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Morphokinetic analysis of development to the morula of mouse embryos fertilized *in vitro* using an incubator with time-lapse cinematography

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Abstract: The preimplantation development of mouse oocytes fertilized *in vitro* was assessed in an incubator with time-lapse cinematography to evaluate morphokinetic development with higher precision. Fast-developing embryos completed compaction at 58 h after insemination and developed to morulae, but 39% of the cultured embryos had not completed compaction at 72 h after insemination. In contrast, at 24, 48, and 96 h after insemination, most cultured embryos were observed to have developed to the 2-cell, 4-cell, and blastocyst stages, respectively. Mouse embryos cultured *in vitro* reached the 2-cell stage at 18 h, the 4-cell stage at 41 h, initiated compaction at 59 h, and formed morulae at 70 h, and blastocysts at 87 h after insemination on average. Interestingly, compacting embryos during development to the morula showed a loosening of compaction due to an increase in the number of blastomeres accompanying cell division. Compaction partially regressed with cell division in compacting embryos, making judgement of the completion of compaction dependent on the timing of observation. Observations at conventional 24-h intervals misjudged the development to the morula. Determination of mouse embryonic development 72 h after insemination may be assessing compacting and compacted morula rather than compacted morula.

Key words: Mice, Compaction, Morula, *In vitro* fertilization, Time-lapse cinematography

Introduction

Since the first successful *in vitro* fertilization (IVF) of mice in 1968 using uterine spermatozoa collected after mating [1], the components of the culture media, the culture condition, and the monitoring system of the embryos being cultured as well as the procedures of IVF have been substantially modified and improved.

In 1969, successful mouse IVF with epididymal spermatozoa in the presence of bovine follicular fluid was reported by Iwamatsu and Chang [2]. In 1971, Toyoda *et al.* [3] established an IVF method for mouse eggs with epididymal spermatozoa in a modified Krebs-Ringer bicarbonate solution supplemented with glucose, Na-pyruvate, and bovine serum albumin (BSA) which had higher fertilization rates and reproducibility. Their study demonstrated that mouse spermatozoa can be capacitated *in vitro* in the chemically defined Toyoda, Yokoyama and Hosi (TYH) medium without fluids from the female reproductive tract. To date, TYH medium has been extensively used as a standard medium for IVF in mice as well as other species of mammals.

Fertilized mouse eggs derived from certain inbred strains or F1 hybrids can be cultured throughout the preimplantation period in a modified Krebs-Ringer bicarbonate solution supplemented with glucose, Na-pyruvate, Na-lactate, and BSA *in vitro*. However, the majority of fertilized mouse eggs derived from random bred strains, such as ICR, arrest their development at the two-cell stage [4]. This phenomenon, called *in vitro* two-cell block, can be overcome by the addition of EDTA-2Na to the culture medium [5–7]. In addition, the development of normal progeny has been confirmed by the subsequent transfer of IVF embryos cultured throughout the preimplantation stage in Whitten medium [4] supplemented with EDTA-2Na [8]. Subsequently, culture media for mouse preimplantation development of embryos have been developed. Chatot *et al.* [9] developed Chatot, Ziomek, and Bavister (CZB) medium which has

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an increased lactate/pyruvate ratio, and contains EDTA and glutamine, but lacks glucose and phosphate. When cultured in this medium, fertilized mouse eggs develop beyond the 2-cell stage and reach the morula but not the blastocyst stage. However, washing embryos in CZB medium containing glucose after 48 h of culture allows their development to the blastocyst stage [9]. The presence of glucose in CZB medium is only necessary from the 4-cell stage; it is detrimental to development in earlier stage mice embryos. Subsequently, the components of CZB were modified by sequential simplex optimization methods to design a medium (SOM) that overcomes the two-cell block in an outbred strain of mouse [10]. A modification of the SOM medium, designated KSOM, since it has an increased concentration of potassium, supports development beyond the 2-cell stage and provides a larger yield of blastocysts in outbred strains of mice [11, 12]. Moreover, KSOM was further modified to $mKSOM^{AA}$ which has an increased concentration of glucose and an additional 19 amino acids [13].

