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Governing cell lineage formation in cloned mouse embryos

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ABSTRACT

Blastomeres of the pre-implantation mouse embryo form trophectoderm and inner cell mass via a process that requires the transcription factors Tead4, Cdx2, Oct4 and Nanog. In mouse morulae cloned by somatic cell nuclear transfer, we observed that the trophectoderm transcription factor Cdx2 is expressed very differently at the protein level compared to time- and stage-matched fertilized counterparts. Protein levels of Cdx2 in cloned embryos appear 'erratic,' i.e. are widely distributed, when plotted as histograms. In contrast to Cdx2, protein levels of the upstream factor Tead4 and of inner cell mass transcription factors Oct4 and Nanog are similar in cloned and fertilized embryos. These observations suggest that trophectoderm formation is initiated but not maintained correctly in cloned mouse morulae, which is consistent with cloned blastocysts' limited implantation and post-implantation success. Because a cell's ability to differentiate is greatly enhanced if it is surrounded by more cells differentiating the same way, a concept designated *community effect* by Gurdon, we reasoned that the insufficient cell numbers often observed in cloned embryos might lead to premature Cdx2 expression and differentiation of blastomeres into trophectoderm. Therefore, we created larger cloned embryos by aggregating them at the 4-cell stage. Homologous aggregation stimulates expression of multiple signaling pathways' components and results in cloned embryos with levels of Cdx2 similar to fertilized embryos. Most of the resultant morulae and blastocysts consist of cells of all three founders, indicating that aggregation increases stability of all of the individual components. We conclude that the induction of pluripotency in cloned embryos is more efficient than previously assumed, and we propose that a minimum cell number is necessary to stabilize pluripotency and inhibit premature expression of Cdx2 in cloned mouse embryos.

Introduction

To support cloned embryo development, somatic nuclei transplanted into enucleated oocytes must activate pluripotency genes and silence somatic genes. These changes are collectively termed *nuclear reprogramming* and need multiple cell cycles to unfold (Eckardt and McLaughlin, 2004; Egli et al., 2007). Experimental measures of oocyte-mediated nuclear reprogramming are important not only for describing this process but also for addressing its mechanisms. These measures include, but are not limited to, rate of embryo cleavage, DNA de-methylation and re-expression of embryonic genes, timing and level of gene expression compared to fertilized embryos and the ability to yield embryonic stem (ES) cells in permissive species. After 3–4 cell cycles, mouse embryos cloned by somatic cell nuclear transfer (NT) fulfill these reprogramming criteria, at least in part (Cavaleri et al., 2008). The resultant morulae have to differentiate their broadly potent blastomeres into pluripotent inner cell mass (ICM) and oligopotent trophectoderm (TE), the progenitors of embryonic and extra-embryonic lineages, respectively. So far, research on cloned embryos has focused primarily on the induction of toti- and pluripotent blastomeres, not on their differentiation.

In fertilized mouse embryos, functional differences among blastomeres are apparent at the 8-cell stage and probably arise at the 4-cell stage (Bischoff et al., 2008; Torres-Padilla et al., 2007). The first two lineages of the mouse embryo, ICM and TE, are marked by the transcription factors Oct4 and Cdx2, respectively. In the majority of cumulus cell cloned mouse embryos, *Oct4* transcription is turned on during the 3rd cell cycle, albeit incorrectly (Bortvin et al., 2003; Sebastiano et al., 2005). At the blastocyst stage, non-ICM restricted *Oct4* expression suggests that the ICM is formed abnormally; Cdx2 protein localization suggests normal TE formation (Kishigami et al., 2006). However, cloned blastocysts give rise to normal ES cells but fail at implantation and early post-implantation development, which seems to contradict the reported gene expression (Brambrink et al., 2006; Jouneau et al., 2006; Wakayama et al., 2006; Wakisaka-Saito et al., 2006). It is likely that neither ICM nor TE is entirely normal in cloned embryos (Amano et al., 2002).

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At the molecular level, functional allocation of blastomeres to ICM and TE lineages involves at least two steps: initial induction with the transcriptional activation of relevant genes and subsequent stabilization of gene expression. It has not been established if errors of *Oct4* and *Cdx2* gene expression in cloned embryos are due to flawed induction or flawed stabilization, or both. These two levels of gene regulation co-exist in a model whereby emerging gene expression patterns need a certain extent of cell–cell interaction to become stabilized (Kupiec, 1997). We thus hypothesize that insufficient cell numbers in cloned embryos (Boiani et al., 2003) could affect overall gene expression and in particular the expression of *Cdx2*, leading to premature maturation of the TE in cloned blastocysts. Indeed, the ability of a cell to differentiate correctly depends on surrounding cells differentiating in the same way; this mechanism has been termed *community effect* (Gurdon, 1988). Initially described in *Xenopus*, the community effect has also been found in mammals (Bauwens et al., 2008; Cossu et al., 1995; Hamazaki et al., 2004).

We have comparatively analyzed cell lineage formation in cloned (NT) and in fertilized (ICSI) mouse embryos using in situ immunofluorescence and confocal microscopy to measure protein levels of Cdx2, Oct4 and Nanog in blastomere nuclei. By plotting the immunofluorescence signals of the nuclei into histograms, we show that expression of Cdx2 has an *erratic*, i.e. widespread distribution, in NT morulae compared to time-matched (72 h) and stage-matched (16 ± 4 cells) ICSI counterparts; the expression distribution of Oct4 and Nanog is indistinguishable in the two types of embryos. Additionally, the expression of Tead4, a transcription factor upstream of Cdx2, is not erratically distributed, suggesting a defect of stabilization and not of induction of the TE in NT morulae. Because cloned embryos have lower cell numbers than fertilized counterparts (Boiani et al., 2003), the lower cell numbers would hinder the correct cell lineage differentiation in the blastocyst if a community effect indeed plays a role in TE formation. In line with this hypothesis, we observe that homologous (i.e. clone–clone) embryo aggregation corrects for erratic expression of Cdx2 and promotes post-implantation development of cloned mouse embryos. Since "most aspects of animal development seem to proceed by the cooperation of several contributory processes, no one of which is individually indispensable" (Gurdon, 1988), it is likely that the process involved in homologous embryo aggregation is a complex and multifaceted one. Transcriptome analysis corroborates this possibility by showing that the components of multiple signaling pathways, particularly those of the Tgf-β pathway, are expressed at higher mRNA levels in aggregated compared to single cloned embryos. In light of the effects of homologous aggregation on gene expression and post-implantation development, we propose that a minimum cell number is necessary to stabilize pluripotency and inhibit premature Cdx2 expression in cloned mouse embryos.

Materials and methods

Mice

Six- to eight-week-old B6C3F1 (C57Bl/6J×C3H/HeN) mice were used as oocyte and cumulus cell donors. Mice expressing *GFP* from the X chromosome (X-GFP) have been described (Hadjantonakis et al., 1998) and were obtained from Jackson Laboratory (Tg(CAG-EGFP) D4Nagy/J). Mice were maintained and used for experiments according to institutional guidelines.

Somatic cell nuclear transfer (NT) and intracytoplasmic sperm injection (ICSI)

Micromanipulations were performed as previously described (Balbach et al., 2007). Since cumulus cell-derived cloned embryos were all female, hemizygous X-GFP male sperm and wild-type oocytes were used for ICSI of B6C3F1 oocytes, and hemizygous F1 females were used as nuclear donor cells for NT. Female embryos, which carried one X-linked GFP, were selected for microarray analysis based on green fluorescence.

In vitro culture of NT and ICSI embryos

In vitro culture of cloned and ICSI embryos was performed as previously described (Balbach et al., 2007). CZB, M16, KSOM(aa) and G1/G2 media were prepared in-house according to original recipes (Boiani et al., 2005). α-MEM was purchased (Sigma cat. no. M4526). All media were supplemented with BSA (2 mg/mL) and gentamicin sulfate (50 μg/mL). For some experiments, culture media were supplemented with Tgf-β1 (2 ng/mL; Paria and Dey, 1990), Bmp4 (10 ng/mL) or Egf (4 ng/mL; Paria and Dey, 1990).

Analysis of ATP content

The ATP content of embryos was measured at different stages of development using a previously reported ATP-dependent luciferase/luciferin reaction system. Ten to 12 embryos were collected in 200 μL milli-Q water, vortexed and placed at 95 °C for 20 minutes to inactivate all ATP converting enzymes and stored at −80 °C until measurement. Samples were incubated in buffer containing $MgCl_2$ 1 mol/L, KCl 2 mol/L and phosphoenolpyruvate 0.2 mol/L at 30 °C for 15 minutes. A luciferase/luciferin cocktail (ENLITEN, Promega) was mixed with the sample, and light emission was detected using a luminometer equipped with a single photon counter (Tecan GENios Pro). ATP levels were calculated by extrapolation to a calibration curve based on known concentrations of ATP (Sigma).

Embryo transfer in vivo

To test for pre-implantation development, embryos were transferred to oviducts of CD1 females, which had been mated to vasectomized males 3 days earlier, and plugged on the day of embryo transfer. Embryo transfer was performed as early as possible at 7 h after incubation in activation medium (6 h) and subsequent wash-off (1 h). To test for post-implantation development, aggregated cloned embryos were transferred to uteri of ICR females that were 2 1/2 days pseudo-pregnant. At 10.5 dpc, females with a weight increase consistent with a pregnancy were sacrificed to count the number of implantations and fetuses.

