

Article

Assisted fertilization and embryonic axis formation in higher primates



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Abstract

In naturally fertilized embryos of various organisms, the spermatozoon provides a localized cue to initiate early embryonic patterning. In mice, the sperm entry point (SEP) may reorient the first cleavage division, which separates the zygote into two halves that follow distinct fates. However, it is unknown whether the mechanical injection of spermatozoa into an oocyte by intracytoplasmic sperm injection (ICSI), a technique commonly used in human assisted reproduction, possesses such a role. Rhesus macaque embryos fertilized by ICSI were examined in order to determine the consequences of placing the spermatozoon at specific positions in the ooplasm and whether this can provide new information about patterning in mammalian eggs. The SEP specified by the injected spermatozoa was most often localized near the first cleavage plane and was mainly distributed along the boundary zone that separates the embryonic and abembryonic parts of the monkey blastocyst. Moreover, the ICSI data, when compared with naturally fertilized mouse embryos, showed a similar outcome in terms of cleavage axes and first embryonic axis specification. As there are no studies to date regarding sperm entry in human oocytes and its influence on embryonic development, this investigation using the rhesus macaque as a clinical model is noteworthy.

Keywords: ICSI, non-human primates, patterning, sperm entry

Introduction

Early embryo patterning in higher primates is an unprecedented research area. There have been no reports either on the subject of the sperm entry point (SEP) or developmental patterning in higher primates. Studies of mouse embryo patterning in relationship to the SEP (Piotrowska and Zernicka-Goetz, 2001; Plusa *et al.*, 2002b; Gray *et al.*, 2004) have directed interest to rhesus macaques, which are more closely related to humans. In human assisted reproduction, intracytoplasmic sperm injection (ICSI) has become the most commonly used technique to achieve fertilization *in vitro*. Although thousands of babies have been born from ICSI-fertilized embryos, little is known of the effect of mechanical injection of spermatozoa into oocytes as opposed to natural fertilization. There is therefore interest in determining whether placing the spermatozoon at specific positions in the monkey ooplasm can be related

to the process of embryonic axis formation in non-human primates (NHP).

Investigating embryo patterning in higher primates is challenging because of a relatively limited source of gametes and embryos. However, studies with primates have unique advantages over those involving rodents for understanding early embryo development in humans. Rhesus macaque oocytes and embryos share high morphological similarity to those of humans, including size, cytoskeletal architecture and cytoplasmic clarity (Hewitson, 2004). Rhesus macaques have also played an important role in the development of human assisted reproductive technology. Assisted reproduction techniques in monkeys are almost identical to those used clinically, and offspring can be produced at similar success rates (Hewitson *et al.*, 1998, 2000). Reproductive techniques

used in human reproduction are well established in rhesus macaques, with efficiency comparable with that of humans, and superior to other NHP (Ron-El *et al.*, 1995; Hewitson *et al.*, 1998). Motivated by prior findings in mice and the resulting open questions in early human embryo development under the influence of emerging reproductive techniques, rhesus macaques were used as a model to investigate the role and consequences of the incoming spermatozoa through ICSI.

Materials and methods

The present study was approved by the Institutional Animal Care and Use Committee (IACUC) at Emory University.

Follicle stimulation regimen

Stimulation of female rhesus macaques exhibiting regular menstrual cycles was induced with exogenous gonadotrophins. Intramuscular injections of recombinant human FSH (r-hFSH; Sereno, Italy) were administered twice daily for 6 days, followed by twice daily injections of r-hFSH and recombinant human LH (r-hLH) (Sereno) and a once daily subcutaneous injection of gonadotrophin-releasing hormone antagonist (Antide; Sereno). On day 7 of stimulation, ultrasonography was performed and an injection of recombinant human chorionic gonadotrophin (r-HCG; Bioqual, MD, USA) was administered for ovulation induction when follicles at 3–4 mm in diameter were observed.

Follicular aspiration by laparoscopy

Follicular aspiration was performed at 27 h post-HCG. Oocytes were aspirated from follicles using a needle suction device lined with Teflon tubing. Multiple individual follicles were aspirated with continuous vacuum, and collection tubes were immediately transported to a dedicated primate laboratory for oocyte recovery and evaluation of the maturation stage.

Collection and evaluation of rhesus macaque oocytes

The contents of each collection tube had been diluted in modified Tyrode solution with albumin, lactate and pyruvate (TALP)-HEPES (Bavister *et al.*, 1983) supplemented with 2 mg/ml hyaluronidase. Oocytes were rinsed and then transferred to pre-equilibrated in-vitro maturation (IVM) medium (modified CMRL-1066; GIBCO no. 289964, modified according to Zheng *et al.*, 2003) prior to the evaluation of maturation. Metaphase II (MII) arrested oocytes, exhibiting expanded cumulus cells, a distinct perivitelline space (PVS) and the first polar body, were selected for subsequent fertilization and bead labelling.