Culture conditions, such as temperature and atmosphere, may also be important factors for the successful preimplantation development of embryos *in vitro* as well as in culture media. *In vitro* mouse embryos are typically cultured in an incubator under an atmosphere of 5% CO_2 in air with saturated humidity at 37°C. The optimum oxygen tension for culturing mammalian embryos has been widely debated by the scientific community. While several laboratories have moved to using 5% as the value for oxygen tension, many modern IVF laboratories still use 20% [14].

Frequent opening and shutting of a CO_2 incubator for microscopic observation of the embryos being cultured might disturb the culture conditions. A CO_2 incubator equipped with a time-lapse monitoring system is a closed system of culture which allows observation of embryos without exposure to the atmosphere [15]. Time-lapse observation has also advanced our understanding of the morphologic mechanisms of the fertilization, development, and behavior of early embryos during the preimplantation period. Previous time-lapse observation studies have investigated pronuclear formation [15, 16], timing of cleavage [16, 17], fragmentation [18–21], compaction [22–27], blastocoel formation [26, 28], and the shape of the inner cell mass [29] in human zygotes/embryos. Embryos that cleave early at the first cleavage have a higher potential for subsequent pre- and post-implantation development [17, 30–32].

Recently, a time-lapse monitoring system has been used to evaluate mouse embryonic development *in vitro*. The first and second cleavages of mouse oocytes fertilized *in vivo* significantly influence the probability of reaching the blastocyst stage in culture [33]. The shorter the time of cleavage to the 2-cell stage, and from the 2-cell to 3-cell stage, the higher is the chance of embryo development to the blastocyst stage [33]. When the time of cleavage and compaction of mouse 2-cell embryos fertilized *in vivo* was evaluated by time-lapse monitoring, the results suggested that the times of the third cleavage and compaction may be useful morphokinetic parameters for predicting developmental potential [34]. Prior to these studies, Sutherland *et al.* [35], for the first time, directly examined the division plane of 8-cell blastomeres during the fourth cleavage of mouse embryos cultured from the late 2-cell stage using time-lapse film cinematography.

Since these mouse studies used embryos fertilized *in vivo*, in the present study, the preimplantation development of mouse oocytes fertilized *in vitro* was assessed. Observation was conducted in a non-humidified incubator with time-lapse cinematography, and the sperm penetration time was accurately controlled in the IVF system [3, 8, 36] to evaluate the morphokinetic development with higher precision.

Materials and Methods

Superovulation was induced in ICR (CLEA, Tokyo, Japan) female mice, 8–14 weeks of age, by intraperitoneal injections of 175 *µ*l of CARD Hyper Ova (Kyudo Co., Ltd., Saga, Japan) and 5 i.u. of hCG (ASKA Pharmaceutical Co., Ltd., Tokyo, Japan) 48 h apart. The female mice were euthanized by cervical dislocation 16 h after hCG injection, and their oviducts were removed and placed in a culture dish (35 mm, Falcon, Corning Incorporated, NY, USA) containing 400 *µ*l of TYH medium (LSI Medience Corporation, Tokyo, Japan) covered with paraffin oil (Nacalai Tesque, Kyoto, Japan). The ampullar region of the oviduct was dissected with a needle, and the eggs surrounded by cumulus cells were introduced to the medium. Spermatozoa were collected from the cauda epididymis of ICR males, 18–26 weeks of age, and suspended in 400 *µ*l of TYH medium under paraffin oil. After preincubation for 2 h in an incubator (CPO_2-2301, HIRASAWA WORKS Inc., Tokyo, Japan) under 37 °C humidified 5% CO_2 in air, a small volume of sperm suspension was added to the medium containing the eggs. The final concentration of spermatozoa was adjusted to 150 cells/*µ*l [3]. Then, 7 h after insemination, the eggs were transferred into 80 *µ*l of KSOM medium supplemented with amino acids (KSOM, Ark Resource, Kumamoto, Japan), covered with paraffin oil and washed twice. All media

Table 1. Results of *in vitro* cultivation of mouse embryos fertilized *in vitro* in a non-humidifying incubator with time-lapse cinematography

Incubator	No. of fertilized eggs cultured	No. (%) of development to:				
		2-cell (24 hr)[1]	4-cell (48 hr)[1]	Morula (72 hr)[1]	Blastocyst	
					(96 hr)[1]	(120 hr)[1]
CPO_2-2301[2] (5% CO_2 in air)	100	96 (96.0)[a]	79 (79.0)[a]	71 (71.0)[a]	84 (84.0)[a]	86 (86.0)[a]
CCM-iBIS[3] (5% CO_2 in air)	395	389 (98.5)[a]	357 (90.4)[b]	226 (57.2)[b]	305 (77.2)[a]	357 (90.4)[a]

