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Early patterning of cloned mouse embryos contributes to post-implantation development

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ABSTRACT

Several research groups have suggested that the embryonic–abembryonic (Em–Ab) axis in the mouse can be predicted by the first cleavage plane of the early embryo. Currently, it is not known whether this early patterning occurs in cloned embryos produced by nuclear transfer and whether it affects development to term. In this work, the relationship between the first cleavage plane and the Em–Ab axis was determined by the labeling of one blastomere in cloned mouse embryos at the 2-cell stage, followed by ex-vivo tracking until the blastocyst stage. The results demonstrate that approximately half of the cloned blastocysts had an Em–Ab axis perpendicular to the initial cleavage plane of the 2-cell stage. These embryos were classified as "orthogonal" and the remainder as "deviant". Additionally, we report here that cloned embryos were significantly more often orthogonal than their naturally fertilized counterparts and overexpressed Sox2. Orthogonal cloned embryos demonstrated a higher rate of post-implantation embryonic development than deviant embryos, but cloned pups did not all survive. These results reveal that the angular relationship between the Em–Ab axis and the first cleavage plane can influence later development and they support the hypothesis that proper early patterning of mammalian embryos is required after nuclear transfer.

Introduction

Due to the lack of in vivo markers, until a few years ago it was not known whether mouse embryos, or any other mammalian embryos, showed any pattern to their development and thus it was not possible to follow cell fate using direct observation. Several studies had suggested that the initial cleavage pattern of the 2-cell stage embryo could be predictive of the Embryonic–abembryonic (Em–Ab) axis set by the asymmetric localization of the inner cell mass (ICM) within the mouse blastocyst cavity (Ciemerych et al., 2000; Fujimori et al., 2003; Gardner, 1997, 2001; Piotrowska and Zernicka-Goetz, 2001) and that embryonic development would not only be regulated by genetics or epigenetics but also by patterning (Zernicka-Goetz, 2006). The first evidence supporting these hypotheses is the fact that an animal–vegetal (A–V) axis, which predicts the fate of amphibian embryos, also exists in the mouse egg after the extrusion of the first polar body (PB) (Gardner, 2001; Zernicka-Goetz, 2002). It has been hypothesized that the maternally inherited DNA and the second polar body would then form most of the animal pole while the vegetal pole would correspond to the rest of the egg. After the first two embryonic divisions, the blastomere that inherits the largest portion of the vegetal pole would then more likely contribute to trophectodermal cells at the blastocyst stage (Piotrowska-Nitsche et al., 2005; Piotrowska-Nitsche and Zernicka-Goetz, 2005). In addition, the blastomere of a 2-cell mouse embryo that retains the sperm entry point divides first and preferentially develops into ICM cells (Piotrowska and Zernicka-Goetz, 2001). Altogether, it appears that, as in amphibian eggs, the A–V axis and the sperm entry point bias the Em–Ab axis of most (70–80%) blastocysts (Gray et al., 2004; Piotrowska and Zernicka-Goetz, 2001; Zernicka-Goetz, 2002). It has also been shown through non-invasive lineage tracing of progenies from 2-cell embryos that the contribution of each 2-cell blastomere to the

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ICM or to the trophectoderm can be anticipated and related to the Em–Ab axis, i.e. that the first cleavage plane is mostly orthogonal to the Em–Ab axis (Fujimori et al., 2003). The frequency of this Em–Ab patterning was shown to depend upon whether one of the two 2-cell blastomeres divided so as to separate the vegetal pole from the animal one (Gardner, 2001; Piotrowska and Zernicka-Goetz, 2001). Finally, it is also noteworthy that the anterior–posterior axis of the body emerging after post-implantation relates to the Em–Ab axis orientation of the blastocyst (Mesnard et al., 2004).

In addition to the morphological data, molecular and epigenetic evidence of the patterning development of mouse embryos has also been reported (Gray et al., 2004; Jedrusik et al., 2008; Torres-Padilla et al., 2007). This is the case for CARM1, a histone–arginine methyltransferase, and for several related histone post-translational modifications (H3R2, H3R17 or H3R26 methylation): differential expression/intensity levels among the 4-cell stage blastomeres promote some to develop as ICM cells (Torres-Padilla et al., 2007).

The existing literature is not in full support of the aforementioned viewpoints and contradicting observations have also been reported. For instance, another lineage-tracing study reported that the cells produced by the first two divisions were not different from each other and that the blastomeres arising from early embryonic stages were equivalent (Motosugi et al., 2005). Based on their results, that same group concluded that embryo asymmetry only emerges at the blastocyst stage with the differentiation of ICM and trophectoderm cells (Motosugi et al., 2005). Alarcon and Marikawa demonstrated, with a different labeling approach, that more than 70% of blastocysts did not show the orthogonal relationship between the first cleavage plane and the Em–Ab axis but were deviant regardless of the mouse strain, B6D2F1 or CD1 (Alarcon and Marikawa, 2003). They also argued that both orthogonal and deviant blastocysts produce normal fetuses at a similar frequency (Alarcon and Marikawa, 2003). Finally, several factors have been reported to affect the developmental fate of mouse embryos before the compaction of blastomeres into the morula. These include the shape of the zona pellucida (Kurotaki et al., 2007; Motosugi et al., 2005) and the topology of apposing pronuclei at the 1-cell stage (Hiiragi and Solter, 2004). However, it is worth noting that regulative and patterning development may both co-exist in embryogenesis (Zernicka-Goetz, 2006).

Cloning by nuclear transfer has been applied successfully to produce cloned animals of various species (Baguisi et al., 1999; Chesne et al., 2002; Wakayama et al., 1998; Wani et al., 2010; Wilmut et al., 1997; Zhou et al., 2003). However, although viable and fertile offspring have been produced through this technique, the success rate is still very low and rarely exceeds five percent. In order to improve this procedure, several research teams have focused on the series of cellular events, including genomic remodeling and epigenetic reprogramming, that occur during early embryogenesis in cloned embryos (Wakayama, 2007; Yang et al., 2007). However, to date, no one has studied the prepatterning of these embryos. It should be noted that cloned embryos lack a sperm entry point, a factor that affects blastomere prepatterning as mentioned above. Instead, cloned embryos are produced by manual transfer of a donor nucleus into an enucleated oocyte. Moreover, cloned embryos are usually activated by a chemical agent such as strontium or calcium ionophore and not by sperm factors as in naturally fertilized embryos.