In-vitro maturation

Oocytes were matured in modified CMRL-1066 culture medium containing 10% heat-inactivated fetal bovine serum (FBS; HyClone Laboratories Inc., Logan, UT, USA) supplemented with 40 µg/ml sodium pyruvate, 150 µg/ml glutamine, 550 µg/ml calcium lactate, 100 ng/ml oestradiol (Sigma) and 3 µg/ml of progesterone (Sigma) for up to 36 h in 35 µl drops of medium under mineral oil at 37°C with 5% CO₂, 5% O₂ and 90% N₂.

Preparation and handling of semen

Rhesus macaque males were trained to routinely produce semen samples by penile electroejaculation. After serial washes, the sperm samples were counted and diluted to a concentration of 2×10^7 sperm/ml. Sperm suspensions were then incubated at room temperature (RT) in air until ICSI. The penile ejaculation procedure has been approved by the IACUC at Emory University.

Fertilization by ICSI

A single spermatozoon was aspirated tail-first into an injection needle from the sperm–polyvinylpyrrolidone (PVP) to the oocyte-containing drop. Oocytes were immobilized with the polar body at the 12 o'clock position and the injection needle was inserted through the zona into the cytoplasm at the 3 o'clock position. The oolemma was breached by gentle cytoplasmic aspiration and the spermatozoon was then released into the oocyte. Oocytes were then transferred to HECM-9 NHP (non-human primate) embryo culture medium (Zheng *et al.*, 2001) at 37°C in a humidified atmosphere of 5% CO₂, 5% O₂ and 90% N₂ until the assessment of fertilization.

Assessment of successful fertilization and in-vitro embryo culture

Fertilization was assessed up to 16 h post-insemination by the appearance of pronuclei. Fertilized oocytes were cultured in HECM-9 medium without FBS until the 4- to 8-cell stage when this was replaced by HECM-9 supplemented with 5% FBS (HyClone Laboratories, Inc.).

Egg surface labelling with fluorescent beads

Rhesus macaque oocytes fertilized by ICSI were used for the labelling of the point of sperm microinjection. Fluorescent (fluorescein-labelled) beads (FB; 2–3 µm diameter; Polysciences, Inc., USA) were placed in TALP-HEPES medium containing 350 µm⁻¹ phytohaemagglutinin (PHA; Sigma) and then transferred to the micromanipulation chamber to allow them to settle on the bottom of the dish for about 30 min. The technique has been modified from previous work with mouse embryos (Piotrowska and Zernicka-Goetz, 2001). Briefly, individual beads were attached to the tip of the ICSI micropipette, introduced through the zona pellucida and placed in contact with the egg's membrane at the exact position of the previously injected spermatozoon. Once the bead adhered, the micropipette was withdrawn. For the control rhesus macaque oocytes, the FB was placed on the egg's surface 90 degrees away from the point of sperm injection, at the same focal plane.

Statistical analysis

All data were subjected to a chi-squared analysis of Statistical Analysis System (SAS, USA), for multiple comparisons. Differences of $P < 0.05$ were considered statistically significant.

Results

To perform this study, the conditions for ICSI and in-vitro culture conditions of the rhesus macaque embryos were first optimized. Among 363 ICSI-derived embryos, 310 formed a male and a female pronucleus, of which 85 reached the blastocyst stage (**Figure 1a**; 27%). The developmental rate was comparable to other reports (Schramm and Bavister, 1996; Hewitson *et al.*, 1998). To determine the exact times of early cleavage divisions following ICSI, the development of 165 embryos was observed and recorded from the moment of fertilization until late blastocyst (**Figure 1b**). The first appearance of the male and female pronuclei was observed at an average of 5 h after fertilization, while the first cleavage division began at 20 h after fertilization (**Figure 1b**, **Table 1**). The establishment of a developmental timeline of rhesus macaque embryos allowed identification of embryos at specific stages in future experiments.