[1]Hours after insemination. [2]Humidifying conventional CO_2 incubator. [3]Non-humidifying incubator with time-lapse cinematography. a-b: Values with different superscripts are significantly different in the same column at $P < 0.05$.

were equilibrated overnight under 37 °C humidified 5% CO_2 in air prior to use. After confirmation of the presence of the second polar body and both male and female pronuclei, the fertilized eggs were cultured for 113 h in a culture dish (LinKID micro DH-004PG, DNP, Tokyo, Japan) containing 60 *µ*l of KSOM covered with paraffin oil at 37 °C in 5% CO_2 in air in a non-humidifying incubator with time-lapse cinematography (CCM-iBIS, ASTEC Co., Ltd., Fukuoka, Japan). The CCM-iBIS had a non-humidifying system and was equipped with a 130 million-pixel CCD camera with a 10×objective lens, red LED lighting, silicone rubber heater with digital PID control, duty controlled gas pressure, and NAS image storage method. The CCD camera for the time-lapse microscopy was set to acquire a single image every 15 min. The parameters of embryonic development were annotated as follows: t2, time of cleavage to 2-cell stage after insemination; t3, time of cleavage to 3-cell stage after insemination; t4, time of cleavage to 4-cell stage after insemination; t5, time of cleavage to 5-cell stage after insemination; tOC, time of the onset of compaction after insemination; tM, time of formation of fully compacted morula after insemination; tB, time of blastocyst formation with blastocoel at half volume of embryo after insemination; tExB, time of formation of expanded blastocyst developed to over 120 *µ*m in diameter after insemination; tHB, time of the onset of hatching from the zona pellucida after insemination. As a control, eggs fertilized *in vitro* were cultured in a dish containing 50 *µ*l of KSOM covered with paraffin oil at 37 °C under a humidified atmosphere of 5% CO_2 in air in a conventional CO_2 incubator (CPO_2-2301). The developmental stages of the embryos were observed under an inverted microscope 24 h apart in the control group.

The animals were kept in a barrier unit at 24 ± 1 °C with a relative humidity of 50 ± 10% under a lighting regimen of 12L/12D (lights on 07:00 to 19:00). They were allowed free access to standard laboratory chow (CA-1, CLEA, Tokyo, Japan) and tap water. All experiments were carried out in accordance with the guidelines for the care and use of animals approved by the Obihiro University of Agriculture and Veterinary Medicine. The authors confirmed that this experiment complied with the ARRIVE guidelines. All animal experimental protocols were approved by the Institutional Animal Ethics Committee of Obihiro University of Agriculture and Veterinary Medicine.

Statistical analyses were performed using JMP software (SAS Institute, NC, USA). All percentages were analyzed using logistic regression and the following model:

$$\ln(\alpha/1-\alpha)=\beta + \text{main factor (type of incubators)}$$

where α=frequency of positive outcomes and β=the intercept. The odds ratios with 95% confidence intervals were calculated. Differences were considered significant at $P < 0.05$. Graph was drawn using DataGraph software (Visual Data Tools, Inc., NC, USA).

Results

The developmental rates to the 4-cell stage at 48 h after insemination of the embryos cultured in CCM-iBIS and in a conventional CO_2 incubator (CPO_2-2301) were 90% (357/395), and 79% (79/100), respectively (Table 1). The differences between the experimental groups were significantly different at $P < 0.05$. The developmental rates of embryos to morulae at 72 h after insemination in CCM-iBIS and CPO_2-2301 were 57% (226/395) and 71% (71/100), respectively. This difference was statistically significant at $P < 0.05$. Developmental rates to the blastocyst stage at 96 h (77%; 305/395) and 120 h (90%; 357/395) after insemination in CCM-iBIS were similar ($P > 0.05$) to those in CPO_2-2301 (84%; 84/100 and 86%; 86/100). There was no significant difference in the developmental rates to the 2-cell stage at 24 h after insemination between the two experimental groups. Table 2 shows the developmental stage of embryos every 24 h after insemination in CCM-iBIS and CPO_2-2301. At 48 h after insemination, 9% and 4% of embryos showed the 5-cell

Table 2. Developmental stage of *in vitro* fertilized mouse embryos at every 24 hr after insemination.

