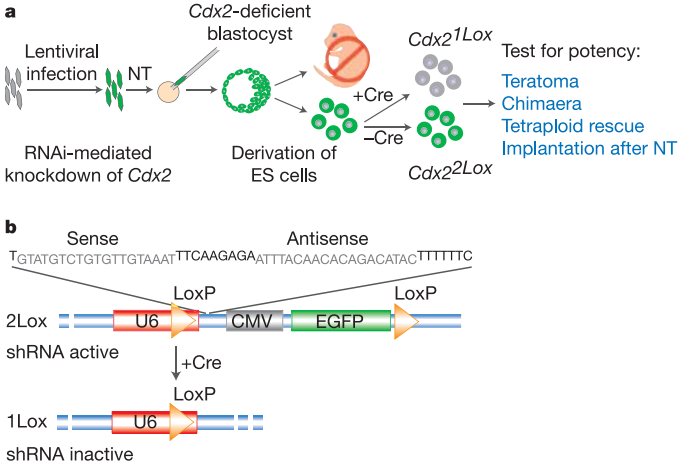

Figure 1 | Derivation of NT-ESCs from *Cdx2*-deficient blastocysts. **a**, Primary tail-tip fibroblasts were infected with a conditional lentiviral RNA interference (RNAi) construct targeting *Cdx2* before nuclear transfer (NT). Blastocysts deficient for *Cdx2* were morphologically abnormal and unable to implant but gave rise to NT-ESCs. After initial expansion of the *Cdx2* knockdown NT-ESCs ($Cdx2^{2Lox}$) we used transient Cre expression to generate subclones ($Cdx2^{1Lox}$) with a deleted hairpin. To test the potency of ES lines before and after 'loop-out' we used teratoma formation, diploid and tetraploid blastocyst injections as well as nuclear transfer. **b**, The conditional RNAi system (pSicoR) has been described previously[6]. The shRNA, which targets nucleotides 1890–1908 located in the 3′ UTR of *Cdx2*, was cloned into the conditional RNAi vector generating pSicoR-$Cdx2^{2Lox}$. This vector carries the *Cdx2* shRNA construct and an enhanced green fluorescence protein (*EGFP*) gene flanked by two LoxP sites (2Lox), which allows for Cre-mediated deletion of the shRNA and the *EGFP* sequences.

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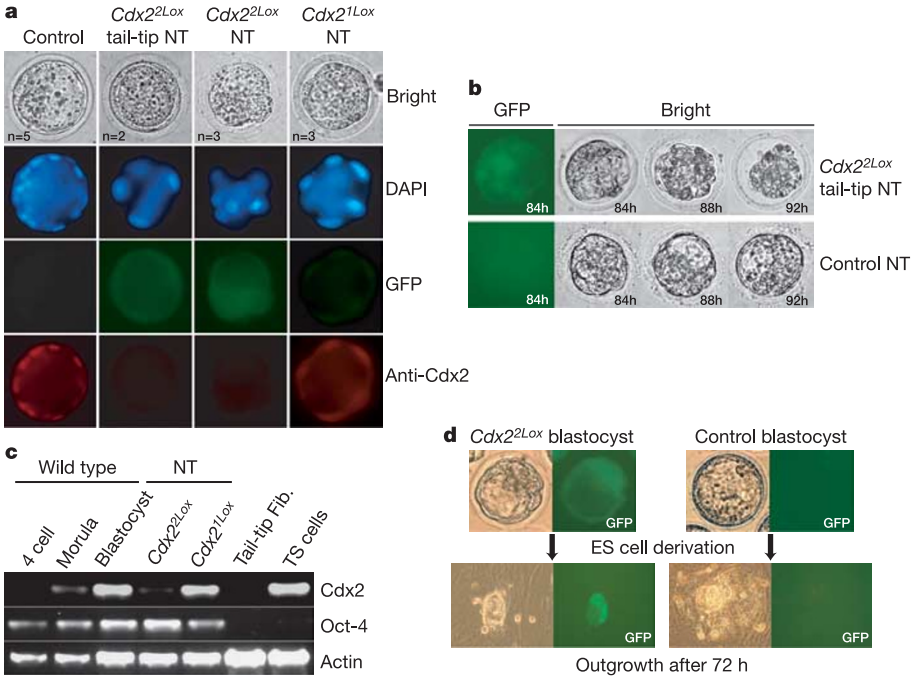

