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—Original Article—

Prediction of blastocyst development and implantation potential *in utero* based on the third cleavage and compaction times in mouse pre-implantation embryos

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Abstract. Cytokinesis and cell division during pre-implantation embryonic development occur as an orchestrated spatiotemporal program. Cleavage, compaction, and blastulation in pre-implantation embryos are essential for successful implantation and pregnancy. Their alteration is associated with chromosomal imbalance and loss of developmental competence. In this study, we evaluated the time of cleavage and compaction as predictors for *in vitro* pre- and peri-implantation development and *in utero* implantation potential by time-lapse monitoring. Mouse 2-cell embryos were collected on 1.5 days *post coitum* (dpc) and were individually cultured to the outgrowth (OG) stage (7.5 dpc). Developmental stages were classified as 3-cell, 4-cell, 8-cell, morula, blastocyst, and OG. Cut-off times for successful blastocyst development were determined by receiver operating characteristic curve analysis. When cut-off times were set as 9 h for the third cleavage from the 2- to 4-cell stage, and 40 h for compaction from the 2-cell to morula stage, blastocyst and OG development rates, respectively, were significantly higher ($P < 0.0001$). Embryos were grouped according to the above cut-off time and transferred to the contralateral uterine horn on 3.5 dpc. Implantation rates *in utero* on 5.5 dpc were significantly higher in early third cleaved (≤ 9 h from 2- to 4-cell) and early compacted embryos (≤ 40 h from 2-cell to morula) than those in delayed embryos ($P < 0.05$). Therefore, the time of the third cleavage from 2- to the 4-cell stage and compaction from 2-cell to morula stage may be a useful morphokinetic parameter for predicting developmental potential, including successful implantation and pregnancy in human *in vitro* fertilization-embryo transfer programs.

Key words: Cleavage, Compaction, Cut-off time, Implantation potential, Time-lapse monitoring

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Mammalian embryos develop sequentially during pre-implantation embryogenesis, which starts with fertilization and ends with blastocyst implantation and coincides with dynamic changes in morphology and zygotic gene expression [1, 2]. Human *in vitro* fertilization-embryo transfer (IVF-ET) techniques are continuously being improved to increase implantation and pregnancy rates with better selection of transfer-competent embryos [3, 4]. Several studies have focused on identifying independent and useful predictors that can be used to select high-quality embryos in IVF laboratories [3–10]. A monitoring system to observe morphology during the course of *in vitro* pre-implantation embryonic development has been proposed [5–11]. Pre-implantation embryos with normal morphology may have chromosomal abnormalities, but can nonetheless reach the blastocyst stage. On the other hand, embryos with good morphology, which are chromosomally normal, may fail to differentiate into blastocysts [12]. To achieve a high implantation rate, transferable embryos are selected by considering various parameters beyond morphokinetics, such as the duration of oocyte activation [13], first cell division [14–17], pronuclear (PN) scoring [18], presence of evenly cleaved blastomeres [19], degree of fragmentation [20, 21], cleavage pattern [9], contraction pattern, and time to hatching [22, 23].

Cytokinesis and cell division during pre-implantation embryonic development occur as a spatiotemporally orchestrated program. [24, 25]. Generally, the time of the first cleavage division from the PN to 2-cell stage is highly variable between embryos within 22–30 h of insemination, whereas that of the second and third cleavages (from the 2- to 4-cell stage) is less variable, and can be detected 32–45 h after insemination [26]. Additionally, the compaction process involves functional changes, with expanding membrane channels serving as intracellular communication pathways [27]. The appearance of cleavage and compaction processes is the most important factor determining successful pre-implantation development [28, 29]. However, there is little information on human pre- and peri-implantation embryos *in vivo* owing to ethical restrictions. Several studies have shown that the first and second cleavage times are correlated with *in vitro* developmental competence and implantation in both humans and

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mice [9, 26, 30]. Many studies have found that transferring early cleaved embryos, rather than those exhibiting delayed development, yields higher implantation and pregnancy rates [31–33]. However, it is difficult to obtain conclusive evidence that implantation is dependent on the overall process of cleavage and compaction in pre-implantation development.

An *in vitro* outgrowth (OG) assay mimics implantation in the uterus, and enables experimental studies on implantation events and mechanisms. This assay has also revealed the relationship between metabolism based on morphokinetics of pre-implantation embryos and implantation potential [34], and has been used as an alternative tool to study the trophoblastic invasion and motility [34–37].

In this study, we showed that monitoring the cleavage and compaction times with a time-lapse imaging system was advantageous for predicting successful blastocyst development and implantation of mouse embryos *in vitro* and *in utero*. We found that the time of the third cleavage to the 4-cell stage and compaction to the morula stage was a useful morphokinetic parameter for predicting the potential of mouse pre-implantation embryos to develop into blastocysts and implant *in utero*. Our results provide important evidence that determining morphokinetic parameters with real-time monitoring could improve implantation and pregnancy rates in human IVF-ET programs.