In the present study, we therefore evaluated the presence of early patterning in cloned mouse embryos and its effects on full-term development. Ultimately, we wanted to assess the utility of a non-invasive method in improving nuclear transfer efficiency. In the experiments we performed, one blastomere of a 2-cell stage embryo was marked with living cell dye DiI as previously described by others (Alarcon and Marikawa, 2003; Piotrowska and Zernicka-Goetz, 2001) to determine whether the orientation of the Ab–Em axis of blastocysts could be predicted by the first cleavage plane in cloned embryos. We report that almost half of the cloned embryos had orthogonal prepatterning—a frequency higher than that of their naturally fertilized counterparts—and that pre-selection of these orthogonal cloned embryos significantly improved post-implantation development. Altogether, we believe that studying the patterning of cloned embryos provides a new viewpoint on developmental dynamics in mammals.

Materials and methods

Collection of embryos and oocytes

Eight-week-old F1 (C57Bl/6 × DBA/2) females were superovulated with PMSG (10 IU) injection followed by hCG (10 IU) injection 48 h later. For naturally fertilized embryos, female mice were mated with males of the same genotype and embryos were flushed from oviducts with Hepes-CZB medium at 40 h after hCG injection. Embryos were then cultured in KSOM medium covered by mineral oil at 37 °C in 5% CO_2 in air. For cloned embryos, metaphase II oocytes were collected from oviducts 13–14 h after hCG injection. Cumulus cells were removed with hyaluronidase (300 IU/ml), and oocytes were washed with Hepes-CZB several times. They were then placed in KSOM medium covered by mineral oil at 37 °C in 5% CO_2 in air until nuclear transfer.

Nuclear transfer

R1 embryonic stem cells cultured for less than 20 passages (gift from Prof. Nagy's laboratory) were used as donor cells. Manipulations (Leica-Narishige Micromanipulators; Leica, Germany) were performed under differential interference contrast at 20x magnification. Twenty to thirty metaphase II oocytes were placed into the chamber containing 1 ml Hepes-CZB medium with 5 μg/ml cytochalasin B covered by mineral oil. Pipettes with an internal diameter of 8–12 μm were used for the injection of the donor nucleus using a Piezo-electric device (P150, PrimeTech Japan). Donor nuclei were removed from metaphasic ES cells by gently aspirating in and out of the injection pipette and then injected into metaphase II oocytes. The metaphase plate of recipient oocytes was removed while withdrawing the pipette from the cytoplasm after injection. One to two hours after injection, reconstructed embryos were activated by 3 h of culture in 10 mM $SrCl_2$ CZB medium without calcium. Subsequently, cloned embryos were cultured in KSOM medium covered by mineral oil at 37 °C in 5% CO_2 in air.

Labeling of blastomeres

Due to its highly lipophilic nature, the carbocyanine dye DiI (Invitrogen) was dissolved in virgin olive oil at 60 °C at a final concentration of 2 mg/ml. When cooled down, the dye solution was backfilled to the microinjection needles and pressed through the tip of the injection needle against the membrane of one of the blastomeres of both cloned and naturally fertilized embryos at the 2-cell stage, with the help of a Femtojet injector (Eppendorf, Germany). A microdroplet of dye could then be absorbed by the blastomere. After labeling, cloned and naturally fertilized embryos were subsequently cultured in KSOM in 5% CO_2 at 37 °C on the microscope stage of an inverted fluorescence microscope (Leica, Germany), specially equipped with an environmental control chamber to maintain optimal

conditions for live cell imaging (temperature and carbon dioxide concentration).

Scoring the blastocysts

Scoring of the blastocysts was performed on naturally fertilized embryos at 102 hphCG injection and cloned embryos at 92 hpa. Blastocysts were placed on the inverted fluorescence microscope equipped with rhodamine filters (Leica, Germany) in a chamber containing 1 ml Hepes-CZB medium covered by mineral oil at 37 °C and held by a holding pipette. Blastocysts were rotated in order to fix the blastocoel cavity floor and the boundary line between the fluorescent and non-fluorescent cells in the same plane. Images were taken with rhodamine filters combined with bright light and analyzed with ImageJ software. The blastocysts were then classified into two groups: (1) "orthogonal" for blastocysts that demonstrated an angular degree between the boundary line of the fluorescent/nonfluorescent cells and the blastocoel cavity floor $\leq 30^{\circ}$ or (2) "deviant" for blastocysts that demonstrated an angular degree of $> 30^{\circ}$. After classification, blastocysts of the two groups were placed in separated culture drops (KSOM in 5% CO_2 at 37 °C).

Embryo transfer

DiI-labeled blastocysts were transferred according to their orthogonal vs. deviant classification into separate pseudopregnant CD1 females on day 2.5 after mating and recovered by Caesarian section on day 17.

Quantitative RT-PCR analysis of gene expression in blastocysts

RNA was extracted from batches of approximatively 25 blastocysts using Qiagen's RNAeasy micro kit (Cat. No. 74004). cDNAs were synthesized by reverse transcription (RT) using the SuperScript™ III Cells Direct cDNA Synthesis System (Invitrogen) and random primer hexamers (Roche Diagnostics) as previously described (Léandri et al., 2009). For each experiment, we performed three replicates. Real-time PCR was carried out with the ABI PRISM7000 Sequence Detection System (Applied Biosystems) as previously described (Wang et al., 2010). The sequences of primers for qPCR were: *Sox2* 5′-CCAGCTCGCAGACCTACATG-3′ (forward primer) and 5′-GAGTGGGAGGAAGAGGTAAC-3′ (reverse primer); *Oct4* (*Pou5f1*) 5′-GGAAGCCGACAACAATGAG-3′ (forward primer) and 5′-TCGGGCACTTCAGAAACAT-3′ (reverse primer); *Cdx2* 5′-TGCCACACTTGGGCTCTC-3′ (forward primer) and 5′-GCTGTGGAGGCTGTTGTTG-3′ (reverse primer); *Nanog* 5′-AGGATGAAGTGCAAGCGGTG-3′ (forward primer) and 5′-GGGATAGCTGCAATGGATGC-3′ (reverse primer). *GAPDH* was used as a reference gene to normalize *Oct4*, *Nanog*, and *Cdx2* expression level. The sequences of GAPDH primers were: 5′-CAGCAACTCCCACTCTTCCAC-3′ (forward primer) and 5′-TGGTCCAGGGTTTCTTACTC-3′ (reverse primer).