Immunofluorescence, confocal imaging and image analysis

Morulae were sampled either stage-matched (16 ± 4 cells) at 72 h (ICSI) or 78 h (NT), or time matched at 72 h (both ICSI and NT, 16 ± 4 cells). Four-cell stage embryos were sampled at 42 h, and 8-cell stage embryos were sampled at 55 h; blastocysts were sampled at 96 h post-activation. Zona pellucida was removed by treatment with warm Tyrode's acidic solution for 20 seconds. Embryos were fixed in 1.5% paraformaldehyde in PBS/Triton-X 100 0.1%/PVP 0.1% for 15 minutes at ambient temperature. After washing in PBT (PBS/Tween-20 0.1%/PVP 0.1%), unspecific binding sites were blocked by incubating in blocking buffer (PBS/donkey serum 5%/BSA 2%/Glycine 2%/Tween-20 0.1%) for 1 h at 4 °C. Embryos were then incubated in a dilution of the primary antibody (anti-Nanog rabbit IgG, Cosmo Bio, Tokyo, Japan, cat. no. REC-RCAB0002P-F; anti-Oct4 goat IgG, Santa Cruz Biotechnology, Heidelberg, Germany, cat. no. sc-8628; anti-Cdx2 mouse $IgG_{1\kappa}$, Emergo Europe, The Hague, Netherlands, cat. no. CDX2-88; anti-β-catenin rabbit antiserum, Sigma-Aldrich, Munich, Germany, cat. no. C2206; anti-Smad4 mouse monoclonal IgG_1, Santa Cruz Biotechnology, Heidelberg, Germany, cat. no. sc-7966; anti-Tead4 mouse IgG, Abnova Corp., Taipei, Taiwan, cat. no. H00007004-B01P) in an extended blocking solution (PBS/donkey serum 1%/BSA 0.5%/

Glycine 0.5%/Tween-20 0.1%) overnight at 4 °C. After a 10-minute wash in PBT, embryos were incubated in a dilution of the secondary antibody (anti-rabbit, -goat, -mouse IgG donkey IgG, conjugated with Alexa Fluor 488, 568, 647, respectively; Invitrogen, Karlsruhe, Germany) in extended blocking solution for 2 h. After washing in PBT, specimens were mounted in microdrops of PBS covered with mineral oil on a thin-bottom plastic dish (Lumox, Greiner Bio One, cat. no. 96077333). Where applicable, nuclei were counterstained with the far-red fluorescent DNA dye Draq5 (Biostatus, Shepshed, UK).

Imaging was performed with an UltraVIEW RS3 spinning disk confocal imaging system (PerkinElmer LAS, Jügesheim, Germany) on an inverted microscope (TE-2000U, Nikon, Düsseldorf, Germany) using a 60× water immersion lens (N.A. 1.2). The light source was a 3-line (488 nm, 568 nm, 647 nm) Argon/Krypton laser (Melles Griot). Optical sections were captured 5 µm apart from each other using a 1.3 megapixel Hamamatsu ORCA ER digital camera. Acquired images were converted to maximum intensity projections using PerkinElmer software and imported into ImageJ (Abramoff et al., 2004). Regions of interest (ROIs) were drawn around non-overlapping parts of nuclei (Fig. S1), and average pixel intensities of individual nuclei were measured. Bleed through of the fluorophores was excluded according to Pawley (2006) by imaging control embryos stained with only one primary antibody while using the three-channel imaging settings used for triple-labeled samples (Fig. S2).

Live-cell labeling of embryos prior to aggregation

The cytoplasm of 4-cell embryos was labeled by bathing zona-free embryos in a suspension of fluorescently labeled latex beads (0.05% w/v, FluoSpheres, Invitrogen cat. no. F10720) in α-MEM without BSA for 10 minutes, followed by washing in the same medium without beads (Fleming and George, 1987).

Embryo aggregation and bisection

Cell number normalization was achieved by aggregating 3 cloned embryos at the 4-cell stage with one another (homologous) as previously described (Boiani et al., 2003). Aggregation was accomplished by arranging zona-free embryos in groups of two or three in a planar scheme in micro-wells. Single cloned embryos (1×) and double (2×) or triple (3×) clone aggregates, as well as aggregated ICSI embryos, were cultured in parallel in separate drops within the same dish. Aggregation of more than three embryos is progressively less effective (Petters and Mettus, 1984). The majority of aggregates formed cavities and developed to blastocyst stage 48 h post-manipulation. To produce fertilized phenocopies of cloned embryos, ICSI-fertilized 2-cell stage embryos were deprived of one blastomere by removing it completely with a needle inserted through the zona pellucida. The remaining blastomere was left to cleave inside the zona.

Illumina bead chip hybridizations

We used ICSI embryos to take into account invasive micromanipulation, and we used two independent biological replicates each consisting of triple (3×) and single (1×) cloned embryos, both without zona pellucida. Since cumulus cell cloned embryos were all female, we used *X* chromosome linked *GFP* transgenic mice to select female ICSI embryos to compare with female NT embryos. Total RNA was isolated using the MicroRNeasy Kit (Qiagen, Hilden, Germany). In order to generate enough RNA for the subsequent microarray analysis, a two-round linear amplification protocol employing a linear amplification kit (Ambion, Austin, TX, USA) was adopted to generate biotin-labeled cRNA, 1.5 µg of which was used for each hybridization reaction. Washing, Cy3-streptavidin staining and scanning were performed on the Illumina BeadStation 500 (Illumina, San Diego, CA, USA) platform using reagents and following protocols supplied by the manufacturer. cRNA samples were hybridized onto Illumina WG-6 BeadChips in three technical replicates. All basic expression data analysis was carried out using the manufacturer's software BeadStudio 1.0. Raw data were background subtracted and normalized using the *rank invariant* algorithm. Normalized data were filtered for significant expression on the basis of negative control beads. Selection for differentially expressed genes was performed on the basis of 2-fold changes plus statistical significance according to the Illumina *t* test error model.

Gene expression difference was considered if variation was more than 2-fold with $p<0.01$. In 3×NT compared to 1×NT, 18 transcripts were consistently higher expressed (↑) and 91 were consistently lower expressed (↓). In order to distinguish enhanced reprogramming from generic changes of gene expression due to an increased number of cells, we compared 1×ICSI with 1×NT embryos. In 1×ICSI, we found 989 genes higher expressed and 2123 genes lower expressed (Table S5). We assumed that for more successful reprogramming, the 989 genes up-regulated in ICSI would need to be up-regulated in NT and that the 2123 genes suppressed in ICSI would need to be suppressed in NT. Therefore, we compared the set of genes responsive to cloned embryo aggregation (18↑, 91↓) with the set of genes that distinguish 1×NT from 1×ICSI (989↑, 2123↓) to find any overlap.

Analysis of gene expression by QRT-PCR (Taqman)

For real-time analysis of gene expression, embryos were harvested, processed and analyzed as previously described (Cavaleri et al., 2008) using the ABI PRISM Sequence Detection System 7900 (Applied BioSystems). Primers for the following genes were designed by the Taqman Assay-on-Demand: *Pou5f1* (*Oct4*, Mm00658129_gH), *Cdx2* (Mm00432449_m1), *Bmp4* (Mm00432087_m1), *β-Actin* (Mm00607939_s1), *Pou2f1* (*Oct1*, Mm00448332_m1), *Tbp* (Mm00446973_m1) and *Hprt1* (Mm00446968_m1). Oligos for *Nanog* amplification were custom designed (PF: 5′-AAC CAG TGG TTG AAT ACT AGC AAT G-3′, PR: 5′-CTG CAA TGG ATG CTG GGA TAC T-3′, Probe: 5′-6FAM-TTC AGA AGG GCT CAG CAC-MGB-3′).

TS cell derivation and culture

Derivation and culture of trophoblast stem (TS) cells were performed as described (Tanaka et al., 1998). In brief, 3.5-dpc blastocysts were placed on mouse embryonic fibroblasts (MEF) in 24-well plates containing TS medium (RPMI 1640 (PAA) containing penicillin/streptomycin 50 µg/mL (PAA), fetal bovine serum 20% (gold, PAA), sodium pyruvate 1 mM (PAA), β-mercaptoethanol 100 µM (Gibco), L-glutamine 2 mM (GlutaMAX-I, Gibco)) supplemented with FGF4 25 ng/mL (1×; PeproTech cat. no. 100-31) and heparin 1 µg/mL (1×; Sigma cat. no. H1349). On day 4, outgrowths had formed, which were disaggregated in the well. Medium was changed every other day (TS medium with 1.5× FGF4/heparin) until TS cell colonies covered about 50% of the well. Cells were then passaged to 35-mm dishes on MEFs. After another passage on MEFs, cells were cultured feeder-free in 70% MEF-conditioned TS medium/30% fresh TS medium with 1× FGF4/heparin and split weekly. Presence of the TS cell marker Cdx2 and absence of the extra-embryonic endoderm and ICM markers Gata4 and Oct4 were confirmed by immunocytochemistry.

Injection of stem cells into blastocysts

Blastocyst injection of ES and TS cells was performed as previously described (Cavaleri et al., 2008).

Statistical analysis

Statistical analysis was performed in JMP 7.0 (SAS Institute).

Table 1
Morula rates after exposure to different media. Values show % blastocyst rates (in brackets *n* starting 1-cell embryos).

	CZB−/+ glucose*	M16	KSOM (aa)	G1/G2	α-MEM
NT	[a]19.4 (36)	[a]30.6 (36)	[a]36.1 (36)	[bc]66.7 (36)	[bcd]76.6 (64)
ICSI	[bcd]76.7 (30)	[d]90.0 (30)	[d]90.0 (30)	[b]60.0 (60)	[cd]88.0 (25)

[abcd]Rates not significantly different share same letter (Fisher's exact test, $p<0.05$).
* CZB without glucose for the first 48 h followed by CZB with glucose.