Materials and Methods

The overall scheme is presented in Fig. 1.

Animal care and embryo collection

Inbred ICR (Institute of Cancer Research) female mice (6–8 weeks old) were induced to superovulate by intraperitoneal injection of 5 IU serum gonadotropin from a pregnant mare (Sigma, St. Louis, MO, USA) followed by injection with 5 IU human chorionic gonadotropin (hCG; Sigma) 46 h later. Superovulated female mice were mated with fertile male mice and euthanized by cervical dislocation 46 h after hCG injection. The day of vaginal plugging was designated as 0.5 days post-coitum (dpc).

Experimental animal protocols were approved by the Eulji University Institutional Animal Care and Use Committee (EUIACUC 12-19).

Embryo collection and time-lapse monitoring of embryo culture to OG stage

Mouse 2-cell embryos were collected from oviducts on 1.5 dpc and cultured in pre-warmed Quinn's Advantage blastocyst medium (SAGE/Origio, Måløv, Denmark) containing serum protein substitute (SAGE/Origio) in an incubator (Sanyo, Osaka, Japan) at 37°C and 5% CO_2. The culture medium was replaced with Dulbecco's modified Eagle medium (Gibco/Life Technologies, Carlsbad, CA, USA) containing 10% fetal bovine serum on 4.5 dpc for culture to the OG stage. Embryogenesis from the 2-cell to the OG stage on 7.5 dpc was observed using a Primo Vision system (Vitrolife, Gothenburg, Sweden) (Fig. 2). The digital camera for the time-lapse microscope was set to acquire a single image every 30 min for 6 days. Stages of embryo development were classified as 3-cell (3C), 4-cell (4C), 8-cell (8C), compacted morula (Mo), early blastocyst (EB), late blastocyst (LB), hatching blastocyst (HB), hatched blastocyst (Hed BL), and OG.

Grouping analysis based on the time of each development stage

Mouse 2-cell embryos were collected on 1.5 dpc and were individually cultured and monitored up to the OG stage (7.5 dpc) by time-lapse imaging. Embryos were classified into success and failure groups based on their development to the blastocyst and OG stages on 4.5 and 7.5 dpc, respectively. Mean cumulative time and time interval between cell divisions was compared between embryos of the success and failure groups, whether or not they developed to the blastocyst and OG stages. Blastocyst development and OG rates were compared with respect to cut-off times determined by the receiver operating characteristic (ROC) curve analysis.

ROC curve analysis

ROC curves were generated using the ROCR library of the R programming language. Linear models were generated using the lm function of R. Area under the ROC curve (AUC) was estimated to assess the ability of sensitivity and specificity (%) to predict development to blastocyst and OG stages; that is, a greater AUC signified superior predictive capacity of the model.

Quantitative analysis of mRNA expression in embryos

We collected cleavage stage embryos (3C, 4C, 8C, and Mo) for quantitative real-time (RT)-PCR analysis. Total mRNA (10 µl) was isolated from five embryos, using an mRNA Direct kit (Dynal Biotech ASA, Oslo, Norway), and 80 µl cDNA was synthesized from 2 µl total mRNA using the PrimeScript 1st strand cDNA Synthesis kit (Takara Bio, Otsu, Japan). A fraction of the reverse-transcribed product (2 µl) was used directly for quantitative RT-PCR, which was carried out in a final reaction volume of 20 µl with SYBR Green (Applied Biosystems, Foster City, CA, USA) and primers amplifying cytokinesis-related genes such as the actin-binding protein anillin (*Anln*) and Ras homolog gene family member A (*RhoA*) and pluripotency markers such as octamer-binding transcription factor (*Oct*)-*4* and caudal type homeobox (*Cdx*)-*2* (Table 1). The internal standard for normalization was *16S rRNA*. Expression levels were determined using a modified version of the $2^{-\Delta\Delta Ct}$ method [38]. Experiments were performed at least three times.

Analysis of in utero implantation after embryo transfer

Implantation rates after embryo transfer were assessed within the cut-off times described above. Embryos were divided into early and delayed groups according to the third cleavage at 9 h from 2C as the 3C and 4C groups, and into another group according to compaction at 40 h from 2C as the 8C and Mo groups. Embryos were transferred on 3.5 dpc into the contralateral uterine horn of pseudopregnant female mice obtained by mating with a vasectomized male. After embryo transfer 2 days later on 5.5 dpc, implantation sites in the uterine horns of pregnant mice were detected by intravenous injection of Chicago Blue dye. Clear blue bands *in utero* were considered sites of successful implantation.