Immunofluorescence and mounting

Embryos were fixed with 4% paraformaldehyde (PFA) in PBS overnight at 4 °C and permeabilized with 0.5% Triton X-100 (30 min, at room temperature-RT). Embryos were then incubated with 2% bovine serum albumin (BSA) in PBS for 1 h. Incubation with rabbit polyclonal anti-H3R2me2 antibody (#05-808 from Upstate, dilution 1:200) or rabbit polyclonal anti-H4R3me2 antibody (ab5823 from Abcam, dilution 1:200) was performed overnight at 4 °C. After two washes with PBS, embryos were incubated 1 h at RT with a FITC-conjugated secondary antibody (Jackson Immunoresearch in West Grove, PA, dilution 1:200) and rinsed again in PBS to remove excess antibodies. All antibodies were diluted in PBS-BSA (2%). DNA counterstaining was performed with PI diluted in PBS (final concentration 10 μg/ml) for 15 min at 37 °C RT. Embryos were then briefly post-fixed (2% PFA, 10 min, RT), deposited on depressed slides (for 3D preservation), and mounted under a coverslip with Citifluor (Citifluor Products).

Microscopy and image analysis

Confocal microscopy was performed with a Zeiss LSM 510 confocal laser scanning microscope equipped with an oil immersion objective (Plan Apochromatic 63 × n.a.1.4) with the 488-and 535-wavelength lasers. Entire embryos were scanned with a distance of 0.37 μm between light optical sections. Maximum intensity projections of Z-stacks were performed using ImageJ software. Nuclei were outlined manually on these projections with ImageJ as well. Mean staining intensities and nuclear areas were then measured for each nucleus. Total fluorescence intensities were calculated for individual nuclei by multiplying the mean fluorescence intensity and nuclear area. Fluorescence levels were then normalized against the blastomere showing the highest level, which was set at 100%.

Statistical analysis

A large number of embryos were analyzed for each group and two to three repeats were performed for every experiment. Results were analyzed using a one-way ANOVA, an independent-sample *t*-test, and a paired-sample *t*-test using SPSS (SigmaStat) software.

Results

DiI labeling method does not affect development of cloned embryos

The cell-lineage tracer DiI was used to label one of the blastomeres of 2-cell stage embryos. Previous studies demonstrated that the DiI method had no influence on the development of naturally fertilized embryos (Alarcon and Marikawa, 2003; Piotrowska and Zernicka-Goetz, 2001), and, in this study, we confirmed that DiI does not alter embryonic development using our methodology either. Indeed, 87% ($n=335$) of naturally fertilized embryos labeled with DiI at the 2-cell stage reached the blastocyst stage, a percentage that was not significantly different from the 95% ($n=42$, $p > 0.5$) of embryos reaching the blastocyst stage in the control group without DiI labeling (Table 1). Similarly, when applied to cloned embryos produced by nuclear transfer, labeling at the 2-cell stage with DiI did not interfere with embryo development to the blastocyst stage. Just over 50% ($n=1311$) of cloned embryos labeled at the 2-cell stage resulted in blastocysts, which was similar to the 48% of cloned blastocysts we usually

Table 1
Development to the blastocyst stage with or without DiI labeling at the 2-cell stage.

	Fertilized embryos		Cloned embryos	
	Non-labeled	Labeled	Non-labeled	Labeled
N° 2-cell	42	335	292	1311
N° Blastocysts (%)[a]	40 (95.2)[b]	292 (87.2)[b]	137 (48.0)[c]	659 (50.4)[c]

[a] Percentages are based on the number of 2-cell embryos.
[b] Labeled and non-labeled embryos in each group of embryos were compared. Values with the same superscripts are not statistically different ($p > 0.5$).
[c] Labeled and non-labeled embryos in each group of embryos were compared. Values with the same superscripts are not statistically different ($p > 0.5$).

obtain after nuclear transfer ($n=137$, $p>0.5$, Table 1). As we usually get more than 90% cleavage to the 2-cell stage with the reconstructed embryos in our laboratory conditions (Zhou et al., 2010), the overall efficiency of this technique was enough to pursue the study.

Labeling with DiI is reliable enough to carry out cell lineage studies

After labeling, naturally fertilized and cloned embryos were monitored until the blastocyst stage with time-lapse microscopy (Fig. 1A and B, respectively). Embryos were placed in culture on the microscope stage of an inverted fluorescence microscope specially equipped with an environmental control chamber (temperature and carbon dioxide concentration controls). Embryos were observed every 14–16 h and sequential images were taken with rhodamine filters at different stages, from 2-cell to blastocyst. As expected, all the progeny cells derived from the labeled blastomeres retained DiI in their membranes and the fluorescent dye did not seem to cross to the progenies of the unlabeled blastomeres. At the early blastocyst stage, we could therefore assess the first cleavage plane as the boundary line between fluorescent and non-fluorescent cells and subsequently used this method of labeling to study lineage inheritance in both naturally fertilized and cloned embryos.

Two-cell cloned embryo blastomeres show a predisposition to follow either an embryonic or abembryonic fate

A number of studies have previously reported that the first cleavage plane of mouse embryos tends to be orthogonal to the Em–Ab axis of early blastocysts (Gardner, 2001; Piotrowska and Zernicka-Goetz, 2001). This Em–Ab axis can be represented by a line drawn at the cavity floor of the blastocoel and the angle with the first cleavage plane (determined by DiI labeling) can be calculated (Fig. 2). The blastocysts in this study were then classified into two groups: (1) "orthogonal" for blastocysts that demonstrated an angular degree between the boundary line of the fluorescent/nonfluorescent cells and the blastocoel cavity floor $\leq 30^{\circ}$ or (2) "deviant" for blastocysts that demonstrated an angular degree $> 30^{\circ}$.

Subsequently, we tested whether the distribution of orthogonal and deviant embryos was significantly different in cloned versus naturally fertilized embryos. In naturally fertilized embryos, only 42% of embryos were classified as orthogonal versus 58% as deviant ($n=236$, $p=0.025$, Table 2). Interestingly, in cloned embryos, we found that the Em–Ab axis was orthogonal to the first cleavage plane in 49% of blastocysts, a ratio significantly higher than in naturally fertilized ones ($n=595$, $p=0.021$, Table 2), suggesting that blastomeres from 2-cell stage cloned embryos have a higher predisposition to separate and form respectively either the Em (embryonic) or Ab (abembryonic) pole.