Whether the actual site of sperm entry during natural fertilization is simply random or dictated by the egg–sperm interactions is largely unknown. On the other hand, the SEP in ICSI is primarily determined by the manipulator. There are no studies to date regarding sperm entry in human oocytes and its influence on embryonic development. Due to the lack of a fertilization cone, the SEP in naturally fertilized monkey embryos could not be identified. Therefore, the SEP of ICSI fertilized embryos was defined as the point at which the injection pipette entered the oocyte. It cannot be assumed that the SEP during natural fertilization is equivalent to the site of sperm injection during ICSI, although it has been shown that mechanical penetration of mammalian oocytes during ICSI triggers fertilization events such as the cortical reaction and subsequent calcium oscillations that are similar to those observed during natural fertilization (Tesarik *et al.*, 1994; Yanagida *et al.*, 2001; Williams, 2002). Sperm entry was followed in order to examine the relationship between the first cleavage plane and the SEP in monkeys. To label the SEP, a PHA-coated fluorescent bead was used. Immediately following ICSI, the bead was introduced under the zona pellucida and placed in contact with the plasma membrane where the pipette penetrated through the oolemma (**Figure 2a**, a–e). The bead remained at its position throughout the preimplantation stages of development (**Figure 2a**, f–j). Development to blastocyst stage was not affected by labelling, with the percentage of SEP-marked embryos reaching blastocyst (29%, 23 out of 79) similar to the control group (27%, 85 out of 310; **Figure 1a**). Some embryos had additional beads trapped in the zona while the egg membrane was labelled. Such double-labelled embryos when observed at the 2-cell ($n = 11$; **Figure 3a**) and the blastocyst stage ($n = 5$; **Figure 3b**), had both beads located at close proximity if not next to each other, which suggests that the bead did not dissociate from the membrane and remained close to the bead located in the zona pellucida. The data in the article is based on the single bead labelled embryos. However, the break in the zona pellucida left after ICSI and the SEP labelling (**Figure 3b**) also served as a point of reference during detailed embryo observation. This break was visible under the objective $\times 40$ when each embryo was rotated to observe the bead position shortly after labelling and later at the 2-cell stage. Although no detailed recording was made, such labelled embryos showed the bead and break

in the zona in close vicinity, and no bead dissociation from the membrane or any pulling of the bead into the cleavage furrow was observed.

To define whether there is any tendency for an ICSI-defined SEP to lie in a particular region of a cleaving embryo, the development of 107 ICSI-derived zygotes was followed with the SEP labelled by a fluorescent bead. Three equal sectors on the surface of 2-cell embryos were defined: central, middle and lateral (**Figure 2b**). The distribution of the bead was significantly different from random expectation ($P < 0.001$; **Figure 3a**). In 71% of embryos ($n = 76/107$) the SEP was at or in close proximity to the cleavage furrow (central), whereas the middle and lateral sectors were labelled in only 15% ($n = 16/107$) and 14% ($n = 15/107$) of embryos respectively (**Figure 4a**, c). The results showed a strong tendency for the SEP orienting the first cleavage plane in ICSI-derived monkey embryos, which is consistent with previous studies on mouse embryos fertilized *in vivo* (Piotrowska and Zernicka-Goetz, 2001; Plusa *et al.*, 2002a; Gray *et al.*, 2004). To determine whether the bead tends to be located at the central sector without any relationship to sperm entry, a set of control experiments was designated in which the fluorescent bead was placed approximately 90° away from the SEP and PB (**Figure 2b**). The resulting 2-cell stage monkey embryos were analysed for the position of the bead as described above. There was no overt tendency for the bead to be positioned in any particular sector when it was not placed at the SEP (**Figure 4a**, d). This study strongly suggested a predisposition for the SEP to be localized in the central region near the cleavage plane in the rhesus macaque 2-cell embryo.

In naturally fertilized mouse embryos, the SEP tends to be located at the first cleavage plane and later at the boundary between the embryonic (Em) and abembryonic (Ab) regions of the blastocyst (Piotrowska and Zernicka-Goetz, 2001). Thus, the location of the SEP marker at this boundary indicates that the position of the first cleavage plane separates the future Em/Ab parts of the blastocyst. Because of the importance of this finding in the establishment of the Em–Ab axis, there was interest in whether the monkey SEP set up by ICSI shares this predisposition and lies within a particular region of the blastocyst. ICSI-derived embryos with the SEP labelled by a fluorescent bead were allowed to develop to blastocyst to determine the distribution of the SEP along the Em–Ab boundary. The SEP marker was scored at the blastocyst stage as lying within either of the three areas: (i) the embryonic (Em) part; (ii) abembryonic (Ab) part; and (iii) the medial part, which was defined as an equatorial zone of four bead diameters in breadth (**Figure 2b**). Of 23 blastocysts that developed, 17 showed the bead located in the medial part (**Figure 4b**, c). These findings revealed that the distribution of the SEP along the Em–Ab boundary of the ICSI-derived rhesus macaque blastocyst is not a random event ($P < 0.02$). This is consistent with studies of the SEP in naturally fertilized mouse embryos (Piotrowska and Zernicka-Goetz, 2001). To be sure that the position of the SEP marker in the medial area of the blastocyst was not determined by the bead itself, control experiments were carried out in which the bead was placed not on the SEP but approximately 90° away from the place of sperm injection (**Figure 2b**). No specific allocation of the bead was found in

the medial region of the blastocyst (**Figure 4b, d**). In such designed control embryos the bead that was placed away from the sperm entry could still relate to the SEP, since it was located at a certain position on the egg surface. This might explain why several embryos displayed the marker in the Ab region (**Figure 4b**).