Incubator	24 hr[1]			48 hr[1]		72 hr[1]		96 hr[1]			120 hr[1]		
	2-cell	3-cell	4-cell	4-cell	5-cell	Com	Morula	BL	ExBL	H-BL	BL	ExBL	H-BL
CPO_2-2301[2] (5% CO_2 in air)	96 (100)[a]	0 (0.0)[a]	0 (0.0)[a]	76 (96.2)[a]	3 (3.8)[a]	71 (100)[4]		56 (66.7)[a]	24 (28.6)[a]	4 (4.8)[a]	6 (7.0)[a]	15 (17.4)[a]	65 (75.6)[a]
CCM-iBIS[3] (5% CO_2 in air)	388 (99.7)[a]	0 (0.0)[a]	1 (0.3)[a]	325 (91.0)[a]	32 (9.0)[a]	142 (38.6)	226 (61.4)	119 (39.0)[b]	27 (8.9)[b]	159 (52.1)[b]	58 (16.2)[b]	33 (9.2)[b]	266 (74.5)[a]

The data indicate the number and proportion of embryos for each developmental stage at each observation time after insemination. [1]Hours after insemination. Com: Initiation of compaction, BL: Blastocyst, ExBL: Expanded Blastocyst, H-BL: Hatching Blastocyst. [2]Humidifying conventional CO_2 incubator. [3]Non-humidifying incubator with time-lapse cinematography. [4]These embryos ("compacting" or "compacted" morula) were indistinguishable. a-b: Values with different superscripts are significantly different in the same column at $P < 0.05$.

stage in CCM-iBIS and CPO_2-2301, respectively. At 96 h after insemination, 61% and 33% of embryos showed expanded blastocysts or hatching blastocysts in CCM-iBIS and CPO_2-2301, respectively with embryos cultured in CCM-iBIS showing a more advanced embryonic stage. Specifically, at 96 h after insemination, 52% of embryos were hatching blastocysts in CCM-iBIS, while only 5% were hatching blastocysts in CPO_2-2301.

Figure 1 shows a histogram of the number of IVF embryos at each developmental stage with the time lapse after insemination. In CCM-iBIS, compaction of fast-developing embryos was completed at 58 h after insemination and these embryos developed to morulae; however, compaction of about 40% of the cultured embryos was still not completed at 72 h after insemination. In contrast, at 24, 48, and 96 h after insemination, most of the embryos were judged to have developed to the 2-cell, 4-cell, and blastocyst stages, respectively.

Figure 2 illustrates the progression of the cleavage of the IVF eggs cultured in CCM-iBIS. The first cleavage occurred 17 h 33 min after insemination. The second cleavage was initiated at 39 h 31 min after insemination. The time between the first and second cleavages was 21 h 57 min. The 3-cell stage lasted for 1 h 16 min. The second cleavage was complete at 40 h 47 min after insemination. At 11 h 18 min after the second cleavage, the third cleavage was initiated at 52 h 5 min after insemination. The compaction of embryos was initiated at 59 h 44 min after insemination and they reached the morula stage at 70 h 28 min after insemination. Interestingly, embryos compacting during development to the morula showed a temporary regression of compaction due to an increase in the number of blastomeres accompanying cell division. Morula developed into blastocysts 16 h 54 min later at 87 h 22 min after insemination.

Discussion

As shown in Table 1, the developmental rate to the 4-cell stage at 48 h after insemination was observed to be significantly higher in the embryos cultured in CCM-iBIS than that of the embryos cultured in CPO_2-2301, However, the developmental rate to the morula at 72 h after insemination of the embryos cultured in CCM-iBIS was significantly lower than that of those cultured in CPO_2-2301. A possible explanation for this is that, in the observations at 24-h intervals under an inverted microscope, at 72 h after insemination, some embryos were judged to be "morula" when their compaction was still incomplete. Whereas, under 15 min time-lapse observations (Figs. 1 and 2), similarly developing embryos would have been judged to be "compacting morula". Embryos cultured in CCM-iBIS showed a more advanced embryonic stage than those cultured in CPO_2-2301 at 96 h after insemination (Table 2). Therefore, the incubator with time-lapse cinematography appears to provide a qualitative improvement of mouse embryos in culture. This may be due to the difference in culture conditions, humidified and non-humidified, and the fact that the embryos were not exposed to the atmosphere during observation in the time-lapse incubator.