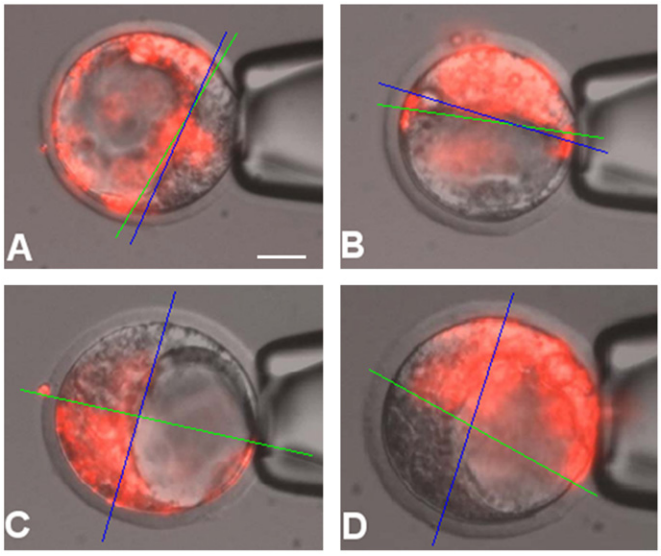

Fig. 2. Scoring of orthogonal and deviant blastocysts. Examples of labeled blastocysts with the first cleavage plane (green) and the blastocoel cavity floor (blue) drawings. According to the angle between these two lines ($\leq 30^{\circ}$ or $> 30^{\circ}$), blastocysts were scored as orthogonal (A/B) or deviant (C/D), respectively. Scale bar: 25 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 2
Relationship between the Em–Ab axis and the first cleavage plane in naturally fertilized and cloned blastocysts.

	No. embryos observed	No. orthogonal blastocysts, (%)[a]	No. deviant blastocysts, (%)[a]
Fertilized embryos	236	100 (42.1 ± 2.2)[b]	136 (57.9 ± 2.2)[c]
Cloned embryos	595	295 (49.1 ± 1.4)[d]	300 (50.9 ± 1.4)r[d]

[a] Percentages are based on the number of 2-cell embryos.
[b,c,d] Independent-sample *t*-tests were used to compare the number of orthogonal vs. deviant embryos between the fertilized and cloned groups. The differences observed were statistically significant except for the comparison of orthogonal vs. deviant blastocysts in the cloned group ($p=0.215$).

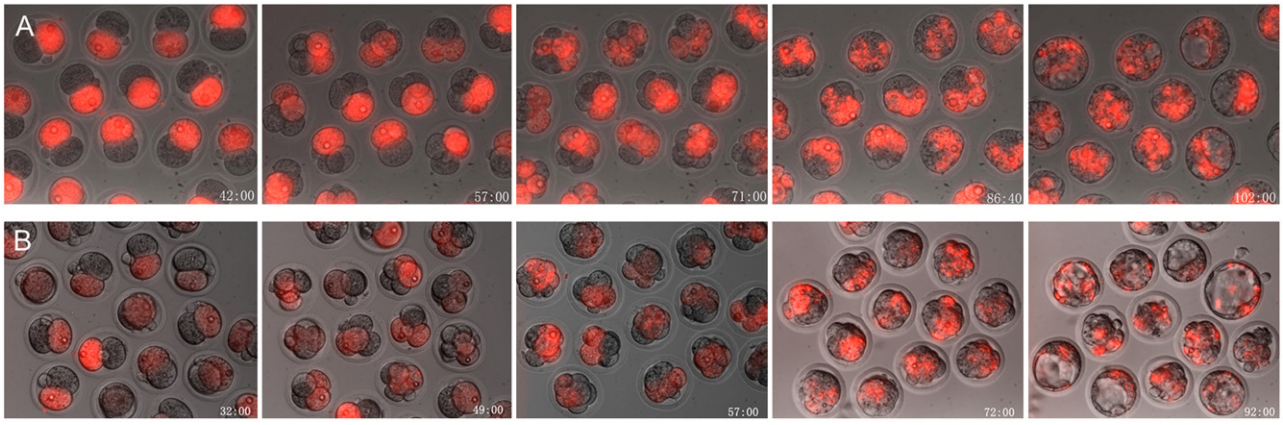

Fig. 1. Sequential live images of embryos labeled with DiI. (A) Naturally fertilized or (B) cloned embryos were regularly observed after DiI labeling at the 2-cell stage. The time in the right corner corresponds to hours post-hCG or post-activation, respectively.

These results also indicate that the developmental pattern was different for cloned mouse embryos as compared to naturally fertilized embryos.

Cloned embryos demonstrate more variability in the levels of histone H3 methylation at arginine 2 (H3R2me2) than naturally fertilized embryos

Based on the research of Torres-Padilla et al. (2007), we suspected that the early variation in H3R2me2 intensity normally observed among blastomeres at the 4-cell stage could be different in cloned embryos. We therefore performed immunodetection of this post-translational histone modification on whole-mount embryos, both for the naturally fertilized and cloned groups. We observed that cloned 4-cell embryos showed marked differences in H3R2me2 levels among their blastomeres ($p=0.02$, $n=13$, Fig. 3A). A similar configuration was observed in naturally fertilized embryos, as expected ($p=0.03$, $n=15$, Fig. 3B). Due to the limitations of the immunostaining and observation procedures, we cannot say whether the total intensities were higher in cloned or naturally fertilized embryos. However, it is clear that the variation among the four blastomeres was greater in clones (Fig. 3C), confirming that blastomeres in cloned embryos are probably more prepatterned than those in naturally fertilized embryos. We also performed immunostaining controls with another histone methylation mark supposed to be equivalent among the nuclei of 4-cell fertilized embryos, H4R3me2 (Torres-Padilla et al., 2007). In that case, we observed that the four blastomeres of cloned embryos did not differ from those naturally fertilized (Fig. 3D and E); H4R3me2 intensities were equivalent in both types of embryos ($p>0.5$, $n=10$ and 11, respectively; Fig. 3F). This suggests that H3R2me2 variation in cloned embryos is not due to poorer development in