Results

Differential reprogramming of Cdx2, Oct4 and Nanog in cloned mouse embryos

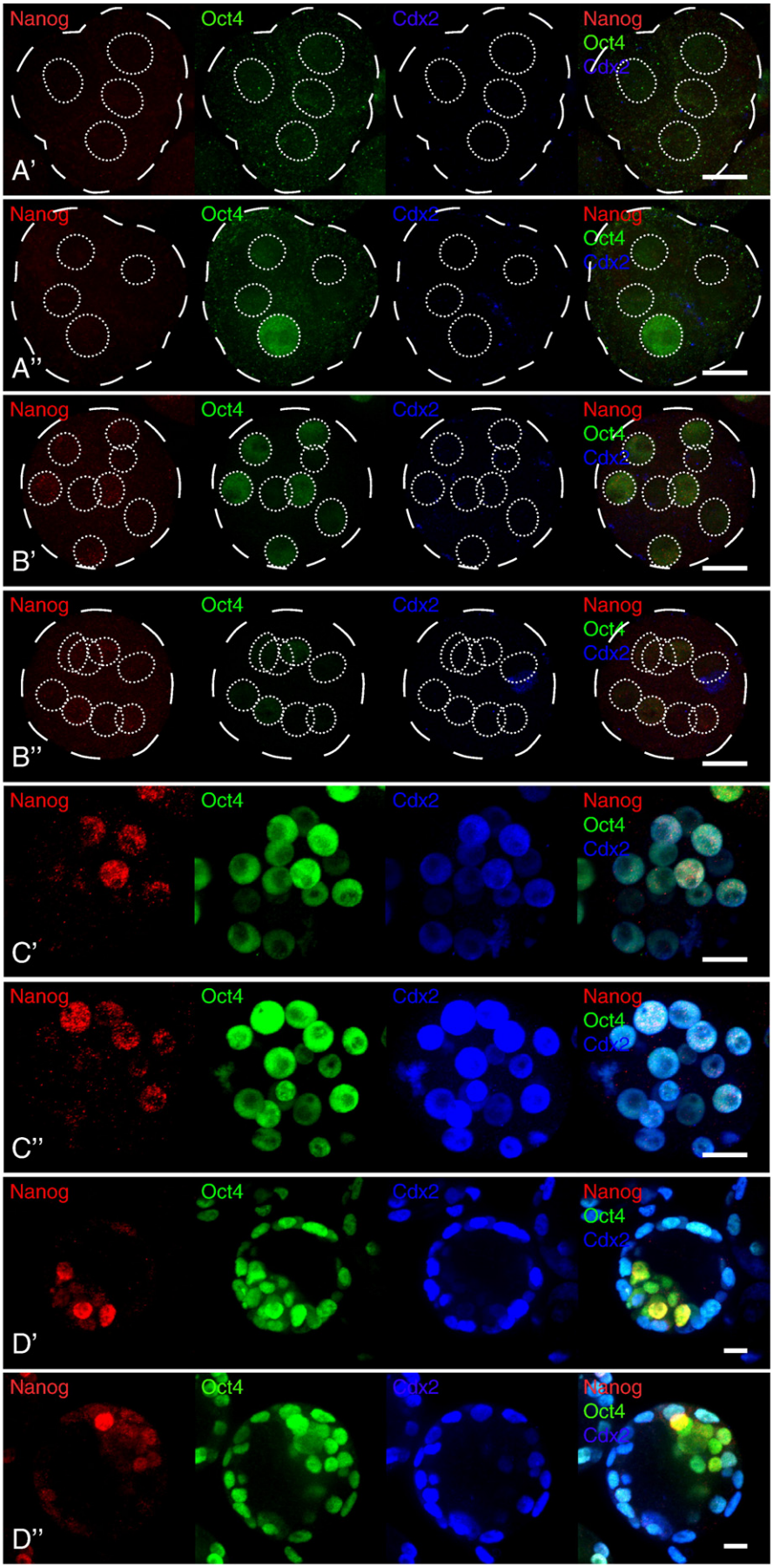
To investigate how the embryonic cell lineages arise after oocyte-mediated nuclear reprogramming, we compared expression of Cdx2, Oct4 and Nanog in cloned (NT) and fertilized (ICSI) mouse embryos by in situ immunofluorescence confocal analysis. We cultured these embryos under multiple conditions since the choice of the optimal medium for cloned embryos is unsettled (Table 1). NT and ICSI embryos that were activated at the same time cleaved synchronously to the 4-cell stage (~42 h). Nanog and Cdx2 proteins were not detected; staining against Oct4 produced a faint signal that was hardly distinguishable from the background (Fig. 1A′, A″; Fig. S3). We ascribe the faint Oct4 signal to maternal inheritance of Oct4 protein or mRNA. At the 8-cell stage (~55 h), Oct4 was detected unambiguously in both ICSI and NT embryos (Fig. 1B′, B″; Fig. S3). After the 8-cell stage, all three proteins – Cdx2, Oct4 and Nanog – were detected in all nuclei (Fig. 1C′, C″ show embryos with representative levels of Cdx2, Oct4 and Nanog; Fig. S3 shows additional features of gene expression besides level, such as negative Oct4-Cdx2 correlation). At 72 h, the two types of embryos exhibited asynchronism, i.e. cloned embryos were lagging behind. In order to match and compare asynchronously dividing NT and ICSI embryos, we sampled them together at 72 h as well as 6 h apart (72 h ICSI, 78 h NT; at these time points 16±4 cells were counted in both groups). We imaged Cdx2, Oct4 and Nanog proteins together in every nucleus of ICSI and NT morulae by confocal immunofluorescence (Fig. 1C′, C″; Fig. S3) without noticing bleed-through (Fig. S2). The mean fluorescence intensities of the individual nuclei of multiple embryos were measured in ImageJ (Fig. S1), tabulated and plotted in a histogram to show the frequency distribution (Fig. S4 shows the case of a single representative embryo). The median and the coefficient of dispersion (see below) were calculated to objectively judge differences of the frequency distributions. While measuring the nuclear immunofluorescence signals, we took the opportunity to record the size of nuclei at the largest cross section in order to appreciate the occurrence and prevalence of arrested blastomeres, which would be reflected in a larger nuclear size compared to blastomeres that cleaved further. The mean nuclear size of NT and ICSI morulae composed of 16±4 cells was not different (108.8±26.9 and 102.8±22.3 square pixels, respectively; Student's *t* test, $p=0.3309$). This argues that the developmental delay of NT embryos is a feature of the whole embryo, not of a subset of its blastomeres.

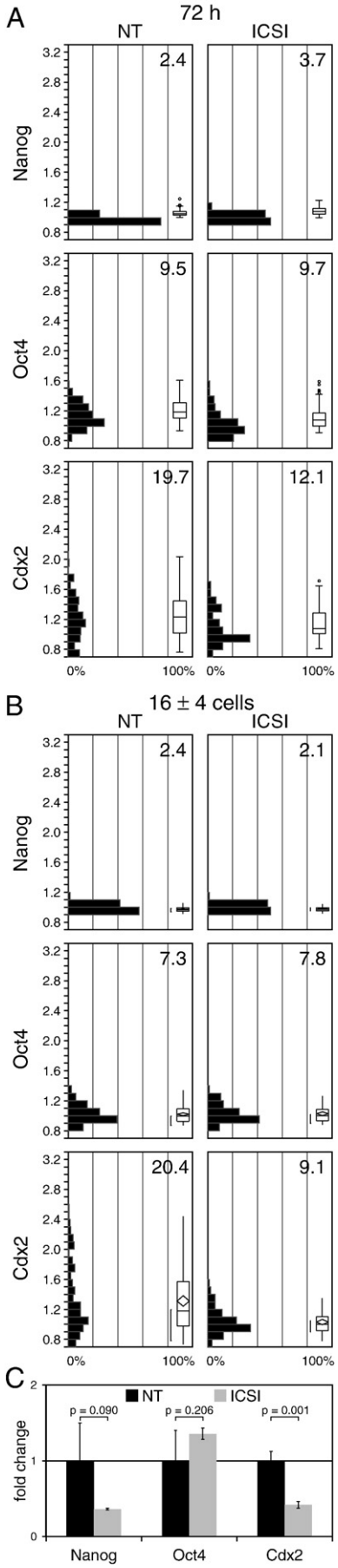
The immunofluorescence intensities of Cdx2, Oct4 and Nanog were not distributed normally in the NT and ICSI morulae (*Shapiro-Wilk* test, $p<0.0001$ for all three transcription factors); therefore, non-parametric statistics were used. For further analysis, we examined morulae that were cultured in α-MEM, which was the most supportive medium of those tested (Table 1). We summarized the different distributions by their coefficient of dispersion (CoD = median absolute deviation/median [%]), which, like the coefficient of variation, is a normalized measure of the dispersion of the distribution. While the distributions of Nanog and Oct4 were similar between the two types of embryos, the distribution of Cdx2 differed markedly (stage-matched embryos; Mann–Whitney *U* test, $p=0.218$, $p=0.606$ and $p<0.0001$, respectively). Cdx2 fluorescence had a narrow peak in ICSI embryos; in NT embryos, fluorescence was dispersed more than twice as wide as in ICSI and fluorescence median and maximum were higher than in ICSI. When sampled at the same time (72 h), the Cdx2 CoD of NT and ICSI morulae was 19.7 and 12.1, respectively (Fig. 2A); for stage-matched samples, the Cdx2 CoD of NT and ICSI morulae was 20.4 and 9.1, respectively (Fig. 2B). We confirmed that these observations were specific for the type of embryo and independent of the culture medium used (Fig. S5). Quantification of the corresponding transcripts by QRT-PCR mirrored the results obtained by immunofluorescence: the average *Cdx2* level was markedly higher in NT, and standard deviations of *Cdx2*, *Oct4* and *Nanog* mRNA values were greater in NT than in ICSI morulae (Fig. 2C). These results confirmed that the observed levels of Cdx2 were controlled through gene transcription and not protein stability. The marked dispersion of Cdx2 mRNA and protein values points to a problem in the differentiation into the extra-embryonic lineage of cloned embryos. In NT and ICSI embryos, the comparable protein levels of Nanog and Oct4 (Fig. 2A, B) suggest that the difference in these genes' mRNA values (Fig. 2C) is corrected at the post-transcriptional level.