Finally, control embryos and SEP-labelled embryos were analysed in terms of whether the bead position localized within three defined zones at the 2-cell stage was predominantly associated with any part of the rhesus macaque blastocyst. For ICSI-SEP (**Table 2**), in the group where the SEP was in the central sector at the 2-cell stage, 16 out of 17 blastocysts (94%) had the SEP in the boundary zone. This indicates a strong relationship between the SEP, the plane of the first cleavage and the Em–Ab boundary of the blastocyst. For ICSI-SEP control (**Table 3**) in the group where the bead was in the central sector at the 2-cell stage, an equal number of embryos had the bead within three defined parts of the blastocyst. Thus, embryos with a bead placed at a distance to the SEP did not show any tendency for the bead to be localized at the Em–Ab boundary.

Discussion

Here the SEP specified by the injected spermatozoa was followed during the development of rhesus macaque embryos resulting from ICSI. The SEP was mostly localized near to the first cleavage plane and was mainly distributed along the boundary zone that separates the embryonic and abembryonic parts of the monkey blastocyst. It is still unclear whether the observed cleavage patterns in the rhesus macaque embryos are universal or influenced by the ICSI procedure. ICSI alone was not compared with conventional IVF or natural fertilization due to the lack of a fertilization cone (the structure formed around the site of sperm entry) and therefore no possibility of identifying the SEP. However the ICSI data, when compared with naturally fertilized mouse embryos, showed a similar outcome in terms of early axis specification, whether the sperm entered the egg by the standard method of ICSI or during natural fertilization (Piotrowska and Zernicka-Goetz, 2001; Plusa *et al.*, 2002a; Gray *et al.*, 2004).

It is also not known if there is any mechanism for the determination of cleavage furrows in primates or how this could relate to the site of ICSI sperm injection. The anterior end