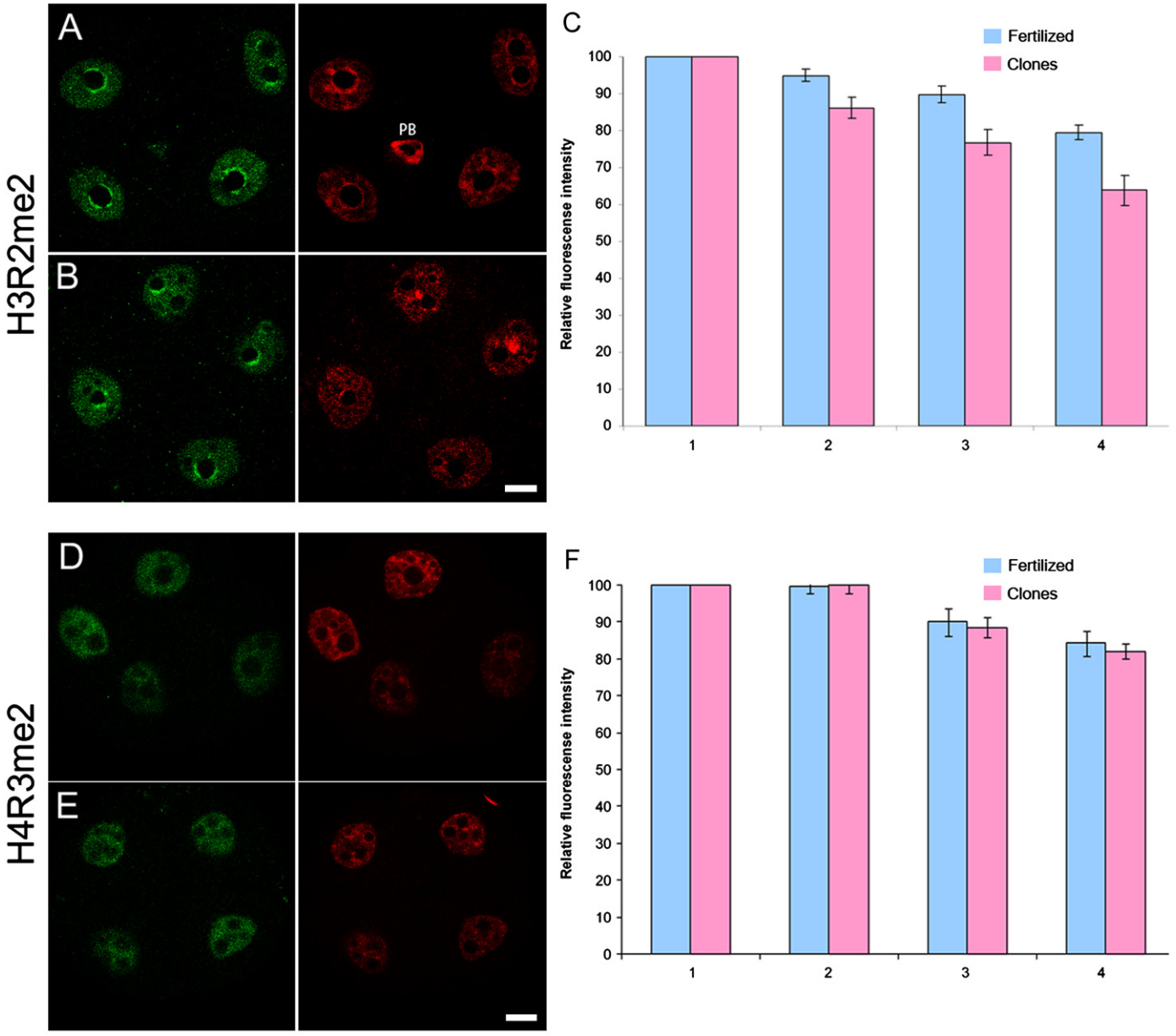

Fig. 3. Immunodetection of modified histones H3R2me2 and H4R3me2. Immunodetection of H3R2me2 or H4R3me2 (green) with DNA counterstaining (red) was performed in cloned (A, D) and naturally fertilized (B, E) 4-cell-stage embryos (single confocal z-sections, PB=polar body). Scale bar: 10 μm. Quantification (C, F) was then performed with ImageJ software on z-projections as described in Materials and Methods. Each bar represents the relative H3R2me2/H4R3me2 fluorescence level of one of the four blastomeres either in naturally fertilized (blue bars) or cloned (pink bars) embryos. Error bars indicate s.d. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

these embryos but to intrinsic epigenetic differences when compared with naturally fertilized ones.

Selection of orthogonal cloned mouse blastocysts is associated with improved development to term

The relationship between the orientation of the Em–Ab axis and the first cleavage plane seems to have no influence on post-implantation development in naturally fertilized embryos (Alarcon and Marikawa, 2003). In this study, we assessed this relationship in cloned mouse embryos produced by nuclear transfer. Subsequent to their classification as orthogonal or deviant, naturally fertilized and cloned blastocysts were transferred into surrogate females. Before transfer, no morphological differences were observed between orthogonal and deviant blastocysts; blastocoel cavities and trophectoderm epithelium were quite similar (Fig. 4B and C). These blastocysts were allowed to develop to term in order to evaluate their developmental potential.

As shown in Table 3, naturally fertilized orthogonal and deviant blastocysts exhibited similar implantation and full-term developmental rates (82% and 24% vs. 86% and 38%, respectively, $n=34$ and 62, $p>0.5$, Table 3). However, this was not the case for cloned embryos: orthogonal blastocysts demonstrated a significantly higher implantation rate than their deviant counterparts (68% vs. 35% %, $n=166$ and 153, $p=0.03$, Table 3). Remarkably, cloned pups were only obtained with the orthogonal group ($n=7$, Fig. 4D). Some offspring obtained from orthogonal cloned blastocysts showed morphological abnormalities, namely abnormal-looking limbs, but three were healthy without any morphological abnormalities. Taken together, these results suggest that the angular relationship between the first cleavage plane and the Em–Ab axis correlates with the potential of cloned embryos to develop into live pups.