Statistical analysis

All experiments were performed at least in triplicate. The statistical

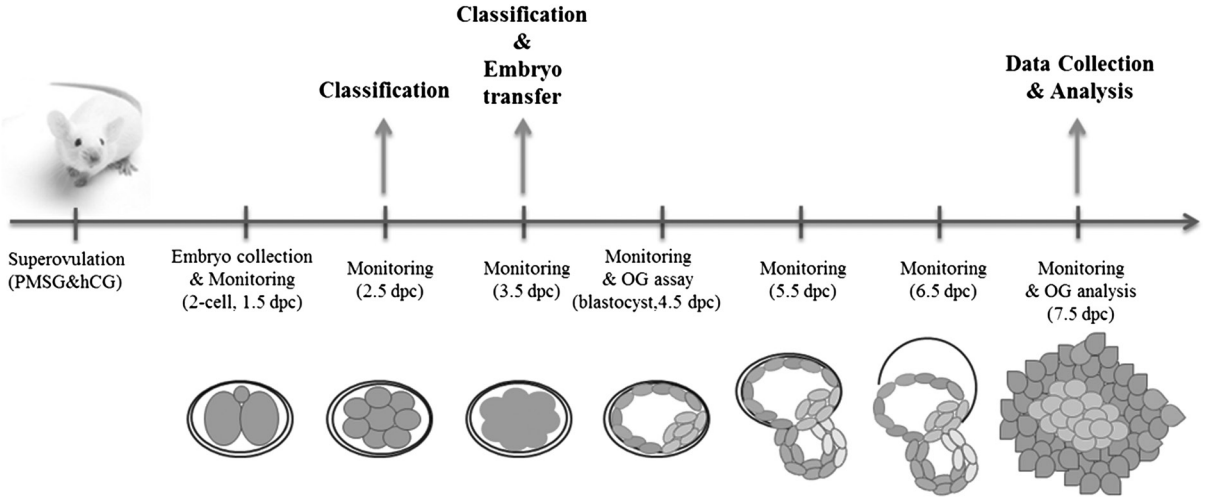

Fig. 1. Schematic illustration of the experiment for investigating the relationship between cleavage and compaction timings and developmental competence. Mouse 2-cell embryos were cultured to blastocyst and outgrowth stages, and embryonic development was monitored for 7 days (from 1.5 to 7.5 dpc). Data were collected at the end of monitoring (7.5 dpc). Classification, embryo transfer, and outgrowth assay were performed. dpc, days *post coitum.*

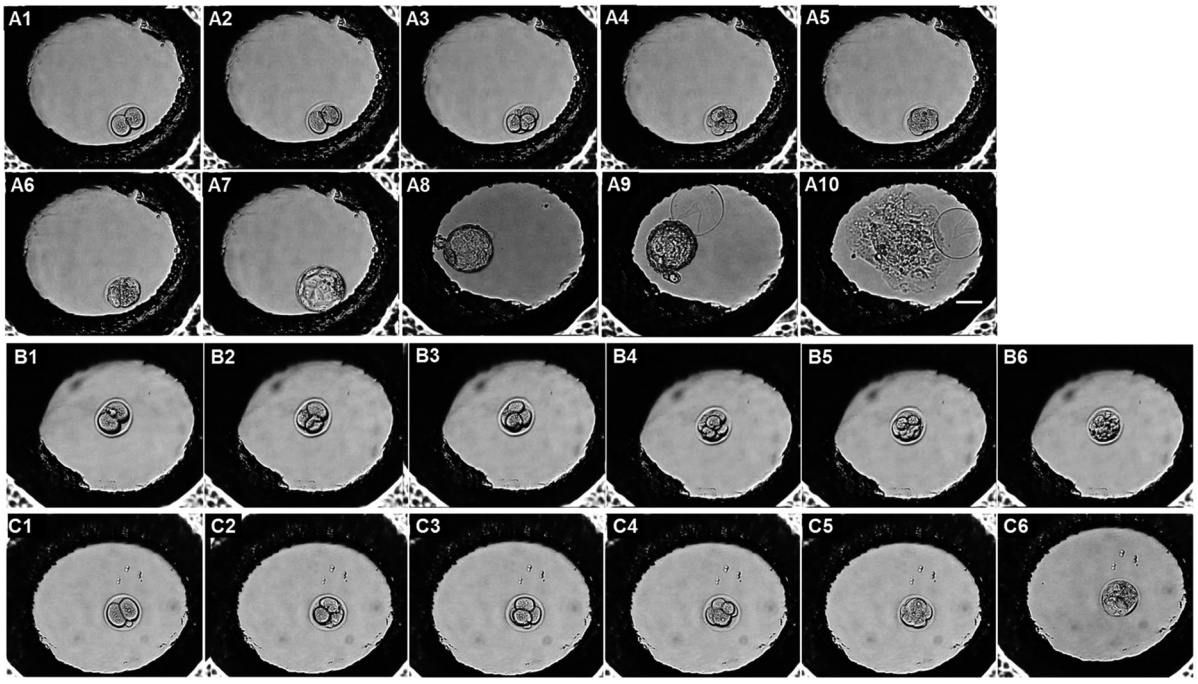

Fig. 2. Time-lapse monitoring of embryos during development. Mouse 2-cell embryos from 1.5 dpc were cultured to the outgrowth (OG) stage (7.5 dpc) in a Primo Vision monitoring system, with images captured every 30 min. (A1–10) The developmental stages were 2-cell, 3-cell, 4-cell, 8-cell, morula, early blastocyst, late blastocyst, hatching blastocyst, hatched blastocyst, and OG. (B, C) Abnormal patterns of embryo development. (B1–6) Embryo arrested at the cleavage stage. (C1–6) Embryo that failed to undergo OG expansion. dpc, days *post coitum.*

significance of two-group comparisons was evaluated by the Student's *t* test or Fisher's exact test, whereas for multiple comparisons, one-way analysis of variance or a χ^2 test was performed. P values < 0.05 were considered statistically significant.