Natural embryo environment is detrimental for reprogramming

It is well known that embryos are affected by culture conditions; suboptimal culture conditions could worsen already inefficient reprogramming after NT (Suzuki et al., 2009; Wrenzycki and Niemann, 2003). Therefore, we sought to provide cloned embryos with the best environment, supposedly the female reproductive tract, to test if reprogramming would be better supported from its onset and enable correct Cdx2 expression. NT, ICSI and parthenogenetic (P) embryos were generated in parallel and transferred into pseudo-pregnant (0.5 dpc) oviducts 7 h after activation (pronuclear stage). Upon flushing from the uterus 3 days later (3.5 dpc), the vast majority of cloned embryos were found arrested at the 1-cell stage, yielding very few blastocysts compared to cloned embryos cultured in parallel (5.3%, $n=264$ versus 40.0%, $n=265$, Fisher's exact test, $p=3.85\times10^{-23}$). Control ICSI embryos developed to blastocyst stage at similar rates in vivo and in vitro (39.4%, $n=188$ versus 40.6%, $n=101$, Fisher's exact test, $p=0.9$), as did P embryos (data not shown). In order to exclude a metabolic cause for the cloned embryos' 1-cell block in vivo, we probed energy substrate requirements. We challenged embryos by replacing D-glucose with metabolically inert L-glucose. Cloned embryos cleaved to 4-cells at the same rate in the presence or absence of D-glucose (63.4%, $n=93$ versus 64.4%, $n=118$, Fisher's exact test, $p=0.886$), and the blastocyst rates of NT and ICSI embryos were reduced to the same extent by glucose deprivation (7.6%, $n=118$ versus 10.1%, $n=69$, Fisher's exact test, $p=0.594$). At the blastocyst stage, cloned embryos had identical ATP content when cultured in D- or L-glucose-containing media, although such content was significantly lower (−45%) compared to ICSI embryos (0.09 pmol/blastocyst). Altogether, these results indicate that, for oocyte-mediated nuclear reprogramming, the benefits of an in vitro environment outweigh the possible harm that may result from suboptimal culture conditions.

Fig. 1. (A′, A″) Immunofluorescence could not detect Nanog (red) nor Cdx2 (blue) in 4-cell stage ICSI (A′) or cloned (A″) embryos, while a faint Oct4 signal (green) may be ascribed to maternal inheritance of Oct4 protein or mRNA. Brightness was increased to make faint signals visible. (B′, B″) At the 8-cell stage, Oct4 signal became stronger in ICSI (B′) and cloned (B″) embryos. Nanog began being expressed weakly. Cdx2 was still undetectable. (C′, C″) Co-immunofluorescence of Nanog, Oct4, Cdx2 in ICSI-fertilized (C′) and NT (C″) morulae. Proteins do not yet follow a distinct spatial pattern. (D′, D″) Immunofluorescence detected Nanog in some cells of the ICM, Oct4 in both ICM and TE (the signal was stronger in the ICM), and Cdx2 in the TE of ICSI (D′) and NT (D″) blastocysts at 96 h of development. Scale bars, 20 μm.


Nanog
Oct4
Cdx2
Nanog
Oct4
Cdx2
A'
A''
B'
B''
C'
C''
D'
D''



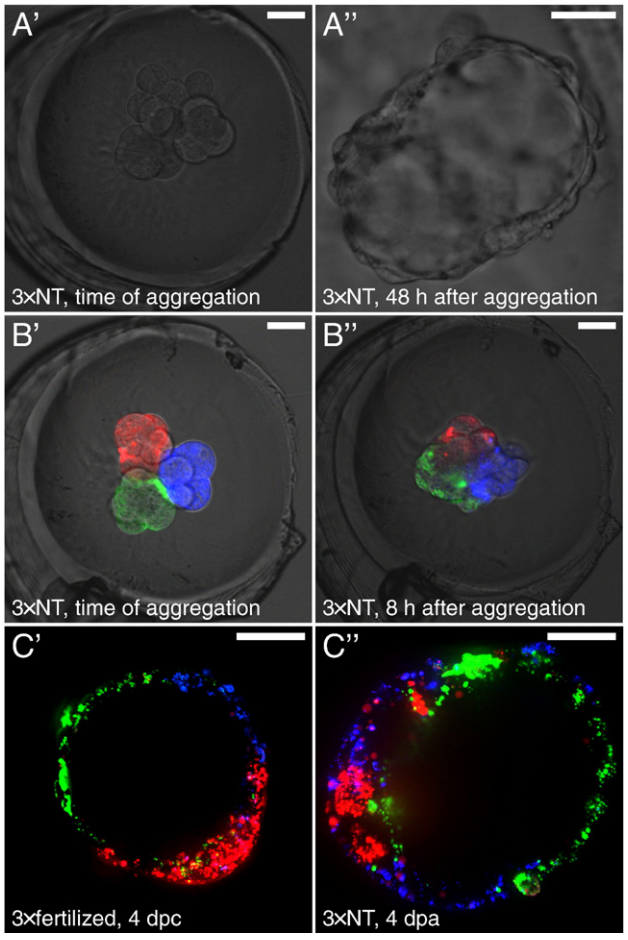


Fig. 3. (A′, A″) Aggregate of three 4-cell cloned embryos at the time of aggregation (A′) and 48 h after aggregation (A″) in a dimple made in a 35-mm plastic dish using a darning needle. (B′) Embryos were labeled with fluorescent latex beads prior to aggregation; (B″) same aggregate, 8 h later. Scale bar, 50 µm. (C′, C″) 3× blastocysts after labeling with fluorescent latex beads and aggregation of individual embryo components at the 4-cell stage; (C′) 3× aggregate of fertilized embryos; (C″) 3×NT aggregate; dpc: days post-coitum; dpa: days post-activation. Scale bar, 50 µm.

Aggregation of cloned embryos normalizes Cdx2 expression

When cloned morulae with erratic Cdx2 expression are allowed to develop further, only a subset form blastocysts that suffer from a reduced normal number of cells and therefore have limited prospects for implantation and post-implantation development. Because colony size has a major impact on differentiation of pluripotent cells (Hamazaki et al., 2004; Wang et al., 2006), we postulated that an increase in cell numbers would return erratic Cdx2 levels to normal without the cells having to go through the bottleneck of the morula-blastocyst transition, which may select for correct gene expression

Fig. 2. Distribution of immunofluorescence intensity levels in NT and ICSI morula-stage embryos cultured in α-MEM. Embryos were stained for Nanog, Oct4 and Cdx2 protein, and immunofluorescence intensity was measured. Gray values were normalized, whereby the median of the ICSI distribution was considered to be 1. The tabulated frequencies of fluorescence intensities of the individual nuclei were then plotted in a histogram to show the density distribution, and the median and the coefficient of dispersion (median absolute deviation/median, [%]) were calculated to objectively judge differences of the density distributions. Percent coefficients of dispersion are shown in the top right corner of each inset. Embryos were sampled at the same time (72 h) (A) or stage-matched at different times, when most embryos had 16±4 cells (ICSI, 72 h; NT, 78 h). A total of 19 NT and 20 ICSI (A), respectively, 32 NT and 33 ICSI (B) embryos were analyzed. (C) Relative mRNA abundances in morulae measured by QRT-PCR, relative to NT morulae. Error bars, SD and *p* values from *t* test indicate if difference between NT and ICSI value are significant.

Table 2
Cell counts of aggregated embryos and non-aggregated controls at 60 h, 72 h, 84 h and 96 h after activation. Values show embryos' median cell count ± median absolute deviation. ZP+: embryos with zona pellucida; if not noted, zona pellucida was removed. n.d., not determined. At least 10 embryos or aggregates were examined for each time point. Cell cycle length (x) was estimated based on the number of cell doublings from the time of aggregation (48 h; 4 or 12 cells for 1× or 3×, respectively) until 96 h using the formula $\frac{number\ of\ cells\ at\ 96\,h}{number\ of\ cells\ at\ 48\,h} = 2^{\frac{96-48}{x}}$.

	1×NT ZP+	1×NT	3×NT	1×ICSI ZP+	1×ICSI	3×ICSI
60 h	n.d.	7 ± 0.5	17 ± 1.0	n.d.	7.5 ± 0.5	18 ± 1.0
72 h	13 ± 3.0	11 ± 4.0	19 ± 2.5	16 ± 0.0	15 ± 1.0	44 ± 3.5
84 h	22 ± 3.5	15 ± 1.5	36 ± 11.5	26 ± 2.0	26 ± 3.0	71 ± 9.5
96 h	39 ± 10.0	46 ± 16.0	67.5 ± 35.0	55 ± 5.5	57 ± 12.5	142.5 ± 29.5
Estimated cell cycle, h	14.6	13.6	19.3	12.7	12.5	13.4

Table 4
Developmental rates of aggregated embryos and non-aggregated controls. More than 75% of embryos developed to 4-cell stage after NT, ICSI or in vivo fertilization. Values show % blastocyst rates (in brackets n starting 4-cell embryos, after aggregation where applicable). ZP+, ZP–: embryos with and without zona pellucida, respectively. 2× and 3× were zona-free.

	NT % (n)	ICSI % (n)	In vivo % (n)
1× ZP+	37.1 (140)*	60.0 (85)**	78.7 (141)
1× ZP−	20.5 (83)	25.0 (24)	83.3 (48)
2×	40.7 (59)*	37.5 (24)	54.2 (48)**
3×	52.3 (130)**	52.9 (51)*	88.6 (105)
No. replicate experiments	7	2	6

* Rate significantly different from 1× ZP− rate (Fisher's exact test, $p<0.05$).
** Difference from 1× ZP− highly significant (Fisher's exact test, $p<0.01$).

patterns. We increased cell numbers by aggregating cloned embryos at the 4-cell stage (Fig. 3A′, A″)—a procedure shown to increase fetal rates 8-fold (Boiani et al., 2003).

Preliminary tests established the need for homologous, i.e. clone–clone, embryo aggregation; in a clone–fertilized aggregate, the fertilized partner overwhelms the clone (Table S1). Jouneau et al. (2006) showed that even tetraploid (4N) fertilized embryos overwhelm an aggregated cloned embryo in 31.0% of the cases, and aggregation of cloned with 4N fertilized embryos did not increase the recovery of embryos with an NT contribution at embryonic day 7. We established that triple (3×) NT aggregates formed blastocysts at a significantly higher rate than single (1×) embryos (Fisher's exact test, $p<0.01$); differences were not highly significant for double (2×) NT embryos (Fisher's exact test, $p<0.05$). However, even 2×NT embryos yielded significantly higher post-implantation development (Table 3), confirming published results (Boiani et al., 2003). Since the positive effect of aggregation was strongest when aggregating three cloned embryos, we used only 3×NT embryos for our subsequent experiments. Of 130 3×NT embryos aggregated at the 4-cell stage, 52% went on to form blastocysts (Table 4), which were dramatically larger in size. As a control, we produced homologous aggregates of ICSI and in vivo fertilized embryos. Of 51 3×ICSI embryos, 53% were successful; nearly all (89%) of the 105 aggregates composed of in vivo fertilized embryos gave rise to blastocysts (Table 4).