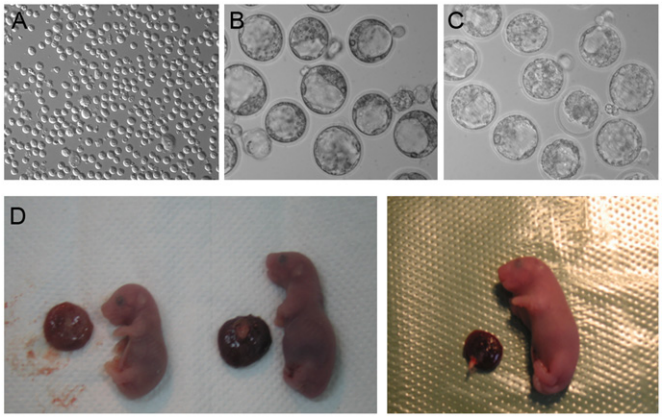

Fig. 4. Development to term of cloned embryos after DiI labeling. (A) Brightfield images showing the R1 donor cell. (B) and (C) Brightfield images showing the cloned orthogonal and deviant blastocysts, respectively. (D) Offspring born from orthogonal blastocysts.

Comparison of pluripotent gene expression between orthogonal and deviant cloned blastocysts

We next analyzed expression of the Cdx2 gene (trophectoderm lineage marker) and of three pluripotency genes: Nanog, Oct4, and Sox2. Fig. 5 shows their relative gene expression, measured by QRT-PCR, in orthogonal cloned blastocysts ($n=77$) relative to deviant cloned blastocysts ($n=79$). No statistical difference was observed except for Sox2 gene expression, which was indeed statistically higher in cloned orthogonal blastocysts as compared to deviant ones ($p<0.05$).

Discussion

Several studies demonstrate that sperm entry position, egg polarity, and plane of first cleavage can bias the Embryonic–abembryonic axis in the blastocyst. As cloned embryos are reconstructed in vitro by the introduction of donor cell nuclei into recipient oocytes through a micromanipulation pipette, they constitute a good model to study the hypothesis of prepatterning in the absence of a sperm entry point and sperm factors. We therefore questioned whether or not cloned embryos have distinct developmental patterning in comparison to naturally fertilized embryos.

The cell tracing method we used is non-invasive and was previously reported to allow, with helpers, normal development to term of naturally fertilized embryos (Piotrowska-Nitsche and

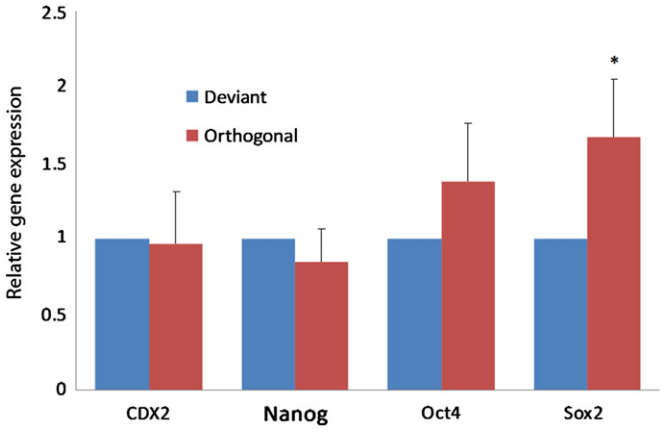

Fig. 5. Comparison of pluripotent gene expression between orthogonal and deviant cloned blastocysts. Cdx2, Nanog, Oct4, and Sox2 gene expression was analyzed in orthogonal and deviant clones by QRT-PCR. This graph shows the relative gene expression in orthogonal relative to deviant cloned blastocysts. Error bars indicate standard deviation. Only Sox2 gene expression was statistically higher in orthogonal blastocysts (p-value <0.05).

Table 3
Postimplantation development of naturally fertilized and cloned embryos according to their classification as having an orthogonal vs. deviant Em–Ab axis.

	Axis relationship to the 1st cleavage plane	No. blastocysts transferred	Day 21 of gestation	
			No. (%), implants	No. (%), pups
Fertilized embryos	Orthogonal	34	28 (82.2 ± 1.1)[a]	8 (23.9 ± 3.9)[a]
	Deviant	62	53 (86.4 ± 5.5)[a]	23 (38.5 ± 4.0)[a]
Cloned embryos	Orthogonal	166	130 (67.9 ± 6.0)[a]	7 (with 3 alive ones)
	Deviant	153	49 (34.7 ± 12.6)[b]	0

Values with the same superscripts are not statistically different ($p>0.5$, independent-sample t-test). The only significant difference was observed for implantation sites of the cloned group ($p=0.03$).

Zernicka-Goetz, 2005). In the present study, we confirm that DiI labeling and regular observations with an appropriate time-lapse microscope allow development to term of the embryos irrespective of their origin (naturally fertilized or cloned) and without helpers.

As previously reported by several groups (Alarcon and Marikawa, 2003; Motosugi et al., 2005), we did not observe orthogonal prepatterning in naturally fertilized embryos. Differences in prepatterning due to mouse strain and culture media have already been documented (Hiiragi et al., 2006) but our results suggest that these cannot be the sole source of discrepancy with those of researchers arguing for orthogonal preferential prepatterning. On the other hand, the method of analysis used to determine the distribution of blastomere descendants at the blastocyst stage seems more controversial (Hiiragi et al., 2006). Other non-invasive lineage-tracing studies might have been more appropriate (Bischoff et al., 2008; Kurotaki et al., 2007), however, due to the difficulty inherent in using the cloned model, we preferred to use the easiest approach and focus on the comparison between naturally fertilized and cloned embryos. Now that a clear difference has been established, further studies to clarify the mechanism and function of asymmetrical division in cloned embryos, as well as more precise methods, will be necessary to track single embryonic cell fate and analyze single-cell global gene expression. Two-photon light scanning microscopy can, for example, be used to achieve high resolution 3D video and less phototoxic studies (McDole et al., 2011; Plachta et al., 2011). Transcription profiling of single-cell gene expression could then provide distribution patterns of informational macromolecules (Guo et al., 2010; Roberts et al., 2011).

Remarkably, we observed that a significantly higher percentage of cloned than naturally fertilized blastocysts had an Em–Ab axis perpendicular to the initial cleavage plane of the 2-cell stage. This result suggests that there might be more prepatterning in cloned embryos and that one of the 2-cell blastomeres has a stronger tendency to contribute either to the embryonic or abembryonic half of the cloned blastocyst.