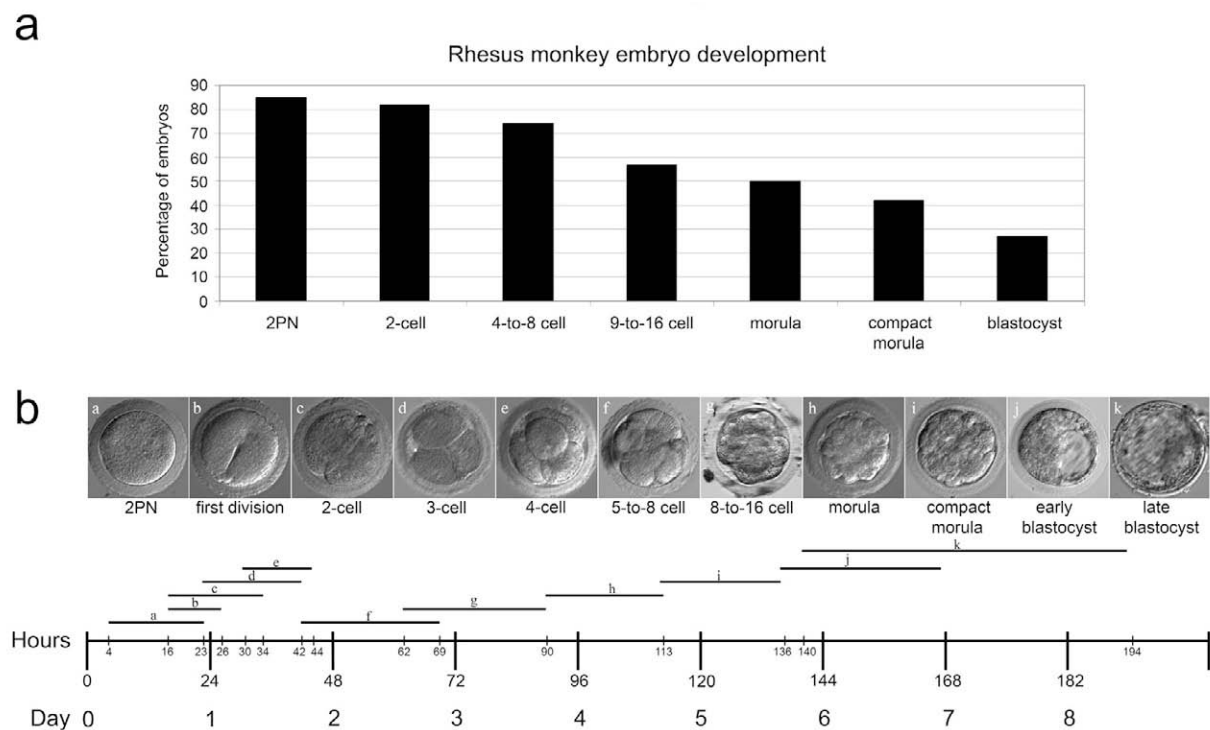


Figure 1. Development and timeline of intracytoplasmic sperm injection (ICSI)-derived rhesus macaque embryos. **(a)** Developmental rate of ICSI embryos. An average of 23 metaphase II oocytes per collection were fertilized by ICSI. Of these, 85% of embryos successfully formed two pronuclei and 27% reached the blastocyst stage. 2PN = two-pronucleate. **(b)** Timeline of ICSI embryo development. Development of rhesus macaque embryos from the time of sperm injection until late blastocyst stage was recorded. Images a–k correspond to the bars above the time line, representing the time interval during which a respective stage of embryos was observed. a = 2PN; b = first division; c = 2-cell; d = 3-cell; e = 4-cell; f = 5- to 8-cell; g = 8- to 16-cell; h = morula; i = compacted morula; j = early blastocyst; k = late blastocyst.

Table 1. The developmental timeline of rhesus macaque embryos. Data based on the observation of 165 oocytes following intracytoplasmic sperm injection (ICSI) and in-vitro culture.

Stage of embryo	Time after ICSI (h)
2PN appearance	5 ± 0.9
First division	20 ± 3.5
Early 2-cell	22 ± 2.7
Late 2-cell	28 ± 4.9
3-cell	32 ± 6.8
4-cell	38 ± 4.8
5- to 8-cell	56 ± 11.6
8- to 16-cell	76 ± 11.3
Morula	105 ± 10.8
Compacted morula	125 ± 11.1
Early blastocyst	152 ± 12.4
Late blastocyst	164 ± 11.2

Values are mean ± SD. PN = pronucleate.