Figure 2 | *Cdx2*-deficient blastocysts and ES cell derivation. **a**, Cdx2 immunostaining of day 3.5–4.5 wild-type and nuclear transfer blastocysts. The following donor cells were used for the nuclear transfer (from second column, left to right): *Cdx2*^{2Lox} tail-tip, *Cdx2*^{2Lox} ES cells, and *Cdx2*^{1Lox} ES cells. **b**, A typical *Cdx2*^{2Lox} tail-tip nuclear transfer blastocyst is shown 84 h after activation of the reconstructed oocytes. *Cdx2*-deficient blastocysts initially cavitated but failed to maintain the blastocoel and collapsed. Below, an expanded nuclear transfer blastocyst derived from control cells is shown. **c**, RT–PCR analysis of normal and *Cdx2*-deficient nuclear transfer pre-implantation morula/blastocysts. Four 4-cell embryos were pooled and RNA was extracted for reverse transcription. All other samples were prepared from single morulae or blastocysts. Tail-tip fibroblasts (lane 6) express neither *Cdx2* nor *Oct-4*. Trophectoderm stem (TS) cells (lane 7) express *Cdx2*, but no *Oct-4*. A faint *Cdx2*-specific band, such as that seen in the blastocyst containing the shRNA construct targeting *Cdx2* shown in the figure, was detected in less than half of the tested embryos; most gave no signal in this test. **d**, Derivation of ES cells from *Cdx2*-deficient blastocysts. A *Cdx2*^{2Lox} tail-tip nuclear transfer-derived blastocyst with its initial outgrowth after 72 h (left) and a wild-type blastocyst (right) with its initial outgrowth are shown.

deficiency of *Cdx2* expression in *Cdx2*^{2Lox} nuclear transfer blastocysts, whereas control morulae and blastocysts showed robust *Cdx2* expression (Fig. 2c).

To assess whether Cdx2 deficiency interfered with post-implantation development, *Cdx2*^{2Lox} nuclear transfer morulae/blastocysts were transferred into the uteri of pseudo-pregnant females. The uteri were removed at embryonic day (E)6.5 and examined for sites of implantation. Figure 3a shows no implantations in the uterus from a foster mother transplanted with five *Cdx2*^{2Lox} nuclear transfer blastocysts, in contrast to a uterus transplanted with five nuclear transfer control blastocysts that resulted in successful implantations (Fig. 3b). As summarized in Table 1, none of the *Cdx2*^{2Lox} nuclear transfer blastocysts formed visible implantation sites (0 out of 40), in contrast to control nuclear transfer blastocysts that were derived from fibroblasts carrying the *CD8* control shRNA (6 out of 15). In addition, no evidence for delayed implantation was obtained, as we failed to detect implantation sites at E7 or E8 in females transplanted with a total of 18 *Cdx2* knockdown nuclear transfer embryos (data not shown). These results demonstrate that nuclear transfer from donor fibroblasts carrying the pSicoR-Cdx2^{2Lox} virus resulted in morphologically abnormal, *Cdx2*-deficient nuclear transfer blastocysts that failed to implant upon transfer into foster mothers.

To investigate whether *Cdx2*-deficient blastocysts can generate ES cells upon explantation in culture, nuclear transfer *Cdx2*^{2Lox} blastocysts were transferred onto feeder cells. Whereas control nuclear transfer blastocysts formed trophoblastic outgrowths characteristic of the trophectoderm lineage, the *Cdx2*^{2Lox} nuclear transfer blastocysts failed to generate any trophoblast cells (Fig. 2d). Consistent with previous observations[4,5], *Cdx2*-deficient blastocysts generated ICM outgrowths that grew into stable, GFP-positive nuclear transfer *Cdx2*^{2Lox} ES cell lines with an efficiency that was comparable to that of nuclear transfer blastocysts derived from wild-type fibroblasts (14% of explanted blastocysts). As a criterion for pluripotency, we tested the ability of the nuclear transfer *Cdx2*^{2Lox} ES cell lines to form chimaeras when injected into diploid blastocysts. The GFP-labelled cells contributed extensively to neonatal chimaeras (Fig. 3c, d) and formed high-grade postnatal chimaeras (Fig. 3i, summarized in Table 2) with high contributions to most tissues (Fig. 3e, f), with the notable exception of the intestine (Fig. 3g), which was entirely composed of *Cdx2*-positive cells derived from the host blastocyst (Fig. 3h). This is in agreement with previous reports, as it has been shown that Cdx2 is required for normal development of the gastro-intestinal tract[7]. We further explored the developmental potency of the NT-ESCs using tetraploid complementation, which represents the most stringent test for pluripotency, as the resulting 'ES mice' are entirely composed of cells derived from the injected ES cells[8]. Consistent with previous results[4], transfer of the *Cdx2*^{2Lox} ES