One hypothesis explaining the spatial relationship of the first cleavage plane relative to the blastocyst axis relies on the physical constraint imposed by the zona pellucida. Several papers have shown that while embryos move or rotate within the zona pellucida, its elongated shape physically constrains placement of the blastocyst cavity to either end (Motosugi et al., 2005; Kurotaki et al., 2007; Alarcón and Marikawa, 2008). We cannot therefore exclude the possibility that the prepatterning we observed in cloned embryos is simply due to physical constraint by the zona pellucida, especially since these embryos had been manipulated (the introduction of the donor cell was performed by piezo-electric pulses making a hole in the zona pellucida). On the other hand, we may also hypothesize that this hole (which remains until the blastocyst stage) increases zona pellucida flexibility and enables the embryo to move or rotate even more. It would be interesting to further investigate this possibility.

Another hypothesis explaining polarity is that the cytoplasmic factors inherited from the oocyte might be non-uniformly distributed among the blastomeres of pre-implantation embryos and thereby determine cell fate as ICM or trophectoderm. The two striking differences between naturally fertilized and cloned embryos that correlate with this hypothesis and could explain the prevalence of orthogonal embryos after cloning are (1) the presence of sperm factors after fertilization and (2) the need for stronger reprogramming of the introduced genome after cloning. Indeed, we cannot exclude the possibility that naturally fertilized embryos are more flexible than cloned embryos because fewer maternal factors are required to generate blastomeres with an open chromatin configuration and sustain pluripotency. The ICM and trophectoderm cells of naturally fertilized embryos could then support post-implantation development irrespective of the initial distribution of oocyte factors, whereas a developmental bias would become more obvious in cloned embryos.

In order to test this idea, we investigated the distribution of arginine methylation on histone H3, as Torres-Padilla et al. reported that the methylation of histone H3 arginine targeted by CARM1 can regulate pluripotency and cell-fate decision in the early mouse embryo (Torres-Padilla et al., 2007). In the present study, we conducted a similar analysis based on the differential level of staining for H3R2me2 among blastomeres of 4-cell stage embryos. Most importantly, we report greater variation in H3R2me2 staining in 4-cell cloned embryos than in naturally fertilized ones. Of course, the cause of this difference in H3 methylation levels between cloned and naturally fertilized embryos requires further study, but we hypothesize that it might affect transcription of key genes involved in the maintenance of cellular pluripotency through chromatin remodeling (as suggested in Torres-Padilla et al., 2007). This hypothesis is sustained by the increased Sox2 gene expression we observed in orthogonal versus deviant cloned blastocysts. As Sox2 is known to participate in the regulation of *Fgf4* expression, required for the establishment and proliferation of extraembryonic ectoderm (Avilion et al., 2003), it would explain why orthogonal cloned blastocysts have a higher likelihood of implanting and developing to term.

We also examined whether the distinct developmental patterning differences between naturally fertilized and cloned embryos could influence the efficiency of full-term development. Embryos were therefore classified according to the angular relationship between the first cleavage plane and the blastocyst Em–Ab axis before their transfer into surrogate mothers. As expected from Alarcon and Marikawa (2003), no difference between orthogonal and deviant blastocysts was observed after implantation in the naturally fertilized group. Remarkably, though, we report here that the orthogonal cloned embryos have a higher developmental potential than the deviant ones. This suggests that, in contrast to naturally fertilized embryos, clones do not have the same ability to sustain full-term development into live offspring as a consequence of their pre-implantation patterning. Again, we can hypothesize that cloned embryos are less flexible than fertilized ones and that the blastomeres do not accommodate as well to their cell fate. Of course, further research is needed to find out which factors play a key role in the prepatterning of cloned embryos, but we believe that this model provides a new opportunity to investigate the exact mechanisms that determine cell fate in the early embryo. To our knowledge, this is the first report of developmental patterning in cloned embryos.

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References

Alarcon, V.B., Marikawa, Y., 2003. Deviation of the blastocyst axis from the first cleavage plane does not affect the quality of mouse postimplantation development. Biol. Reprod. 69, 1208–1212.

Alarcón, V.B., Marikawa, Y., 2008. Spatial alignment of the mouse blastocyst axis across the first cleavage plane is caused by mechanical constraint rather than developmental bias among blastomeres. Mol. Reprod. Dev. 75, 1143–1153.

Avilion, A.A., Nicolis, S.K., Pevny, L.H., Perez, L., Vivian, N., Lovell-Badge, R., 2003. Multipotent cell lineages in early mouse development depend on SOX2 function. Genes Dev. 17, 126–140.

Bischoff, M., Parfitt, D.E., Zernicka-Goetz, M., 2008. Formation of the embryonic–abembryonic axis of the mouse blastocyst: relationships between orientation of early cleavage divisions and pattern of symmetric/asymmetric divisions. Development 135, 953–962.

Baguisi, A., Behboodi, E., Melican, D.T., Pollock, J.S., Destrempes, M.M., Cammuso, C., Williams, J.L., Nims, S.D., Porter, C.A., Midura, P., Palacios, M.J., Ayres, S.L., Denniston, R.S., Hayes, M.L., Ziomek, C.A., Meade, H.M., Godke, R.A., Gavin, W.G., Overstrom, E.W., Echelard, Y., 1999. Production of goats by somatic cell nuclear transfer. Nat. Biotechnol. 17, 456–461.

Chesne, P., Adenot, P.G., Viglietta, C., Baratte, M., Boulanger, L., Renard, J.P., 2002. Cloned rabbits produced by nuclear transfer from adult somatic cells. Nat. Biotechnol. 20, 366–369.

Ciemerych, M.A., Mesnard, D., Zernicka-Goetz, M., 2000. Animal and vegetal poles of the mouse egg predict the polarity of the embryonic axis, yet are nonessential for development. Development 127, 3467–3474.

Fujimori, T., Kurotaki, Y., Miyazaki, J., Nabeshima, Y., 2003. Analysis of cell lineage in two- and four-cell mouse embryos. Development 130, 5113–5122.

Gardner, R.L., 1997. The early blastocyst is bilaterally symmetrical and its axis of symmetry is aligned with the animal–vegetal axis of the zygote in the mouse. Development 124, 289–301.

Gardner, R.L., 2001. Specification of embryonic axes begins before cleavage in normal mouse development. Development 128, 839–847.