Results

Differences in mean times of each developmental stage are related to the potential for development to the blastocyst stage

Mouse 2-cell embryos were cultured to the OG stage for 6 days

Table 1. List of primers used for quantiative RT-PCR and expected product sizes

Gene	GenBank accession number	Primer sequences (5'–3')	Product size (bp)
Anln	NM_028390.3	F: TGGGGCTGAGCAGATGGTCG	274
		R: TCCGGGACTGGCCATAACTGAAGA	
RhoA	NM_016802.4	F: CATTGACAGCCCTGATAGTT	120
		R: TCGTCATTCCGAAGGTCCTT	
Oct-4	NM_013633.3	F: TCAGGTTGGACTGGGCCTAGT	100
		R: GGAGGTTCCCTCTGAGTTGCTT	
Cdx-2	NM_007673.3	F: CGCAGAACTTTGTCAGTCCTCCGCAGTACC	254
		R: GTATTCGGCGGGGCTGCTGTAGCCCATAGC	
16S rRNA	XM_003688749.3	F: AGATGATGCAGCCGCGC	163
		R: GCTACCAGGGCCTTTGAGATGGA	

Table 2. Mean times for each developmental stage with respect to developmental potential to blastocyst and outgrowth stages

Blastocyst development		Success (149/171)	Failure (22/171)	Blastocyst development		Success (149/171)	Failure (22/171)
		Cumulative time (h)				Time interval (h)	
From 2C to	3C	6.2 ± 0.3 [a]	11.1 ± 0.7 [d]	From	2C to 3C	6.2 ± 0.3 [a]	11.1 ± 0.7 [d]
	4C	7.5 ± 0.3 [a]	13.6 ± 0.9 [d]		3C to 4C	1.3 ± 0.1 [a]	2.5 ± 0.5 [d]
	8C	21.4 ± 0.4 [a]	33.8 ± 3.0 [d]		4C to 8C	13.9 ± 0.3 [a]	21.1 ± 2.2 [d]
	Mo	31.3 ± 0.5 [a]	52.8 ± 6.8 [d]		8C to Mo	10.0 ± 0.4 [a]	18.8 ± 4.6 [d]
	EB	51.7 ± 0.9 [a]	68.5 ± 20.0 [b]		Mo to EB	20.3 ± 0.7 [a]	21.0 ± 3.0 [a]
Outgrowth development		Success (95/160)	Failure (65/160)	Outgrowth development		Success (95/160)	Failure (65/160)
		Cumulative time (h)				Time interval (h)	
From 2C to	3C	5.8 ± 0.3 [a]	8.0 ± 0.5 [d]	From	2C to 3C	5.8 ± 0.3 [a]	8.0 ± 0.5 [d]
	4C	6.9 ± 0.3 [a]	9.8 ± 0.5 [d]		3C to 4C	1.1 ± 0.1 [a]	1.8 ± 0.2 [c]
	8C	20.0 ± 0.5 [a]	25.0 ± 0.9 [d]		4C to 8C	13.1 ± 0.3 [a]	16.0 ± 0.6 [d]
	Mo	30.7 ± 0.6 [a]	34.6 ± 1.3 [c]		8C to Mo	10.6 ± 0.5 [a]	10.0 ± 0.8 [a]
	EB	48.3 ± 0.7 [a]	57.3 ± 1.8 [d]		Mo to EB	17.7 ± 0.6 [a]	24.3 ± 1.4 [d]
	LB	59.2 ± 0.9 [a]	66.6 ± 2.5 [d]		EB to LB	10.8 ± 0.5 [a]	12.4 ± 1.8 [a]
	HB	71.3 ± 1.5 [a]	72.6 ± 3.1 [a]		LB to HB	12.9 ± 1.0 [a]	11.4 ± 1.9 [a]
	Hed BL	92.3 ± 1.5 [a]	95.0 ± 4.8 [a]		HB to Hed BL	21.1 ± 1.8 [a]	28.5 ± 5.1 [a]

Data are shown as mean ± SEM. 2C, 2-cell; 3C, 3-cell; 4C, 4-cell; 8C, 8-cell; Mo, morula; EB, early blastocyst; LB, late blastocyst; HB, hatching blastocyst; Hed BL, hatched blastocyst. [a,b,c,d] Different superscripts indicate statistically significant differences ([b] $P < 0.05$; [c] $P < 0.01$; [d] $P < 0.001$).