Table 1 | Survival of clones to blastocyst and post-implantation stage after nuclear transfer from different donor cells

Donor cells	pSicoR genotype	Number of clones with pseudo-pronuclei	Number of morulae/blastocysts	Number of implants at E6.5 (number of foster mothers)
Fibroblasts	*Cdx2*^{2Lox}	211	40	0 (7)
Fibroblasts	*CD8*^{2Lox}	76	15	6 (3)
ES cells	*Cdx2*^{2Lox}	177	18	0 (4)
ES cells	*Cdx2*^{1Lox}	199	22	11 (5)
ES cells	*CD8*^{2Lox}	103	15	7 (3)

Shown are the number of reconstructed oocytes with pseudo-pronuclei after 5–6 h of activation. Morula/blastocyst transfers were done on day 3.5. pSicoR-CD8^{2Lox} fibroblasts carry a shRNA against *CD8* (ref. 6).

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Table 2 | Developmental potential of ES cells deficient (*Cdx2*^{2Lox}) or proficient (*Cdx2*^{1Lox}) for *Cdx2* expression

	Injected into 4N blastocysts			Injected into 2N blastocysts	
		Embryos live at			
Donor ES cells	Number injected	E14	Term	Number injected	Number of chimaeras
Cdx2^{2Lox}	112	0	0	65	12/23
Cdx2^{1Lox}	36	ND	6	ND	ND

Thirty-three sites of implantation were detected in the tetraploid *Cdx2*^{2Lox} recipients, but no or only reabsorbed embryos (at 14.5) were present. Twelve of the 23 born diploid pups were chimaeras. ND, not done.

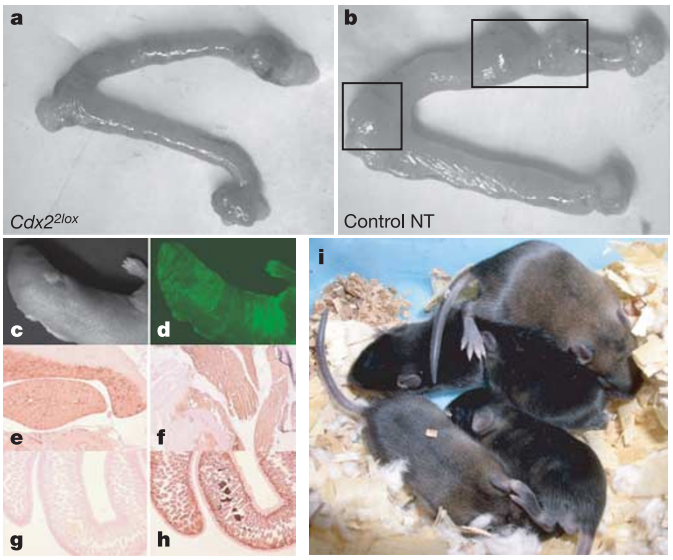

Figure 3 | *Cdx2*-deficient cells maintain developmental potential but are unable to implant after nuclear transfer. **a**, **b**, In each example shown, five nuclear transfer blastocysts were transferred at day 3.5 into the uterus of a day 2.5 pseudo-pregnant female. **a**, *Cdx2*-deficient blastocysts fail to implant. A representative uterus isolated at day 6.5 is shown. No deciduae were detectable from transplanted *Cdx2*-deficient blastocysts. **b**, Control nuclear transfer blastocysts showed normal implantation sites at day 6.5. **c**, Bright-field image of a postnatal *Cdx2*^{2Lox} ES chimaera. **d**, GFP signal indicates a contribution from *Cdx2*^{2Lox} ES cells. **e**–**g**, Histological sections and anti-GFP staining from a newborn *Cdx2*^{2Lox} chimaera. There was a contribution to the liver (endoderm; **e**) and muscle (mesoderm; **f**) but not to the intestine (**g**). **h**, Anti-Cdx2 staining of the intestine shown in **g**. **i**, Coat colour contribution of *Cdx2*^{2Lox} ES cells. Recipient blastocysts have a C57BL/6 × DBA/2 F_1 background and the *Cdx2*^{2Lox} ES cells a C57BL/6 × 129SvJae background. The presence of agouti (129/SvJae) fur indicates donor cell contribution. A litter with one wild type (black mouse below the top agouti), two low-contribution (middle) and two high-contribution chimaeras are shown.