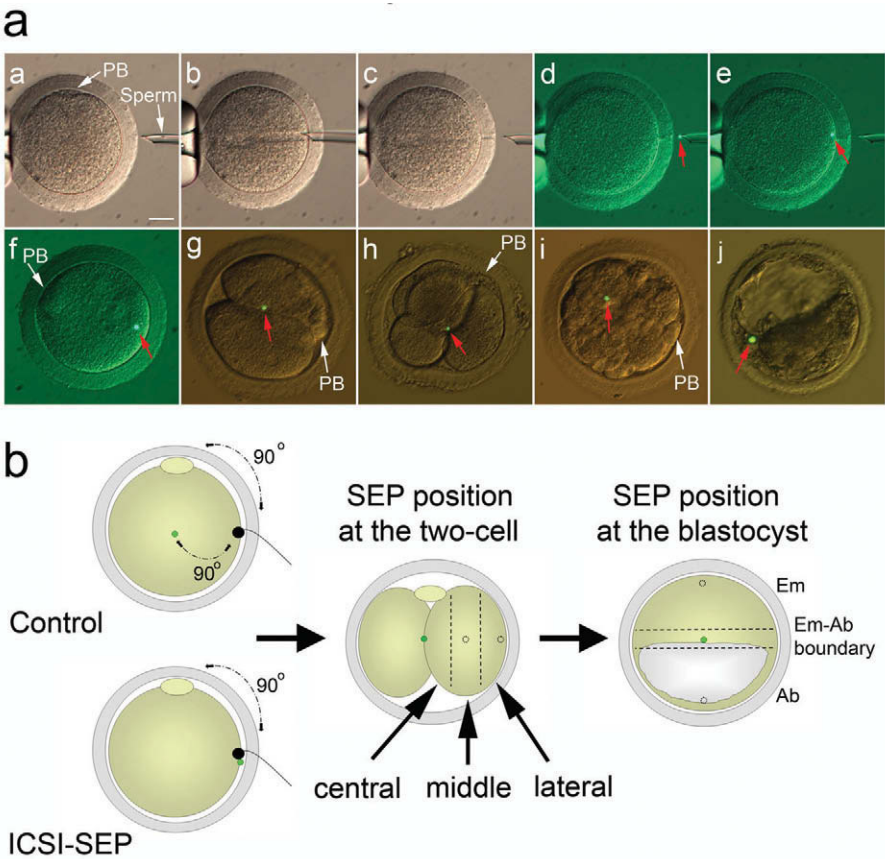


Figure 2. Labelling of the sperm entry point (SEP) in intracytoplasmic sperm injection (ICSI)-derived rhesus macaque embryos. (a) SEP was labelled with a fluorescent bead. Mature rhesus macaque oocytes were fertilized by ICSI (a–c). The position of the sperm injection was marked by a bead (d–e). The phytohaemagglutinin-coated bead remained attached to the membrane close to the male pronucleus 18 h after ICSI (f) and over the following days (g–j) until the blastocyst stage. The red arrow indicates the green fluorescent bead. PB = polar body. Scale bar: 25 μ m. (b) Two groups of ICSI-derived embryos were labelled: Control = bead placed at a point 90° from both the SEP and the PB; SEP = bead placed at the SEP. Embryos in both groups were scored according to the position of the bead in defined sectors a: (i) the 2-cell stage (equal-sized central, middle and lateral sectors) and; (ii) the blastocyst stage (embryonic (Em) and abembryonic (Ab) parts separated by an Em–Ab boundary zone).

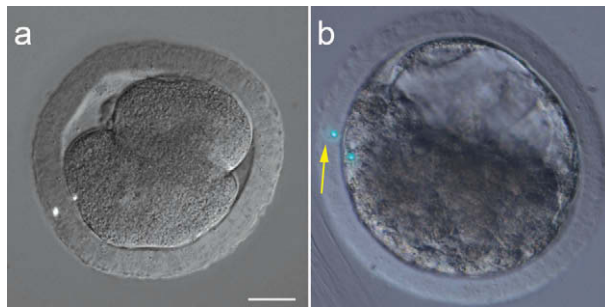


Figure 3. Intracytoplasmic sperm injection (ICSI)-derived rhesus macaque embryos labelled by the fluorescent bead at the egg membrane and co-labelled at the zona pellucida. (a) 2-cell stage; (b) blastocyst stage. Note break in the zona pellucida left after ICSI and bead labelling (b, yellow arrow). Scale bar: 20 µm.