(from 1.5 to 7.5 dpc), and developmental stages were classified as 3C, 4C, 8C, Mo, EB, LB, HB, Hed BL, and OG, as shown in Fig. 2. Cultured embryos developed to blastocyst (87.1%, 149/171) and OG (53.2%, 91/171) stages. Mean cumulative times of each stage were shorter in embryos of the group with successful blastocyst development as compared to that in the failure group (Table 2). Mean time intervals between cell divisions to the blastocyst stage, except for Mo and EB, were significantly shorter than those in the failure group ($P < 0.001$).

Differences in mean times of each developmental stage are related to the potential for development to the OG stage

The mean cumulative times and time intervals to reach the OG stage on 7.5 dpc from each developmental stage were compared between embryos of the success and failure groups (Table 2). Mean cumulative times for each developmental stage to the blastocyst stage were significantly shorter than those for embryos in the failure group ($P < 0.01$). On the other hand, mean cumulative times after hatching were similar between the two groups ($P > 0.05$). The mean time intervals between 2C to 3C, 3C to 4C, 4C to 8C, and Mo to EB were significantly shorter than those in the group that failed to reach OG stage ($P < 0.01$).

Sensitivity and specificity of developmental potential to reach blastocyst stage determined based on the time intervals of each development stage

The cut-off time for the highest sensitivity and specificity at each developmental stage was set according to the ROC analysis. When the cut-off time was set as 9 h from 2C to 4C, sensitivity and specificity of blastocyst development were 86.4% and 72.8%,

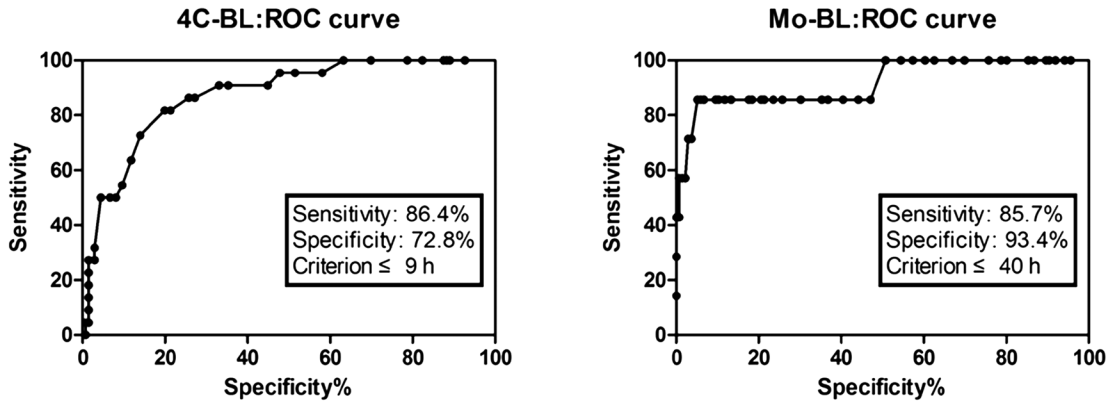

Fig. 3. ROC curve analysis of sensitivity and specificity of developmental potential to the blastocyst stage according to cut-off times from 2- to 4-cell and morula stages. Cut-off values corresponding to true-positive and false-negative rates are shown on the curve. To predict blastocyst development: AUC_{ROC} for sensitivity score = 86.4; AUC_{ROC} for specificity score = 72.8 within 9 h from 2- to 4-cell stage; AUC_{ROC} for sensitivity score = 85.7; AUC_{ROC} for specificity score = 93.4 within 40 h from 2-cell to morula stage.

Table 3. Developmental potential to the blastocyst and outgrowth stages according to cut-off times from 2-to 4-cell and morula stages

Development to the blastocyst stage					
		Blastocyst rate (n)			Blastocyst rate (n)
Cut-off time (2C to 4C)	≤ 9 h	97.3% [a] (109/112)	Cut-off time (2C to Mo)	≤ 40 h	89.5% [a] (136/152)
	> 9 h	67.8% [b] (40/59)		> 40 h	68.4% [b] (13/19)
Development to the outgrowth stage					
		Outgrowth rate (n)			Outgrowth rate (n)
Cut-off time (2C to 4C)	≤ 9 h	66.1% [a] (74/112)	Cut-off time (2C to Mo)	≤ 40 h	57.2% [a] (87/152)
	> 9 h	32.2% [b] (19/59)		> 40 h	31.6% [b] (6/19)

2C, 2-cell; 4C, 4-cell; Mo, morula. [a,b] Different superscripts indicate statistically significant differences ([b] $P < 0.001$).

respectively. A cut-off time of 40 h from 2C to Mo showed the highest sensitivity and specificity of blastocyst development, 85.7% and 93.4%, respectively (Fig. 3).