cells resulted in no live embryos at E14 (Table 2). These data indicate that nuclear transfer using pSicoR-Cdx2^{2Lox} fibroblasts generates abnormal blastocysts that are inherently unable to implant and grow into a fetus but are able to generate pluripotent ES cells that have a diminished developmental potency as compared to wild-type ES cells.

To assess whether NT-ESCs derived from *Cdx2*-deficient blastocysts could have the same pluripotency as wild-type ES cells, we investigated whether the block to normal developmental potential could be relieved by reversing the effects of the *Cdx2* gene knockdown. Normal *Cdx2* gene function was restored in *Cdx2*^{2Lox} ES cells by transient transfection of a Cre plasmid, resulting in the deletion of the *Cdx2* shRNA and *EGFP* marker gene (*Cdx2*^{2Lox} to *Cdx2*^{1Lox}; compare Fig. 1b), and rendering the cells Cdx2 competent and GFP negative. Nuclear transfer from *Cdx2*^{1Lox} donor cells generated GFP-negative normal-appearing nuclear transfer blastocysts that expressed wild-type levels of Cdx2, as shown by immunostaining (Fig. 2a, right column) and RT–PCR (Fig. 2c, lane 5). To test whether deletion of the shRNA would restore pluripotency, the *Cdx2*^{1Lox} ES cells were injected into tetraploid blastocysts. As shown in Table 2, *Cdx2*^{1Lox} ES cells efficiently generated ES mice in contrast to the *Cdx2*^{2Lox} ES cells that were unable to give rise to ES mice. These results show that deletion of the *Cdx2* shRNA sequences creates ES cells that can generate all somatic tissues including normal intestinal cells, which cannot be derived from the *Cdx2*^{2Lox} parental ES cells (compare Fig. 3g, h). Finally, to test whether totipotency of *Cdx2*^{1Lox} ES cell nuclei was recovered, we transplanted *Cdx2*^{1Lox} blastocysts derived by nuclear transfer using *Cdx2*^{1Lox} donor ES cells into pseudo-regnant foster mothers. As summarized in Table 1, normal-sized implants were detected at E6.5. These results confirm that *Cdx2* deficiency was responsible for the failure of clones to generate functional blastocysts and exclude other genetic alterations acquired during *in vitro* manipulation of the cells in the characteristic block to implantation. Most importantly, our data demonstrate that ES cells competent to generate all lineages can be derived from abnormal nuclear transfer blastocysts.

The ethical controversy surrounding nuclear transplantation arises from the necessity to destroy the reconstructed human blastocyst in order to obtain embryonic stem cells that can be used for biomedical research and therapy (for different view points see refs 9, 10). All available evidence is consistent with the conclusion that after nuclear transfer, the reconstructed embryos lack the potential to develop into normal human beings with any acceptable or practical efficiency[11]. Despite the incompatibility with normal human development, the utility and promise of nuclear transfer is that embryonic stem cells derived by nuclear transfer have the same biological and molecular characteristics and the same therapeutic potential as those derived from fertilized embryos[11,12]. Altered nuclear transfer further cripples an already compromised blastocyst and eliminates the developmental potential to implant into the uterus to establish the fetal–maternal connection[3]. The genetic manipulations of the somatic donor cells that are required to generate such an inherently abnormal blastocyst are simple and straightforward. Our data indicate that the *Cdx2*-deficient blastocyst derived by nuclear transfer from a genetically engineered somatic cell is morphologically abnormal and lacks functional cells of the trophectoderm lineage, consistent with previous results with embryos from mutant animals[5]. Because the gene is expressed before the blastocyst stage[5], *Cdx2*-deficient clones are molecularly abnormal already at pre-blastocyst stages before an overtly abnormal phenotype becomes apparent. By reversing the *Cdx2* deficiency we demonstrate that fully competent ES cells can be derived from the inherently abnormal product of nuclear transfer using *Cdx2*-deficient donor cells.