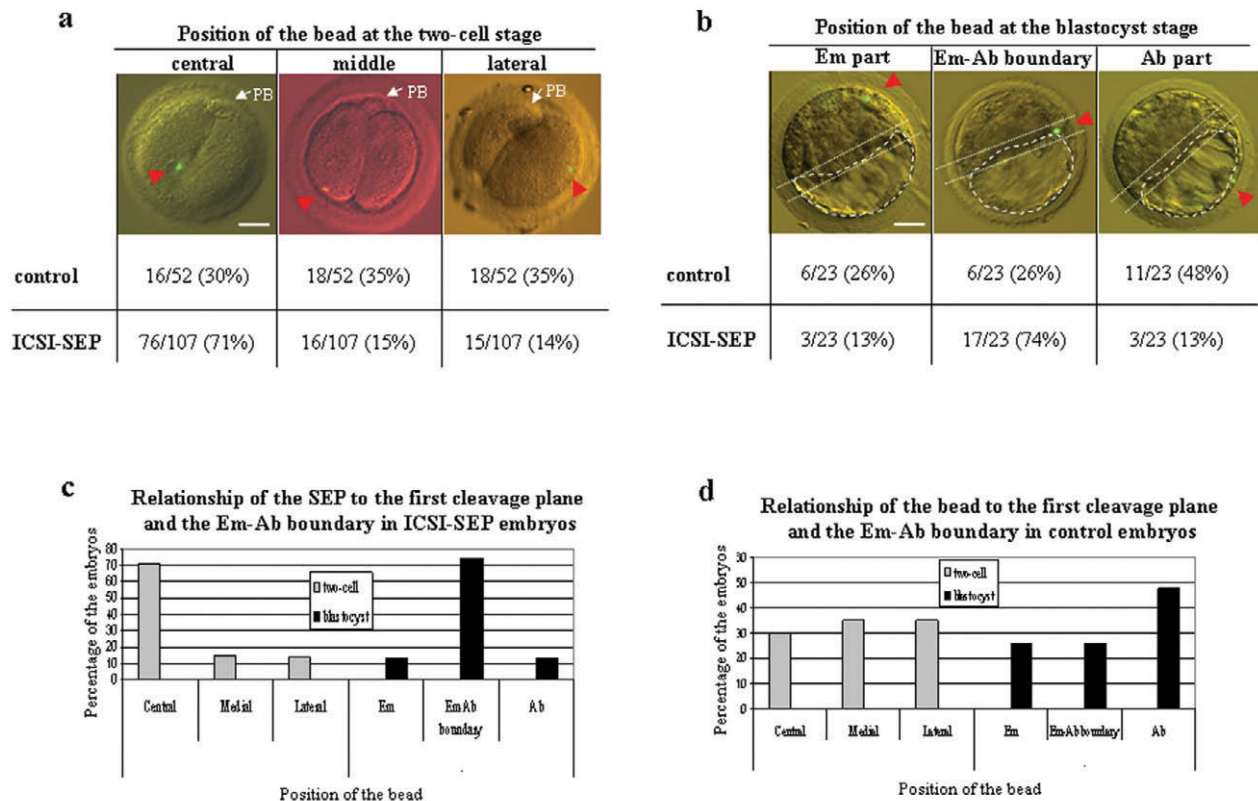


Figure 4. Relationship of the sperm entry point (SEP) to the first cleavage plane and the embryonic–abembryonic (Em–Ab) axis of the blastocyst in intracytoplasmic sperm injection (ICSI)-derived rhesus macaque embryos. (a) The distribution of the bead at the 2-cell stage in two groups (ICSI–SEP and control) of ICSI-derived embryos. In the control group the randomly placed bead was equally distributed between three defined sectors, while in the ICSI–SEP group the bead marking the SEP was predominantly localized in the central sector of the 2-cell embryo ($P < 0.001$), close to the cleavage furrow. The red arrow head indicates the green fluorescent bead. PB = polar body. Scale bar: 25 µm. (b) The distribution of the bead at the blastocyst stage in the two groups (ICSI–SEP and control) of ICSI-derived embryos. Rhesus macaque eggs were marked with beads at the SEP (ICSI–SEP) and randomly (control). At the blastocyst stage, the position of the bead was scored depending on whether it lay in the embryonic (Em), medial (Em–Ab boundary), or abembryonic (Ab) region. Representative micrographs of rhesus macaque blastocysts are shown in which the limits of the medial regions are indicated by dashed white lines. The distribution of the SEP was significantly different when compared with the control group ($P < 0.01$), showing a strong relationship between the SEP and Em–Ab boundary of the blastocyst. The red arrow head indicates the green fluorescent bead. Scale bar: 25 µm. (c) Relationship between the SEP, the first cleavage plane and the Em–Ab boundary zone of the blastocyst. The SEP was significantly differently distributed within three sectors at the 2-cell ($P < 0.001$) and the blastocyst ($P < 0.02$) stage embryo when compared with expected equal distribution within those sectors. (d) Relationship between the randomly placed bead, the first cleavage plane and the Em–Ab boundary zone of the blastocyst. The bead placed away from the SEP (control) was similarly distributed within defined sectors at the 2-cell and the blastocyst stage when compared with expected equal distribution within those sectors.

Table 2. Position of the intracytoplasmic sperm injection sperm entry point (ICSI-SEP) at the 2-cell stage according to its position at the blastocyst stage.

Position of the SEP at the blastocyst stage	Position of the SEP at the 2-cell stage			Total
	Central	Middle	Lateral	
Embryonic part	0	1	2	3
Embryonic–abembryonic boundary	16 ^a	1	0	17
Abembryonic part	1	1	1	3
Total				23

^aA SEP in the central sector of the 2-cell-stage embryo was predominantly localized to the embryonic–abembryonic (Em–Ab) boundary zone of the blastocyst, which was significantly different when compared with the total distribution (chi-squared test, $P = 0.003$).

Table 3. Position of the bead (ICSI-SEP control) at the 2-cell stage according to its position at the blastocyst stage^a.

Position of the bead at the 2-cell stage	Position of the bead at the blastocyst stage			Total
	Central	Middle	Lateral	
Embryonic part	2	2	2	6
Embryonic–abembryonic boundary	2	2	2	6
Abembryonic part	2	3	6	11
Total				23

^aA bead placed at a distance from the sperm entry point (SEP) did not show any tendency to be localized at one part of the blastocyst and the bead distribution was not significantly different when compared to total distribution (chi-squared test).

of the mouse sperm tail was not found to be related to the plane of first cleavage (Davies and Gardner, 2002), possibly for the reason that the penetrating sperm does not represent the original position of sperm entry. The SEP, defined as a position at which the naturally fertilizing spermatozoon interacts with the plasma membrane to enter the egg, has been found to be associated with the earlier-dividing 2-cell blastomere in mice, which additionally has a tendency to contribute to the embryonic hemisphere of the blastocyst (Bennett, 1982; Piotrowska and Zernicka-Goetz, 2001). Moreover, parthenogenetic embryos tend not to show the same predominance of the early dividing blastomere to the embryonic region (Piotrowska and Zernicka-Goetz, 2002). This might suggest that the SEP somehow drives the contribution of the early dividing cell toward the embryonic region. Thus, the SEP can be an important indicator of the future axial development of the embryo, because it defines a plane of cleavage that can separate the embryo into distinct halves, as well as polarizing these halves (Piotrowska *et al.*, 2001; Plusa *et al.*, 2002b; Fujimori *et al.*, 2003; Piotrowska-Nitsche and Zernicka-Goetz, 2005). However, there is current debate regarding the existence of pre-patterning and when symmetry is broken in the mouse embryo (Hiragi and Solter, 2004; Zernicka-Goetz, 2005, 2006; Lawrence and Levine, 2006; Gardner, 2007; Kurotaki *et al.*, 2007). One belief is that there is no specification of the axis in the egg and that the mouse embryo is totally symmetric with identical cells dividing randomly so the blastocyst cavity forms at a random site (Alarcon and Marikawa, 2003; Hiragi and Solter, 2004; Motosugi *et al.*, 2005). Others support the view that the orientation of the first cleavage division is related to the embryonic–abembryonic axis of the mouse blastocyst and that some differences between cells can be identified before cell