Aggregated embryos were cultured to the morula stage and subjected to triple immunofluorescence (Cdx2, Oct4, Nanog) to measure effects of the enlarged cell community on pluripotency and TE differentiation. In the aggregated NT embryos, median immunofluorescence intensity decreased from 1× to 2×NT as well as from 2× to 3×NT (Nanog: −0.1% and −4.8%*; Oct4: −4.3%* and −17.6%*; Cdx2: −19.3%* and −18.8%*, from 1× to 2×NT and from 2× to 3×NT, respectively; Mann–Whitney U test, * $p<0.01$). Also the CoD, a measure of dispersion of the distribution, for Cdx2 decreased with increased cell count (1×NT, 24.7; 2×NT, 15.9; 3×NT, 10.1; 1×ICSI, 9.1). We sought to exclude the possibility that this pattern was due to dispersion of light, which is related to the length of the optical path (aggregate embryo diameter 3×>2×>1×, hence light intensity 3×<2×<1×). Therefore, we measured fluorescence levels of the nuclear DNA dye Draq5 in aggregated fertilized embryos. Unlike the transcription factor distributions (Fig. 4A), the Draq5 distributions were equally dispersed (CoD: 1×, 8.3; 2×, 8.4; 3× 8.2), and intensity shifts were minimal compared to changes in the transcription factor intensities (1× to 2×: −0.06%; 2× to 3×: −3.3%; Mann–Whitney U test, $p>0.05$, $p>0.01$, respectively). Thus, we conclude that the measured changes of Cdx2, Oct4 and Nanog after aggregation are biologically regulated and were not the result of technical restraints. In particular, after aggregation of cloned embryos, Cdx2 protein attained a level comparable to that of non-aggregated ICSI embryos (3×NT median was 99.0 ± 10.0% of ICSI median; Mann–Whitney U test, $p=0.226$; Fig. 4A).

Next we verified whether the beneficial effect of cloned embryo aggregation is indeed mediated by cell contact. It is well known that embryos cultured together develop better than those cultured in individual drops of culture medium (Paria and Dey, 1990). We therefore tested whether the distribution of Cdx2 in cloned embryos is corrected only when embryos are aggregated after removal of the zona pellucida but not when cultured close together while still zona enclosed. We observed that culturing three zona-enclosed cloned embryos together in the same microvolume did not change the level or distribution of Cdx2 expression (individual culture vs. co-culture, Cdx2 median level +0.7%, Mann–Whitney U test, $p=0.3279$; CoD 11.3% vs. 15.3%).

Tead4, a transcription factor upstream of Cdx2, has been shown to be required for trophectoderm (TE) induction in mouse embryos (Nishioka et al., 2008). Therefore, we checked whether Tead4 is also dysregulated in cloned embryos; however, we observed only a slightly different Tead4 distribution between aggregated and non-aggregated cloned embryos (Tead4 median level, +4.0%; $p=0.02331$; CoD 8.8% vs. 8.5% in 1×NT vs. 3×NT). From these data, we conclude that erratic Cdx2 expression in cloned mouse embryos may not be ascribed to Tead4 alone, suggesting that the observed defect of Cdx2 is one of stabilization and not activation.

Next we asked what happens to the gene expression of clones after the morula stage. We analyzed the three transcription factors Cdx2, Oct4 and Nanog in single (1×) and aggregated (3×) cloned and fertilized blastocysts at 96 h post-activation (Fig. 1D′, D″; Fig. S3). For this analysis, we only included blastocyst-stage embryos with at least 32 cells (median cell number: 1×NT, 56; 1×ICSI, 53.5; 3×NT, 87; 3×ICSI, 145; Table 2). All types of blastocysts displayed the dispersed Cdx2 distribution (Fig. 4B). This confirms that 1×NT morulae indeed initiate pluripotency properly but fail to restrict it; this may result in premature over-expression of TE-specific genes.

Normalization of Cdx2 expression is a synergistic effect of the aggregated embryo components

There are two possibilities to explain our results: a mechanism like a community effect (Gurdon, 1988) corrects expression of *Cdx2* embryo-wide to a level typical for fertilized embryos, or those embryos

Table 3
Post-implantation developmental rates of aggregated cloned embryos and non-aggregated controls. Zona pellucida was removed from non-aggregated controls. Values show n embryos/fetuses at the respective stage (rate in brackets).

	Fetuses 10.5 dpc	Decidua	Transferred in vivo
1×NT	2 (2.1%)	94 (58.0%)	162
2×NT	13 (13.4%)*	97 (48.5%)	200

* The rate of fetal formation at 10.5 dpc of 2×NT was highly significantly different from non-aggregated controls ($p=0.00719$).

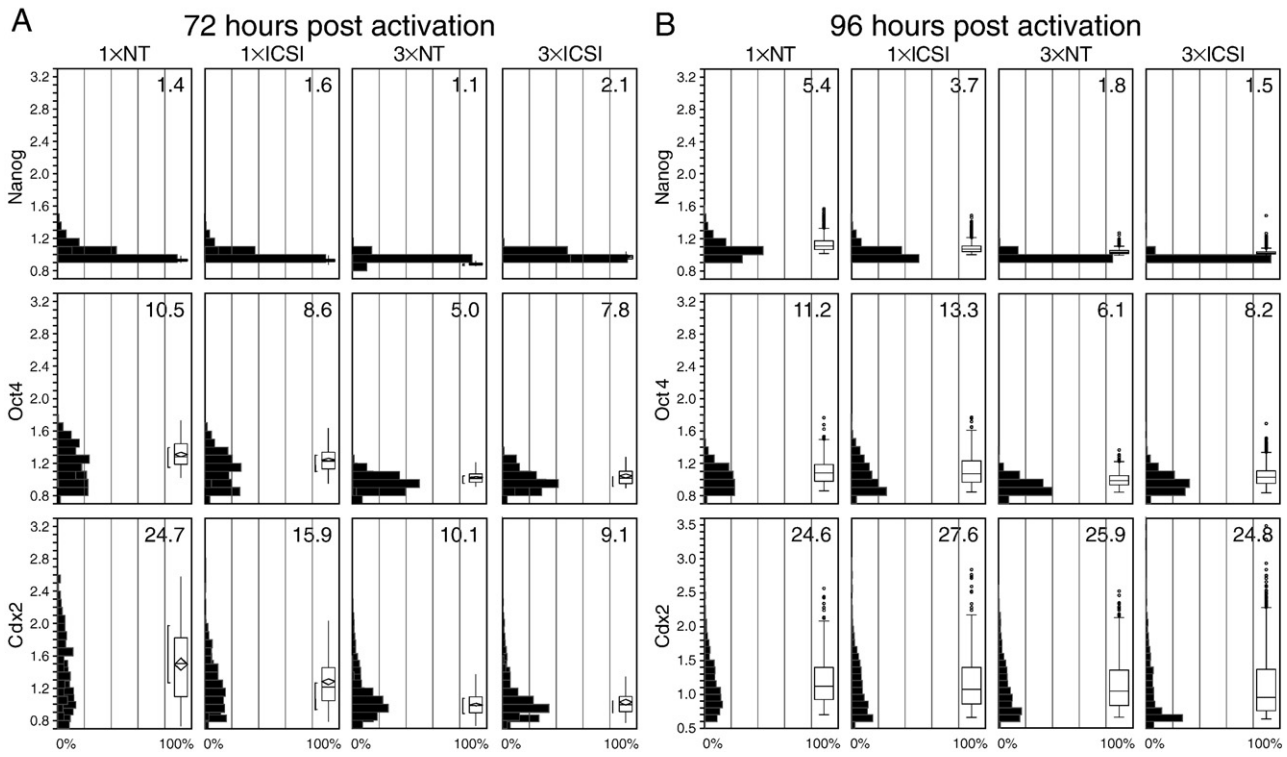


Fig. 4. Immunofluorescence intensity distribution of Nanog, Oct4, Cdx2 protein in aggregated embryos (A) 72 h and (B) 96 h post-activation. Gray values were normalized, whereby the median of the ICSI distribution was considered to be 1. The tabulated frequencies of fluorescence intensities of the individual nuclei were then plotted in a histogram to show the density distribution, and the median and the coefficient of dispersion (median absolute deviation/median, [%]) were calculated to objectively judge differences of the density distributions. Percent coefficients of dispersion are shown in the top right corner of each inset. A total of 28 NT embryos (A) and 24 NT and 37 ICSI embryos (B) were analyzed.

that are already poised to express normal levels of *Cdx2* overgrow the other founder embryos in the aggregate. To distinguish these possibilities, we determined the composition of aggregates by tracking cells of different embryo origin and compared it with the predicted embryo composition of 3×NT blastocysts calculated by binomial distribution (Zhou et al., 2008). On the assumption that aggregation does not alter developmental competence of the individual components differently from each other, the probability P that an aggregate of n embryos comprises k successful founders, which have an individual probability to develop p, can be calculated following a binomial distribution:

$$P(X = k) = \binom{n}{k} p^k (1-p)^{n-k}$$

Given $p = 0.20$ for 1×NT embryos after removal of the zona pellucida (Table 4), we would expect blastocysts to form in 3×NT aggregates in 48.8% of the cases. Indeed, the actual blastocyst rate of 52% is very close but does not match the rate predicted by the binomial distribution. We sought to track the contribution of founder embryos to aggregates at the single cell level by counting their nuclei. Live cloned embryo fluorescent imaging did not succeed; GFP and RFP fluorescent histone markers, injected as mRNA into pronuclear-stage embryos to differentially label the nuclei of the individual embryos before aggregation, proved to interfere with cleavage to different extents (not shown). Therefore, a time-lapse cell tracking study would have lead to a biased result. Instead, we labeled the individual embryos before aggregation using red, green and blue latex beads, which are taken up by microphagocytosis and retained in the cytoplasm of the blastomeres without affecting developmental ability (Fleming and George, 1987) (Fig. 3B′, B″). Although the total cell numbers of 2×NT and 3×NT embryo aggregates increased less than proportionally to the number of embryos forming the aggregate (Table 2), contribution from multiple embryos was always the case: 60% of the 3×NT blastocysts were made up of three embryos, and 40% were made up of two embryos (Table 5). The absence of a third color in the latter confirms that there was not a release of beads from one cell or embryo and re-uptake by another. Aggregates of ICSI and in vivo fertilized embryos yielded 96% and 100% tri-color blastocysts, respectively, showing that virtually all embryos had become integrated. The compositions we observed deviate substantially from the compositions predicted by the binomial distribution: 1.6%, 19.7% and 78.7% of NT aggregates should be composed of three, two and single embryos, respectively. Aggregation thus dramatically increases viability of all of the individual components. This indicates that, after aggregation, normalization of Cdx2 expression is mediated by a community effect and excludes the possibility that only founder embryos with normal gene expression survive.