Prediction of potential for developing to the blastocyst stage within 9 and 40 h of the set cut-off time

All monitored embryos were grouped according to the above cut-off times of 9 h and 40 h. We found that 97.3% (109/112) of embryos taking ≤ 9 h from 2C to 4C developed into blastocysts, whereas only 67.8% (40/59) of those taking > 9 h from 2C to 4C successfully developed ($P < 0.001$). In addition, 89.5% (136/152) of embryos taking ≤ 40 h from 2C to Mo developed to blastocysts whereas 68.4% (13/19) of those taking > 40 h successfully developed ($P < 0.001$, Table 3). When applying the above cut-off times, OG rates were significantly higher in early cleaved and compacted embryos than in delayed embryos (Table 3).

Gene expression within the cut-off times for cleavage and compaction events

The mRNAs levels of the cytokinesis-related genes *Anln* and *RhoA*, the pluripotency marker *Oct-4,* and the trophoblast marker *Cdx-2* were determined by quantitative RT-PCR. Embryos were divided into two groups according to the cut-off times of 9 h and 40 h from 2C to 4C and Mo, respectively (Fig. 4). Embryos taking ≤ 9 h from 2C to 4C showed lower *Anln* expression than those taking > 9 h from 2C to 4C. In addition, embryos that underwent compaction within 40 h from 2C to Mo expressed a lower level of *Anln* mRNA than those that compacted after > 40 h from 2C to Mo ($P < 0.01$, Fig. 5). There were no differences in other genes among groups.

Enhanced implantation rates in utero based on cut-off times of third cleavage and compaction events

Embryos were divided into two groups (from 2C to 3C or 4C at 9 h) and two other groups (from 2C to 8C or Mo at 40 h) based on ROC curve analysis. On 3.5 dpc, embryos from each group were transferred to the contralateral uterine horn of pseudopregnant females. Implantation sites were detected by intravenous injection of Chicago blue dye on 5.5 dpc. Implantation rates were higher for transferred embryos with a cut-off time of ≤ 9 h from 2C to 4C than for those with a cut-off time of > 9 h (78.6 ± 8.7% *vs*. 42.9 ± 14.0%, $P < 0.01$). In addition, implantation rates were higher for transferred embryos with a cut-off time of ≤ 40 h from 2C to Mo compared to

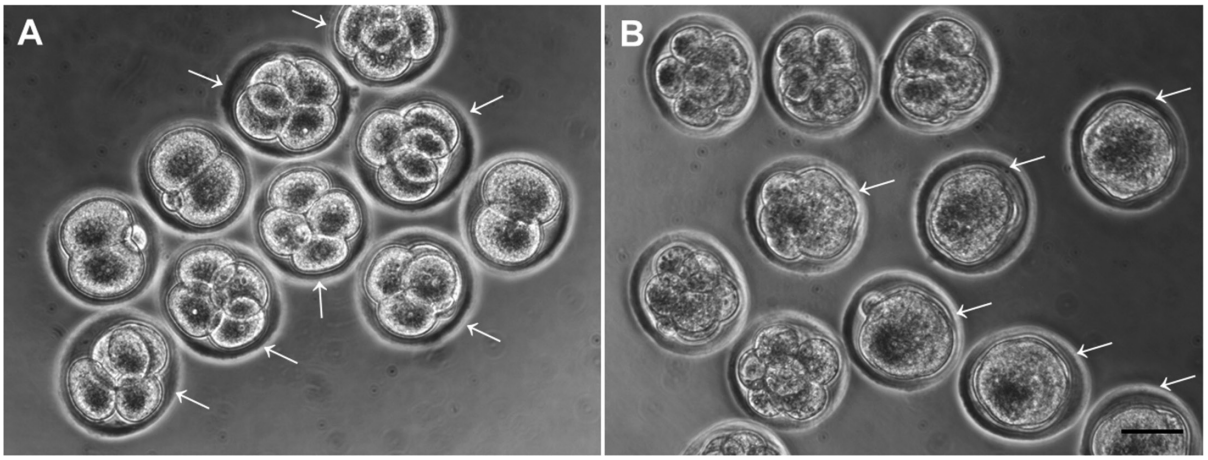

Fig. 4. Embryos at each time point of cleavage and compaction events. Mouse 2-cell embryos were cultured and classified as early-developed or delayed embryos at 9 h from 2-cell (A) and 40 h from 2-cell (B). Arrows indicate early cleaved or compacted embryos. Scale bars = 50 μm.