Gray, D., Plusa, B., Piotrowska, K., Na, J., Tom, B., Glover, D.M., Zernicka-Goetz, M., 2004. First cleavage of the mouse embryo responds to change in egg shape at fertilization. Curr. Biol. 14, 397–405.

Guo, G., Huss, M., Tong, G.Q., Wang, C., Li Sun, L., Clarke, N.D., Robson, P., 2010. Resolution of cell fate decisions revealed by single-cell gene expression analysis from zygote to blastocyst. Dev. Cell 18, 675–685.

Hiiragi, T., Alarcon, V.B., Fujimori, T., Louvet-Vallee, S., Maleszewski, M., Marikawa, Y., Maro, B., Solter, D., 2006. Where do we stand now? Mouse early embryo patterning meeting in Freiburg Germany (2005). Int. J. Dev. Biol. 50, 581–586 (Discussion 586–587).

Hiiragi, T., Solter, D., 2004. First cleavage plane of the mouse egg is not predetermined but defined by the topology of the two apposing pronuclei. Nature 430, 360–364.

Jedrusik, A., Parfitt, D.E., Guo, G., Skamagki, M., Grabarek, J.B., Johnson, M.H., Robson, P., Zernicka-Goetz, M., 2008. Role of Cdx2 and cell polarity in cell allocation and specification of trophectoderm and inner cell mass in the mouse embryo. Genes Dev. 22, 2692–2706.

Kurotaki, Y., Hatta, K., Nakao, K., Nabeshima, Y., Fujimori, T., 2007. Blastocyst axis is specified independently of early cell lineage but aligns with the ZP shape. Science 316, 719–723.

Léandri, R.D., Archilla, C., Bui, L.C., Peynot, N., Liu, Z., Cabau, C., Chastellier, A., Renard, J.P., Duranthon, V., 2009. Revealing the dynamics of gene expression during embryonic genome activation and first differentiation in the rabbit embryo with a dedicated array screening. Physiol. Genom. 36, 98–113.

McDole, K., Xiong, Y., Iglesias, P.A., Zheng, Y., 2011. Lineage mapping the pre-implantation mouse embryo by two-photon microscopy, new insights into the segregation of cell fates. Dev. Biol. 355, 239–249.

Mesnard, D., Filipe, M., Belo, J.A., Zernicka-Goetz, M., 2004. The anterior–posterior axis emerges respecting the morphology of the mouse embryo that changes and aligns with the uterus before gastrulation. Curr. Biol. 14, 184–196.

Motosugi, N., Bauer, T., Polanski, Z., Solter, D., Hiiragi, T., 2005. Polarity of the mouse embryo is established at blastocyst and is not prepatterned. Genes Dev. 19, 1081–1092.

Piotrowska, K., Zernicka-Goetz, M., 2001. Role for sperm in spatial patterning of the early mouse embryo. Nature 409, 517–521.

Piotrowska-Nitsche, K., Perea-Gomez, A., Haraguchi, S., Zernicka-Goetz, M., 2005. Four-cell stage mouse blastomeres have different developmental properties. Development 132, 479–490.

Piotrowska-Nitsche, K., Zernicka-Goetz, M., 2005. Spatial arrangement of individual 4-cell stage blastomeres and the order in which they are generated correlate with blastocyst pattern in the mouse embryo. Mech. Dev. 122, 487–500.

Plachta, N., Bollenbach, T., Pease, S., Fraser, S.E., Pantazis, P., 2011. Oct4 kinetics predict cell lineage patterning in the early mammalian embryo. Nat. Cell Biol. 13, 117–123.

Roberts, R.M., Katayama, M., Magnuson, S.R., Falduto, M.T., Torres, K.E., 2011. Transcript profiling of individual twin blastomeres derived by splitting two-cell stage murine embryos. Biol. Reprod. 84, 487–494.

Torres-Padilla, M.E., Parfitt, D.E., Kouzarides, T., Zernicka-Goetz, M., 2007. Histone arginine methylation regulates pluripotency in the early mouse embryo. Nature 445, 214–218.

Wakayama, T., 2007. Production of cloned mice and ES cells from adult somatic cells by nuclear transfer: How to improve cloning efficiency? J. Reprod. Dev. 53, 13–26.

Wakayama, T., Perry, A.C., Zuccotti, M., Johnson, K.R., Yanagimachi, R., 1998. Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei. Nature 394, 369–374.

Wang, Y., Hai, T., Liu, Z., Zhou, S., Lv, Z., Ding, C., Liu, L., Niu, Y., Zhao, X., Tong, M., Wang, L., Jouneau, A., Zhang, X., Ji, W., Zhou, Q., 2010. HSPC117 deficiency in cloned embryos causes placental abnormality and fetal death. Biochem. Biophys. Res. Commun. 397, 407–412.

Wani, N.A., Wernery, U., Hassan, F.A., Wernery, R., Skidmore, J.A., 2010. Production of the first cloned camel by somatic cell nuclear transfer. Biol. Reprod. 82, 373–379.

Wilmut, I., Schnieke, A.E., McWhir, J., Kind, A.J., Campbell, K.H., 1997. Viable offspring derived from fetal and adult mammalian cells. Nature 385, 810–813.

Yang, X., Smith, S.L., Tian, X.C., Lewin, H.A., Renard, J.P., Wakayama, T., 2007. Nuclear reprogramming of cloned embryos and its implications for therapeutic cloning. Nat. Genet. 39, 295–302.

Zernicka-Goetz, M., 2002. Patterning of the embryo: the first spatial decisions in the life of a mouse. Development 129, 815–829.

Zernicka-Goetz, M., 2006. The first cell-fate decisions in the mouse embryo: destiny is a matter of both chance and choice. Curr. Opin. Genet. Dev. 16, 406–412.

Zhou, S., Ding, C., Zhao, X., Wang, E., Dai, X., Liu, L., Li, W., Liu, Z., Wan, H., Feng, C., Hai, T, Wang, L., Zhou, Q., 2010. Successful generation of cloned mice using nuclear transfer from induced pluripotent stem cells. Cell Res. 20, 850–853.

Zhou, Q., Renard, J.P., Le Friec, G., Brochard, V., Beaujean, N., Cherifi, Y., Fraichard, A., Cozzi, J., 2003. Generation of fertile cloned rats by regulating oocyte activation. Science 302, 1179.