To determine if a community effect (Gurdon, 1988) also applies to normal pre-implantation development, we performed the converse experiment in ICSI-fertilized embryos, which do not lack in cell number and whose Cdx2 distribution is normal. It is known that

Table 5
Composition of aggregate embryos observed and expected from binomial probability. Values show fraction of total blastocysts of the respective type (in brackets n total blastocysts analyzed). NA, not applicable.

	3×NT		3×ICSI	
	observed (n)	expected	observed (n)	expected
All 3 colors detected in blastocyst	60.0% (20)	1.6%	97.4% (38)	2.7%
2 or more colors detected in blastocyst	100.0% (20)	21.3%	100.0% (38)	27.0%
2 or 3 colors in ICM	40.0% (20)	NA	73.7 (38)	NA

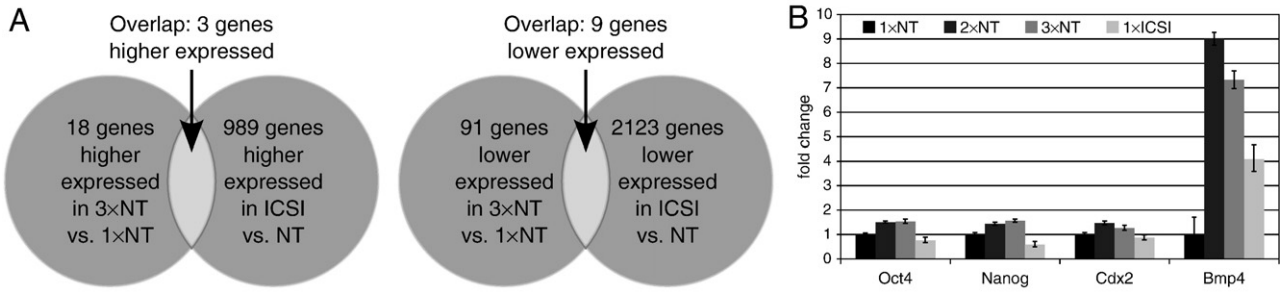


Fig. 5. Using the Illumina microarray platform, we probed 45,200 transcripts covering >19,100 genes in the NCBI RefSeq database. Gene expression difference was considered if variation was more than 2-fold with $p<0.01$. In 3×NT compared to 1×NT, 18 transcripts were consistently higher expressed (↑) and 91 were consistently lower expressed (↓). In order to distinguish enhanced reprogramming from generic changes of gene expression due to an increased number of cells, we compared 1×ICSI with 1×NT embryos and found in 1×ICSI 989 genes higher expressed and 2123 genes lower expressed. We assumed that for more successful reprogramming, the 989 genes up-regulated in ICSI would need to be up-regulated in NT and the 2123 genes suppressed in ICSI would need to be suppressed in NT. (A) Therefore, we compared the set of genes responsive to cloned embryo aggregation (18↑, 91↓) with the set of genes that distinguish 1×NT from 1×ICSI (989↑, 2123↓). The overlap was held to reveal those genes that promote reprogramming in the context of aggregation. This overlap consisted of 3 genes that were up-regulated and 9 genes that were down-regulated as a result of aggregation (Table 6). (B) QRT-PCR confirmation of select genes after microarray analysis, values figured as relative to 1×NT blastocysts. Error bars, SD.

bisected fertilized embryos can develop into blastocysts and to term when transplantated into uteri of foster mothers, but they display morphological aberrancies at the blastocyst stage, such as a lack of an inner cell mass (Tarkowski, 1959), and their developmental success is greatly reduced (Papaioannou et al., 1989; Papaioannou and Ebert, 1995; Tsunoda and McLaren, 1983). To mimic the cell deficit observed in cloned embryos at the blastocyst stage, we reduced the total cell number of embryos fertilized by ICSI by pulling one blastomere out of the zona pellucida at the 2-cell stage. Cell reduction of the ICSI embryos at the 2-cell stage resulted in morulae with half the expected number of cells (12.2 ± 2.2 versus 24.7 ± 4.3 cells ± SD) and a reduction in blastocyst rates by 60.1% (14/37 versus 29/30; Fisher's exact test, $p=3.1\times10^{-7}$). Cdx2 levels decreased in bisected ICSI morulae (bisected vs. whole ICSI −9.6%, Mann–Whitney *U* test, $p=0.0001$; CoD 12.5% vs. 11.2%) while the distributions of Nanog and Oct4 were not affected by cell reduction (data not shown). Expression of Cdx2, Nanog and Oct4 in ICSI embryos remains the same after cell number increase (data not shown). This argues that a lower cell number is less detrimental for ICSI than for cloned embryos, and that mechanisms of Cdx2 regulation are not entirely equal between the two.

Signaling pathways mediate the effect of cloned embryo aggregation

Growth factors and signaling pathways are established mediators of the community effect. In *Xenopus* myogenesis, the community effect can be mimicked in vitro by provision of eFGF in the culture medium (Standley et al., 2001). If the mechanism underlying the effect of aggregation is indeed a community effect, then growth factors and signaling pathways should test positive for more use. We examined the expression of genes encoding components of signaling pathways in 3×NT embryos. To this end, we used the Illumina microarray platform to compare the transcriptomes of 3×NT and 1×NT blastocysts, both without zona pellucida. The Illumina microarray includes 45,200 transcripts covering >19,100 genes in the NCBI RefSeq database. DAVID analysis of KEGG pathway components indicated that Hedgehog ($n=8$ genes), Igf ($n=16$ genes) and Wnt ($n=13$ genes) pathways were significantly changed ($p<0.05$) by aggregation, but the Hippo pathway was not (Table S2). To restrict the field and to test if aggregation activates a signaling pathway that mediates the observed effect, we cross-compared the transcriptomes of 1×NT and 3×NT with those of ICSI-fertilized embryos (Fig. 5A). The overlap of the genes responsive to clone aggregation (3×NT vs. 1×NT) and those distinguishing ICSI from NT blastocysts consisted of 3 genes that were up-regulated and 9 genes that were down-regulated (Fig. 5A; Table 6), and we consider that those 12 genes promote cloned embryo development as an effect of aggregation (see Illumina bead chip hybridizations section; Fig. 5A). Notably, the DAVID analysis pointed to a role of the Hedgehog pathway, and subsequent cross-comparison analysis exposed the Hedgehog target gene *Bmp4*, which encodes a growth factor. To confirm the ability of our microarray analysis to resolve small differences in expression level, we performed QRT-PCR for *Bmp4* and *Cdx2*, *Oct4* and *Nanog*. The data confirmed only minor changes in *Cdx2*, *Oct4* and *Nanog* as opposed to a marked and significant change in *Bmp4* at the blastocyst stage (Fig. 5B).

Bmp4 is a member of the transforming growth factor β (Tgf-β) superfamily of secreted polypeptides and is up-regulated upon TE differentiation (Erlebacher et al., 2004). Another member of the Tgf-β superfamily, Tgf-β1, has a key role in TE maintenance (Tolkunova et al., 2006). Since our data show that cloned embryos fail to regulate TE properly, we explored the potential of Tgf-β signaling in the regulation of *Cdx2* expression in cloned embryos. We supplemented the culture medium with Tgf-β1, Bmp4 or Egf throughout in vitro culture. Analysis of blastocyst formation in ICSI and cloned embryos did not show a developmental response to recombinant growth factors; blastocysts did not form at a different rate or time (Table S3). To bypass possible availability issues, we transplanted cloned blastocysts with trophoblast stem (TS) cells or with androgenetic embryonic stem (ES) cells, both of which contribute to Bmp4-producing extra-embryonic tissues. However, we failed to observe any improvement after implantation, although the donor cells integrated correctly (Fig. S6; Table S4). These results indicate that aggregation embodies the full set of conditions to

Table 6
Illumina microarray. Genes up-regulated or down-regulated more than 50% following embryo aggregation (*t* test, $p<0.01$). Values sorted by increasing 3×/1× ratio.

Symbol	Accession	Definition	Ratio ICSI/NT	Ratio 3×/1×
Slc16a9	NM_025807.1	Solute carrier family 16, member 9	0.37	0.34
4932413L07Rik	AK077025	Hypothetical protein	0.35	0.35
4931419K03Rik	NM_172499.1	RIKEN cDNA 4931419K03	0.20	0.36
Sqle	NM_009270.2	Squalene epoxidase	0.42	0.41
Zmpste24	NM_172700.1	Zinc metalloproteinase, STE24 homolog	0.45	0.41
D6Ertd253e	NM_178608.2	Chr 6, ERATO Doi 253, expressed	0.34	0.43
Foxa3	NM_008260.1	Forkhead box A3	0.05	0.45
Tex2	NM_198292.2	Testis expressed gene 2	0.35	0.45
9330186A19Rik	NM_178781.2	RIKEN cDNA 9330186A19	0.30	0.45
Bmp4	NM_007554.1	Bone morphogenetic protein 4	8.60	2.22
Foxq1	NM_008239.3	Forkhead box Q1	15.95	2.45
Rpl38	NM_023372.1	Ribosomal protein L38	2.28	3.11

activate signaling pathways and correct the Cdx2 phenotype of cloned embryos. Since Tgf-β signals induce translocation of Smad4 from the cytoplasm to the nucleus where it then acts as a transcription factor, we examined this response in situ. Smad4 responded to aggregation by an increase in both total and nuclear amount. This response was specific for Smad4; β-catenin, part of the unrelated Wnt signaling pathway, did not change upon aggregation (Fig. 6).