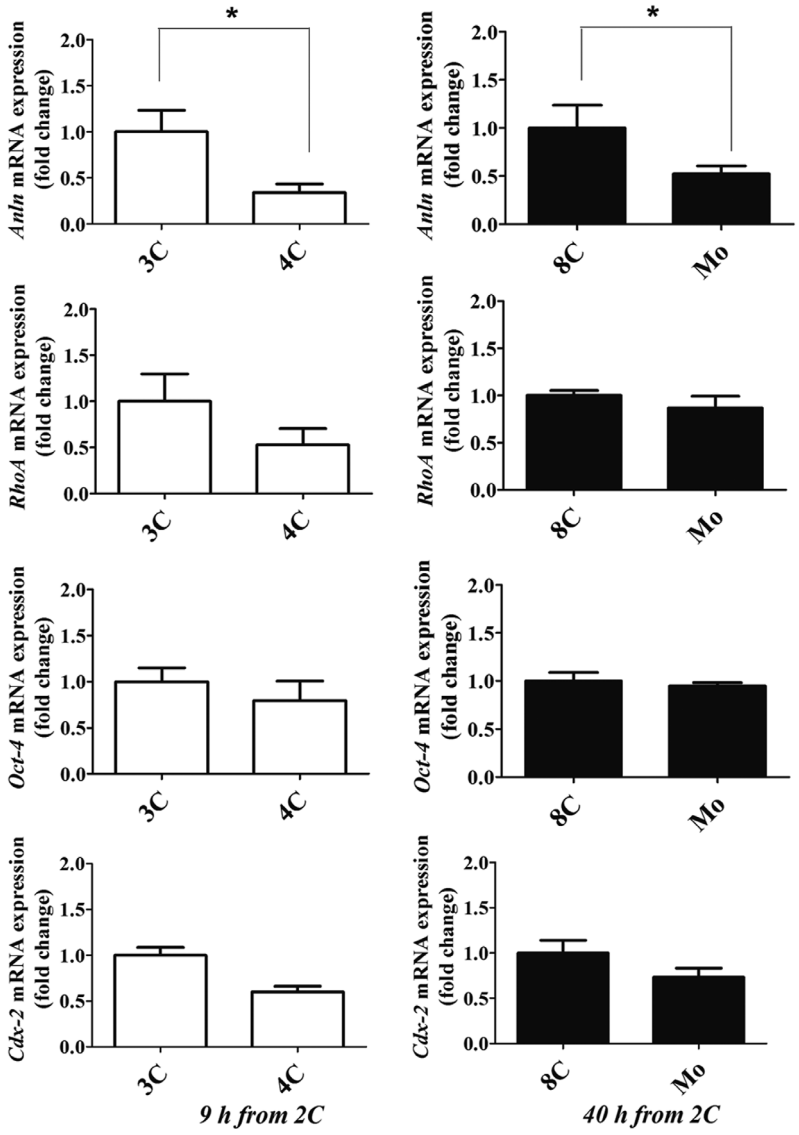

Fig. 5. Gene expression at each time point of cleavage and compaction events. Mouse 2-cell embryos were cultured and classified as 3- or 4-cell at 9 h from 2-cell, and 8-cell and morula at 40 h from 2-cell. mRNA expression of *Anln*, *RhoA*, *Oct-4*, and *Cdx-2* was compared between early and delayed embryos within each cut-off time. Data are shown as mean ± SEM. * $P < 0.05$.

Table 4. Implantation rates *in utero* after embryo transfer

Group of transferred embryos	9 h from 2C		40 h from 2C	
	3C	4C	8C	Mo
No. of transferred embryos	42	42	48	48
No. of implanted embryos (mean number)	18 (2.6 ± 0.8) [a]	33 (4.7 ± 0.5) [b]	16 (2.0 ± 0.4) [a]	26 (3.3 ± 0.6) [b]
Implantation rate	42.9 ± 14.0% [a]	78.6 ± 8.7% [b]	33.3 ± 6.3% [a]	54.2 ± 9.3% [b]

Data are shown as mean ± SEM. 2C, 2-cell; 3C, 3-cell; 4C, 4-cell; 8C. 8-cell; Mo, morula. Embryos were classified as 3C and 4C at 9 h from 2C, respectively, and as 8C and Mo at 40 h from 2C, then transferred to the contralateral uterine horn on 3.5 dpc. [a,b] Different superscripts indicate statistical differences ([b] $P < 0.05$).

those with a cut-off time of > 40 h (54.2 ± 9.3% *vs*. 33.3 ± 6.3%, $P < 0.01$) (Table 4).

Discussion

Embryo selection based on morphological grading and biochemical markers is not always associated with higher implantation rates. High-quality blastocysts lead to high pregnancy and implantation rates, but some still do not implant into the uterus; this is related to embryonic biochemical factors such as O_2 consumption [39, 40], pyruvate and glucose uptake [34, 41], lactate production [42], the secretion of factors such as platelet-activating factor and insulin-like growth factor [43], and the activity of the enzymes involved in acid and carbohydrate metabolism pathways [44, 45]. However, measuring these parameters typically requires special instruments and training, limiting their practical application. Therefore, more efficient methods for assessing embryo viability and development competence based on alternative criteria are needed.

Some researchers have proposed PN morphological scoring to select viable embryos for transfer in human assisted reproductive technology (ART) programs. PN morphology has a significant effect on embryo developmental potential *in vitro*, which therefore affects day 3 and 5 morphological scores and the ability of these embryos to develop to the blastocyst stage [18, 46]. However, one study reported that PN scoring is not correlated with implantation and pregnancy rates [47]. As such, the question of which markers are useful for selecting good-quality embryos remains controversial.