commitment (Gardner, 1997, 2001; Piotrowska and Zernicka-Goetz, 2001; Piotrowska *et al.*, 2001; Fujimori *et al.* 2003; Plusa *et al.*, 2005). Time-lapse three-dimensional study, which offers better lineage analysis versus two-dimensional observations, recently provided support for cell labelling lineage experiments that showed that in the majority of embryos individual blastomeres give rise to distinct blastocyst regions (Bischoff *et al.*, 2008).

The way in which embryos develop into blastocysts might be influenced by more than one factor, one of which could be the SEP. The suggested role of the zona pellucida as another important factor contributing to the specification of the embryonic axis (Kurotaki *et al.*, 2007), was not supported by a recent study showing that the embryonic–abembryonic axis of the mouse blastocyst develops independently of the zona pellucida (Gardner, 2007). If the zona does not influence development, perhaps the shape of the embryo could, as experimentally elongated embryos adopt their new shape and divide along their new imposed axis (Gray *et al.*, 2004; Plusa *et al.*, 2005; Gardner and Davis, 2006). In humans some gene products, like leptin and STAT3, are not randomly positioned within the ooplasm (Antczak and Van Blerkom, 1997), while the distribution of myosin IIA is restricted to the cortical region directly over the meiotic spindle in human and rhesus oocytes (Hewitson *et al.*, 1999). This polarity might prove invaluable in positioning polarized molecules and influencing cleavage axes. Moreover, another study provides evidence linking the meiotic spindle location to the first cleavage plane and resulting early embryo development parameters in human embryos (Cooke *et al.*, 2003). It is unclear how and when embryonic cells are

allocated and committed. After fertilization, an embryonic ooplasm is partitioned in the first three cell divisions by specific cleavage planes (Gulyas, 1975; Gardner, 2002; Piotrowska-Nitsche and Zernicka-Goetz, 2005a,b) that, in mice, lead to the earliest epigenetic modifications (Torres-Padilla *et al.*, 2007) and differences in pluripotency between blastomeres at the 4-cell stage that affect the embryo development (Piotrowska-Nitsche *et al.*, 2005a,b). In human blastomeres, the distribution of marker genes such as *Oct-4* and β -*HCG* indicates that their allocation follows this specific cleavage pattern (Cauffman *et al.*, 2005; Hansis 2006) and those blastomeres might differ in their potency. The nature of early blastomere differentiation in the human 4-cell embryo and its consequences for embryonic development has been discussed and analysed (for reviews, see Edwards and Beard, 1997, 1999; Hansis and Edwards, 2003; Edward and Hansis, 2005).

It is not known whether in rhesus macaques the polar body associates the cleavage plane and boundary zone at the blastocyst stage, as is the case in mice (Gardner, 1997; Piotrowska *et al.*, 2001; Gardner, 2002; Gray *et al.*, 2004; Piotrowska-Nitsche and Zernicka-Goetz, 2005; Plusa *et al.*, 2005). In primates, the ICSI-derived cleaving embryo produces small cytoplasm fragments around the polar body that makes it difficult to distinguish the polar body (Ebner *et al.*, 2003; Hnida and Ziebe, 2004). Therefore, it was not possible to link the sperm entry point to the polar body during early embryo development. Because of the importance of the polar body in embryonic axis formation in a naturally fertilized mouse embryo, it would be very interesting to study this relationship in rhesus macaques as a non-human primate model.

Whether embryos of mammals other than rodents can become asymmetric upon fertilization is unknown. A molecular link between fertilization and embryonic axis formation in *Caenorhabditis elegans* (Jenkins *et al.*, 2006) suggests that spermatozoa of other species, including humans, might play a role as a localized cue for polarity. Sperm entry might be one of the factors that influence this process. Since there are no studies to date regarding sperm entry in human oocytes and its influence on embryonic development this investigation using the rhesus macaque as a clinical model is noteworthy. The nature of embryonic axis specification in relation to assisted reproduction techniques is an interesting question of both clinical and basic importance. It is hoped that this analysis provides an extension of the other rhesus ICSI studies to date, and will encourage new directions for this field.

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