Discussion

Post-implantation development of cloned embryos is limited, possibly due to flawed induction of pluripotency by oocyte-mediated nuclear reprogramming, flawed restriction of pluripotency (i.e. differentiation), or both. Cloned mouse embryos express mRNA and protein of the pluripotency factor Oct4 at levels close to normal, even though the upstream DNA control regions of the *Oct4* gene are hypermethylated (Cavaleri et al., 2008). Most cloned mouse embryos fail in vivo at gastrulation (Jouneau et al., 2006); however, cloned blastocysts yield normal ES cells in vitro. Taken together, these observations suggest that, in spite of the prevalent interest of current reprogramming studies on the induction of pluripotency, differentiation is also affected in cloned embryos. We examined the prerequisites of pluripotency and differentiation – the expression of master regulators of inner cell mass (Nanog, Oct4) and trophectoderm (Tead4, Cdx2) – at the morula stage. Cloned embryos express Cdx2 erratically compared to time- and stage-matched ICSI embryos (72 h and 16±4 cells, respectively); Cdx2 values are far more widespread and the median level is markedly higher in cloned morulae than in fertilized counterparts. The key finding of our study is that this pronounced defect in *Cdx2* gene expression can be corrected by increasing the total cell number of cloned mouse embryos without the need for genetic manipulation. By increasing the total cell number of cloned morulae by homologous embryo aggregation, expression of Cdx2 is corrected; in ICSI embryos, expression of Cdx2, Nanog and Oct4 remains the same after cell number increase. This finding suggests that cell number is a parameter in the regulation of the extra-embryonic lineage gene expression in cloned embryos.

We analyzed Cdx2 and the other lineage regulators Oct4 and Nanog at the morula stage. In order to appreciate their sub-cellular localization, we analyzed them at the single cell level. Because transcripts do not guarantee the presence of the corresponding protein, we used immunofluorescence to detect Cdx2, Oct4 and Nanog proteins. Following synchronous activation, NT and ICSI embryos showed Oct4 but not Nanog or Cdx2 nuclear signal at the 8-cell stage, which is consistent with the report of Suwinska et al. (2008) but at odds with the report of Ralston and Rossant (2008). It should be noted that these studies are based on different embryo production systems (in vivo, in vitro), whereby the age of the embryo is not always known with certainty (in vivo fertilization) and the pace of cleavage is not the same (different culture media). It should also be considered that the expression timing of lineage markers may differ at the mRNA and protein level (Guo et al., 2010). Thus, using micromanipulation to produce mouse embryos side by side is a very important aspect of our study, given our aim to investigate cell lineage formation after cloning as compared to fertilization. In spite of the synchronous activation and synchronous progression to the 4-cell stage, NT and ICSI embryos develop asynchronously after the 8-cell stage. Normal development shows that certain processes in mouse embryogenesis, e.g. cavitation occur according to the amount of time that has passed since oocyte activation. Since normal development is very unlike cloned embryo development, this time dependency of developmental processes

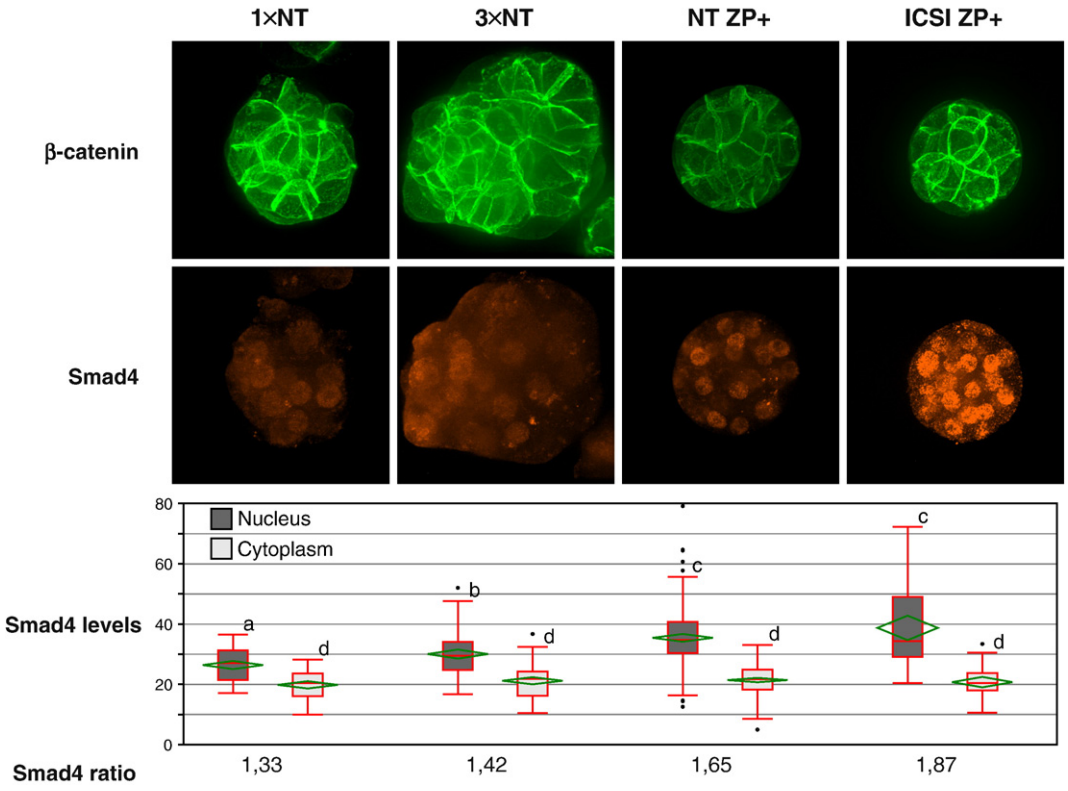


Fig. 6. Upon aggregation, Smad4 relocates to the nucleus while β-catenin is unresponsive. Top row: immunostaining shows β-catenin localization at the cell membrane (maximum projection of confocal images). Second row: representative images of immunostaining for Smad4 (maximum projection of confocal images). Third row: the nuclear portion of Smad4 immunosignal increased after aggregation of NT embryos, while the signal was highest before removing the zona pellucida (ZP+). Values of Smad4 levels refer to average pixel intensity above background. Different letters (a, b, c, d) indicate significant difference of average immunofluorescent signal (Mann–Whitney *U* test, $p<0.05$). Bottom row shows ratio of Smad4 signal in the nucleus over the cytoplasm.

may or may not apply equally to cloning. We took this aspect into account by collecting and analyzing cloned and fertilized embryos at the same time (72 h; different cell number) and also 6 h apart from each other (16 ± 4 cells in both NT embryos at 78 h and ICSI embryos at 72 h). The morula stage, defined here as 72–78 h into development or 16 ± 4 cells, is known to us as the earliest stage at which to predict fetal success of NT embryos using the Oct4-GFP transgene (Cavaleri et al., 2008) and it is reached by 70% of NT embryos. Induction of pluripotency, as measured by Oct4 and Nanog protein distributions, is indistinguishable between NT and ICSI embryos, regardless of the culture medium used. However, restriction of pluripotency, as measured by Cdx2 protein distributions and mRNA levels, differs in cloned and fertilized embryos; in NT morulae, Cdx2 values were more widespread, and the median level was markedly higher, both at the time-set (72 h) and cell number-set (16 ± 4 cells) embryo sampling point. In contrast to Cdx2, the upstream factor Tead4 was not affected.

We established that the erratic pattern of Cdx2 expression in cloned embryos was not an artifact of suboptimal in vitro culture conditions. Providing NT embryos with the natural environment of the female genital tract not only did not allow for better Cdx2 gene expression, but it also prevented cloned embryos from undergoing cleavage. This phenotype is even more notable since cloned mouse embryos retain the preference of the nucleus donor cells for high glucose concentration (Han et al., 2008) – the Fallopian tube fluid contains glucose at a level comparable to α-MEM (5.19 mM versus 5.56 mM, respectively; Gardner and Leese, 1990) – and yet cloned embryos fail in the Fallopian tube. Our functional assay of cloned embryo metabolism (glucose utilization and ATP levels) indeed demonstrated only a minor role of metabolism. The detrimental effect of the in vivo environment on reprogramming should not be surprising. Indeed, in vitro culture is a driving force in certain reprogramming platforms (Page et al., 2009). Moreover, natural induction of pluripotency in vivo must be restrained as it could have harmful consequences, e.g. neoplasia. We propose that there may be reprogramming-inhibitory signaling molecules in the oviduct; this could for example involve phosphoinositide-3 kinase (PI3K) signaling; forced activation of its downstream effector Akt has been shown to inhibit reprogramming in cloned embryos (Nakamura et al., 2008). In fact, it has been proposed recently that reprogramming in epiblast stem cells is dependent on suppression of extrinsic growth factor stimuli (Guo et al., 2009). Our data emphasize that reprogramming by nuclear transplantation is not a natural event, thus calling into question the validity of likening cloned to fertilized embryos.