Early cleavage of pre-implantation embryos has been suggested as an additional criterion for embryo selection, as it has been found to be correlated with implantation potential and enhanced viability compared to embryos that exhibit delayed cleavage [5, 15, 48, 49]. The time of the first cleavage alters gene expression associated with implantation potential, and slowly cleaving embryos exhibit reduced viability, chromosomal abnormalities, and decreased oxygen consumption at the blastocyst stage, thereby lowering hatching and pregnancy rates [50, 51]. A higher mitochondrial DNA copy number is also associated with a higher rate of zygotic cleavage in humans and pigs [52, 53]. In addition, chromosomal imbalances, such as aneuploidy, frequently occur in delayed-cleaved embryos that comprise unequal blastomeres [19, 54]; the first cleavage from PN to 2C may be affected by intrinsic factors within the oocyte, the sperm, or both [26].

Compaction is an important event during pre-implantation embryo development, and embryos with apparently normal cleavage do not always reach morula and blastocyst stages at the appropriate time. When embryos are compacted at the 4-cell stage, they may miss the chance to develop further, which requires differentiation into the inner cell mass and trophectoderm [12]. Partial compaction is also a negative indicator of developmental competence and pregnancy rate [55]. Embryos that undergo compaction and cavitation become distinguishable and reflect embryo commitment to the next stage of development [56]. It has been reported that embryo quality on day 2 may be linked to the ability to undergo compaction on day 3 [57].

Here, we compared the mean times of cleavage and compaction between embryos that successfully reached the blastocyst stage and those that failed to do so. Embryo with shorter mean cleavage and compaction times showed increased development and higher outgrowth rates compared to embryos with longer times. These results demonstrate that embryos with early third cleavage and compaction events had higher potential for development to the blastocyst stage and consequent implantation *in vitro* and *in utero*.

ROC curve analysis is a well-established method for identifying precise diagnostic points. Sensitivity and specificity are key parameters used to determine the accuracy and efficiency of diagnostic tests [58, 59]. In the context of the present study, sensitivity was defined as the proportion of embryos that developed successfully to the blastocyst and OG stages, whereas specificity was defined as the proportion of embryos that failed to reach these stages. We predicted blastocyst developmental competence and implantation potential based on ROC analysis. Embryos that underwent third cleavage division and compaction within the cut-off time had the highest sensitivity (86.4%) and specificity (93.4%) for blastocyst development. Although we predicted a high rate of OG development, there was no meaningful data in the ROC analysis; that is, several cut-off times were unsatisfactory for establishing OG potential. Therefore, there is still room for improvement in this regard; further studies with larger sample sizes are needed to optimize specificity and sensitivity.

With the exception of *Anln* gene expression, the expression of other genes examined in the different embryo groups was similar regardless of cut-off time. *Anln* is implicated in cytoskeletal dynamics during cellularization and cytokinesis and interacts with *RhoA*, which regulates cytoskeletal dynamics, cell cycle progression, and cell transformation [60]. *Anln*, which is a maternal transcript, is highly expressed at the zygote stage, with mRNA expression declining during development to blastocyst stage [9, 61]. *Oct-4* is a key marker of the pluripotency regulatory network involved in the self-renewal of undifferentiated embryonic stem cells via a reciprocal interaction with *Cdx-2* [62]. We also analyzed other pluripotency

genes such as *Nanog*, *Sox-2*, and *c-Myc*, and found that these genes showed similar expression patterns regardless of cut-off time (data not shown). In this study, only the *Anln* level differed between embryos with early and delayed development due to the shortened cleavage interval. Cleavage and compaction times may be related to cytokinesis-related gene expression in pre-implantation embryos [24]. We did not compare the mRNA expression in early and delayed groups when they reached to same stage. Further investigation will be required to analyze mRNA expression in blastocysts from early and delayed groups.

In embryo transfer experiments, we obtained the highest implantation rate (78.6 ± 8.7%) in embryos with a cut-off time of ≤ 9 h from 2C to 4C. We also found that Mo at ≤ 40 h from 2C had higher implantation potential *in utero* compared to the potential of embryos at 8C. Our results suggest that embryos with third cleavage at 9 h and compaction at 40 h not only have higher probability of developing into blastocysts, but also higher implantation potential. It is well-known that embryos within appropriate times of the third cleavage and compaction exhibit better developmental competence than those that undergo late cleavage and delayed compaction. Indeed, transferrable 4-cell embryos with uniform blastomeres were manually selected 2 days after insemination in human IVF-ET programs [63–65].

In conclusion, our study showed that the cumulative time from 2C to 4C and from 2C to Mo predicts blastocyst development and implantation potential *in utero*. Specifically, embryos exhibiting short third cleavage intervals had a higher probability of blastocyst formation, OG, and implantation *in utero*. In addition, the compaction time was related to further embryonic developmental competence and implantation potential. Our results provide evidence that analyzing morphokinetic parameters by real-time monitoring may improve the efficacy of selection of transferrable embryos with high implantation potential in human IVF-ET programs.

Conflict of interest: The authors declare that they have no conflicts of interest.

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