If ANT was ever contemplated as an approach for the generation of human ES cells by nuclear transfer, the following issues need to be considered. (1) Although *CDX2* is expressed in the trophectoderm of human blastocysts[13] and derivatives of hES cells[14] its expression pattern in the human fetus has not been determined and it is unknown whether it has an identical effect on placentation as in mouse. Because the effect of gene inhibition on human placentation cannot be directly tested, surrogate assays such as *in vitro* differentiation of ES cells are required to assess the effect of *CDX2* deficiency on human trophoblast development. (2) The use of retroviral vectors for gene transduction[15] raises the possibility of insertional mutagenesis and the activation of oncogenes leading to leukaemia[16]. However, this probably does not represent a serious problem in ANT because, in contrast to the gene therapy trials using retroviral infection of bone marrow cells, viral integration into fibroblasts does not lead to a proliferative advantage and selective outgrowth of infected cells due to an activated oncogene. Also, because all nuclear

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transfer ES cells are clonal, it would be easy to ensure by DNA analysis that proviral integration was not in the vicinity of an oncogene.

The results reported in this paper provide proof of principle that inhibition of genes important for trophoblast function can prevent placentation without interfering with ES cell potency, and may thus provide a scientific basis for the ongoing debate surrounding the nuclear transfer technology. However, because the *Cdx2*-deficient embryo is not obviously abnormal before the onset of *Cdx2* expression, this approach may not solve the ethical dilemma. Moreover, research with primate or human cells will be required to assess whether *CDX2* is an optimal target for human application. Finally, we want to emphasize that ANT is a modification but not an alternative to nuclear transfer, as the approach requires additional manipulations of the donor cells that may complicate the logistics of production and safety assessment of patient-specific ES cell lines for therapy.

METHODS

Cloning and design of shRNAs. shRNAs were designed using the pSico-Oligomaker 1.5 (developed by A. Ventura, Jacks Lab), which is freely available at http://web.mit.edu/ccr/labs/jacks/protocols/pSico.html. Cloning into pSicoR was done as described on the website.

Generation of lentivirus and Cre-mediated recombination. Lentivirus was generated as described before[6]. The number of integrations was determined by Southern blot analysis. Genomic DNA was digested with *Xba*I (single cut in the viral backbone) and probed with an EGFP probe.

Cre-mediated recombination was achieved by transiently transfecting a Cre-recombinase-containing plasmid. Briefly, after introducing the Cre plasmid into the ES cells by electroporation, cells were cultured for 24 h (not longer, to avoid random integration of plasmid) in ES medium plus puromycin. GFP-negative subclones were picked, expanded and tested for recombination ($Cdx2^{1Lox}$).

Immunohistochemistry and RT–PCR. Cdx2 staining of blastocysts was done as described previously[5]. The protocol is available on the Rossant laboratory website (http://www.mshri.on.ca/rossant/protocols/immunoStain.html). Monoclonal anti-Cdx2 (CDX2-88, BioGenex) was used for all Cdx2 stainings. RT–PCR was done with a one-step RT–PCR kit (Qiagen) using the following primers: β-actin 5′-GGCCCAGAGCAAGAGAGGTATCC-3′ (forward) and 5′-ACGCACGATTTCCCTCTCAGC-3′ (reverse); *Oct-4* (333 bp) 5′-GGATGGCA TACTGTGGACCT-3′ (forward) and 5′-AGATGGTGGTCTGGCTGAAC-3′ (reverse); and *Cdx2* (225 bp) 5′-AAACCTGTGCGAGTGGATG-3′ (forward) and 5′-CTGCGGTTCTGAAACCAAAT-3′ (reverse). β-actin reverse transcription primer was published by ref. 5, and *Oct-4* and *Cdx2* primers were designed using PRIMER3.

NT, embryo transfer, ES cell derivation and 2N/4N blastocyst injections. Nuclear transfer was done as described previously[8,17]. Nuclear transfer embryos were transferred at day 3.5 (morula/blastocyst stage) into the uteri of day 2.5 pseudo-pregnant recipient females. For ES cell derivation, the zona pellucida was removed using acidic tyrode (AT) solution and blastocysts were explanted on irradiated feeders in ES medium plus MEK1 inhibitor (PD98059). Diploid and tetraploid blastocyst injections were done as described previously[18].

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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