Nishioka et al. (2009) recently reported that Hippo signaling is the principal mechanism regulating *Cdx2* expression and thereby trophectoderm formation in fertilized mouse embryos. The authors suggest that Hippo signaling down-regulates *Cdx2* in inside cells of the morula, but not in outside cells. Therefore, embryos with a smaller number of cells are expected to present higher levels of Cdx2 because the smaller number of total cells disproportionally affects the number of inner cells. While cloned embryos have smaller cell numbers than fertilized counterparts overall (Table 2; Boiani et al., 2003), this factor was controlled in our experimental design (see below). Cdx2 levels in cloned mouse embryos have previously been reported to be normal (Kishigami et al., 2006). However, Kishigami et al. (2006) only looked at blastocyst-stage embryos and without quantifying Cdx2 levels. Many cloned embryos do not pass the morula-blastocyst transition. Thus, an analysis of only those embryos that form blastocysts implies an unintended selection for correct gene expression patterns. It is tempting, on the basis of Nishioka et al. (2009), to envision a link between the higher levels of Cdx2 and a higher outer-to-inner cell ratio, which increases as the embryo volume decreases in embryos with smaller cell numbers, such as cloned embryos (Boiani et al., 2003). Since the cloned and fertilized embryos of our study were compared not only on the basis of equal time in development (72 h) but also on the basis of similar cell counts (16 ± 4 cells), it is noteworthy that cloned embryos still displayed increased median Cdx2 levels and dramatically higher maximum Cdx2 levels. It follows that a distorted outer-to-inner cell ratio does not apply here and may not be the reason for the peculiar Cdx2 expression pattern of cloned embryos. We conclude that there must be another mechanism regulating *Cdx2* expression in cloned embryos, or the thresholds in cloned and fertilized embryos for down-regulation of *Cdx2* by the Hippo pathway must be different. Indeed, in fertilized embryos, we observed a slight disturbance of Cdx2 protein levels after 50% reduction of cell number, which was accomplished by bisection at the 2-cell stage. However, the magnitude of the change was much smaller than the variance of the Cdx2 distribution observed in cloned embryos prior to aggregation. This argues that the aberrant Cdx2 distribution in cloned morula-stage embryos is not solely caused by small cell number but is a phenotype of cloned embryos. After progression to the blastocyst stage (≥32 cells), fertilized embryos also acquired the dispersed Cdx2 distribution, which at the morula stage was only observed in cloned embryos. However, the differential ability for implantation and post-implantation development shows that the trophectoderm (TE) of NT embryos, while outwardly similar to that of fertilized embryos, is impaired. TE lineage markers such as Eomes have been proposed to respond positively to the elevation of *Cdx2* expression in outside cells of the mouse morula (Ralston and Rossant, 2008). It follows that if the blastomeres of cloned morulae up-regulate *Cdx2* earlier than they should, the TE undergoes premature maturation and is already 'aged' by the time they come into contact with the endometrium. The view that cloned embryos suffer from premature differentiation is gaining momentum (Zhang et al., 2009).

The reason for the erratic pattern of *Cdx2* expression in cloned morulae had not previously been addressed. We know from Gurdon's seminal work that only large enough groups or colonies of cells may differentiate properly (Gurdon, 1988). This community effect was originally described in *Xenopus* myogenesis, but it also applies to differentiation of mouse pluripotent stem cells (Hamazaki et al., 2004; Wang et al., 2006). Whether cloned or fertilized, pre-implantation embryos can be considered as colonies of cells, but the former have lower cell counts than the latter (39 ± 10.0 versus 55 ± 5.5 cells at the blastocyst stage; Table 2). This prompts the question as to whether a lower cell count is simply an outcome or whether it exerts regulatory effects on reprogramming. To explore if colony size also matters in cloned embryo differentiation, we increased the cell number of NT embryos. It should be noted that such an increase may be achieved in different ways leading to different outcomes (Jouneau et al., 2006): e.g. blastomeres can be added to cleavage stages by embryo aggregation, or ES/TS cells can be added to the blastocyst stage by injection inside the cavity or underneath the zona pellucida. We observed that in a fertilized/cloned (heterologous) embryo aggregate, the fertilized overwhelms the cloned partner (Table S1). We also considered using tetraploid (4N) fertilized embryos as partners for cloned embryo aggregation; however, Jouneau et al. (2006) showed that even tetraploid fertilized embryos overwhelm an aggregated cloned embryo in 31.0% of the cases, and aggregation of cloned with 4N fertilized embryos did not increase the recovery of embryos with an NT contribution at embryonic day 7. Therefore, we performed homologous aggregation of cloned embryos with each other at the 4-cell stage, thereby increasing the cell number of the cloned embryos without introducing genetic or phenotypic diversity.

After aggregation of three NT embryos with each other, the pattern of Cdx2 expression changes to resemble that of fertilized embryos while already correct Oct4 and Nanog expression patterns do not change. Although the total number of cells increases less than proportionally to the number of embryos, all three of the founder embryos contributed to 3×NT blastocysts in 60% of the aggregates; the remaining 40% of blastocysts were comprised of two founders, as determined by latex bead tracking. These values exceed by far the

binomial probability that all three founders contribute to the aggregate, calculated to be only 1.6%. This proves that aggregation dramatically increases stability of the founders and excludes the possibility that, after aggregation, only founder embryos with (already) normal gene expression survive. The observation that the total number of cells increases less than proportionally to the number of embryos forming the aggregate warrants future investigation. Possible explanations include asynchrony of the individual embryos and a homeostatic mechanism whereby the individual embryos slow down their cell cycle in order to compensate for the increased biomass of the aggregate. In addition, the core cells of the aggregate may encounter limited diffusion of nutrients, ions and oxygen.

In order to validate the functional relevance of disturbed *Cdx2* gene expression, we demonstrated a 6-fold increase of development to mid-gestation after aggregation of two cloned embryos (2×NT), confirming our previously published observation that homologous cloned embryo aggregation increased fetal rates 8-fold (Boiani et al., 2003). However, the mechanism underlying this community effect has not been explored, until now. Inspired by the observation that eFGF can mimic the effect of an enlarged cell community (Standley et al., 2001) and that *Cdx2* expression is a target of the Hippo signaling pathway (Nishioka et al., 2009), we sought to unravel which molecules mediated aggregation's ability to normalize *Cdx2* expression. It has been suggested that mouse embryos, which indeed respond to growth factors (Paria and Dey, 1990), can even change the fate of their cells in response to the activation of a single pathway (Chazaud et al., 2006). We analyzed the transcriptome of aggregated cloned embryos (Illumina platform) to discern which changes of gene expression are associated with normalization of Cdx2. Direct pathway activity may not necessarily be evident from the transcriptome analysis because signals are also transduced by phosphorylation and other post-translational modifications of existing pathway molecules without changes in the level of transcriptional activity of the encoding genes. However, the downstream effects of any signaling pathway are changes in gene expression, which are detectable by mRNA analysis. By performing a DAVID analysis of KEGG pathway components, we found that the aggregation of cloned embryos alters the expression of several components of signaling pathways. While no changes of Hippo signaling targets were detected, Hedgehog was one of the pathways most responsive to aggregation with 8 positive counts and an overall increase of 2.72 ($p = 0.025$). *Bmp4*, which we detected amongst other Hedgehog pathway targets, encodes a growth factor that belongs to the transforming growth factor β (Tgf-β) superfamily. It was also among 12 genes that we found differentially expressed in 3×NT versus 1×NT embryos and in NT versus ICSI embryos, confirmed by QRT-PCR. The importance of Bmp4 in development is apparent from consequences of lacking Bmp4 at gastrulation (Fujiwara et al., 2001), the same stage when cloned embryos fail due to defects of the extra-embryonic lineages (Jouneau et al., 2006). Moreover, *Bmp4* is up-regulated upon TE differentiation (Erlebacher et al., 2004). Another member of the Tgf-β superfamily, Tgf-β1, is expressed in mouse embryos from the 2-cell stage on (Rappolee et al., 1988) and has a key role in TE maintenance (Tolkunova et al., 2006). Additionally, Tgf-β1 has been shown to facilitate, in combination with EGF, development of fertilized mouse embryos to blastocyst in vitro without increasing cell number, suggesting a participation in differentiation of the pre-implantation embryo (Paria and Dey, 1990).

We sought to directly manipulate the Tgf-β pathway in cloned embryos as a substitute for aggregation. In accordance with the reports of Standley et al. (2001) and Dadi et al. (2007), we directly supplied cloned embryos with signaling molecules (Egf, Tgf-β1, Bmp4), but this failed to change let alone normalize *Cdx2* expression or to increase developmental potential. Direct provision of recombinant factors likely lacks both spatial and temporal specificity, suggesting the use of in situ sources for Bmp4. However, transplantation of cloned blastocysts with androgenetic ES cells or biparental TS cells did not result in improved development although the donor cells were correctly integrated. We consider that the community effect requires a group of similar cells that synchronously undergo differentiation, while ES and TS cells are already restricted in their potency, i.e. no longer totipotent. Additionally, aggregation may involve activation of other pathways of the Tgf-β superfamily.

Based on the principle of pathway convergence, the analysis of Smad4 allows one to detect the activation of several pathways of the Tgf-β superfamily (Whitman, 1998). Therefore, we decided to examine the effect of aggregation on the nucleus/cytoplasmic localization of Smad4. As a control we examined β-catenin, target of the unrelated Wnt signaling pathway. Both β-catenin and Smad4 signaling are essential for embryo patterning after implantation; therefore, defective outcomes have not yet been selected by morula or blastocyst stage and can be observed in our study. Aggregation indeed induces nucleus-cytoplasmic redistribution of Smad4 but not β-catenin, which is invariably excluded from the nuclei of cloned embryos and remains localized at the cell periphery.

In conclusion, we provided evidence that induction of pluripotency genes in cloned mouse embryos is more efficient than previously assumed; however, levels of Cdx2 are erratic, suggesting that cloned morulae prematurely restrict pluripotency for differentiation. This defect is corrected by homologous aggregation of cloned embryos through a complex process that involves signaling pathways to the nucleus. It has been proposed that emerging gene expression patterns need a certain extent of cell–cell interaction to become stabilized (Kupiec, 1997). Our findings show that a minimum cell number is indeed necessary for stabilization of reprogrammed gene expression patterns and correct restriction of pluripotency in cloned embryos. The relevance of our findings is not confined to oocyte-mediated reprogramming, as signaling pathways and colony size have been suggested to also be involved in reprogramming and differentiation of ES cell lines (Bauwens et al., 2008; Guo et al., 2009).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2010.04.012.

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