Although Sutherland *et al.* [35], Karnaukhov *et al.* [37], Pribenszky *et al.* [33], and Kim *et al.* [34] used time-lapse monitoring systems to evaluate mouse embryonic development *in vitro*, they used zygotes or 2-cell stage embryos derived from fertilization *in vivo*. For the evaluation of preimplantation development *in vitro* with higher precision, analysis of *in vitro* fertilized eggs with synchronous ovulation and fertilization is recommended [3, 36]. In this study, we precisely determined the timing of cleavage in IVF mouse embryos during preimplantation development using time-lapse cinematography (Table 2, Figs.

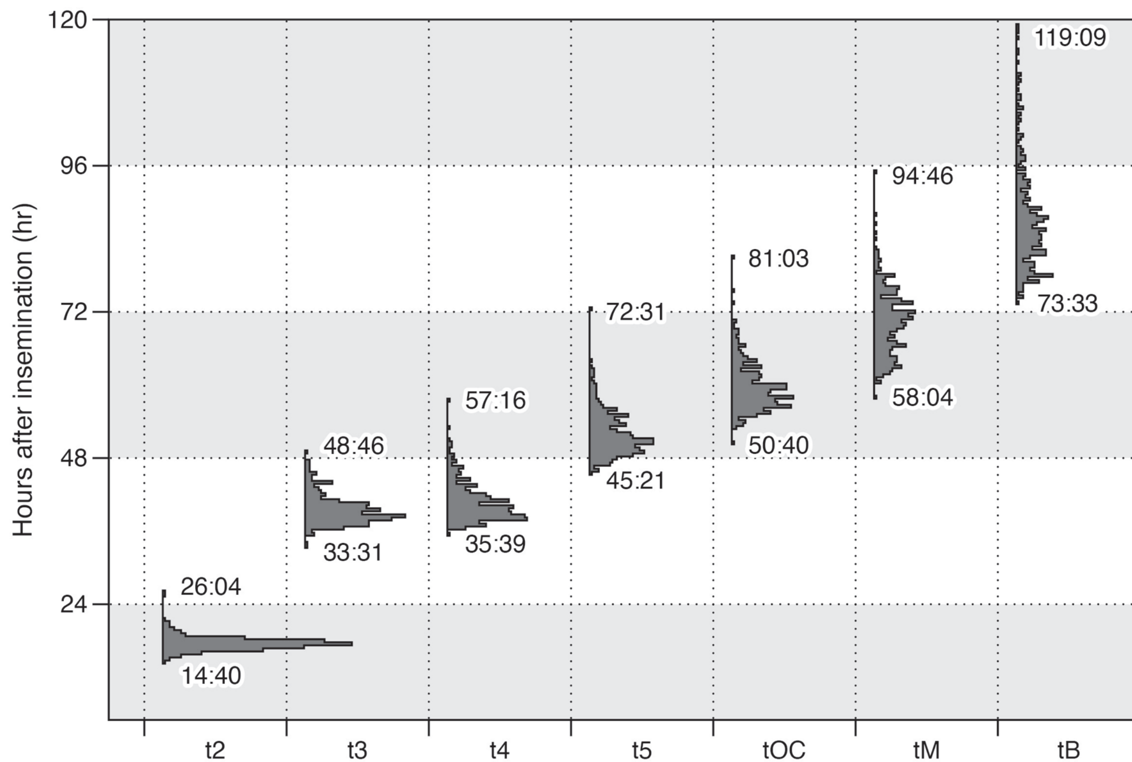

Fig. 1. Histogram of the number of embryos fertilized *in vitro* at each developmental stage with the lapse of time after insemination. Values show the range of times to cleavage of each developmental stage. Embryonic development is annotated as follows: t2, time of cleavage to the 2-cell stage after insemination; t3, time of cleavage to the 3-cell stage after insemination; t4, time of cleavage to the 4-cell stage after insemination; t5, time of cleavage to the 5-cell stage after insemination; tOC, time of the onset of compaction after insemination; tM, time of formation of fully compacted morula after insemination; tB, time of formation of blastocyst with blastocoel at half volume of the embryo after insemination.

1 and 2). As shown in Fig. 2, when IVF mouse zygotes were cultured in CCM-iBIS, they developed to the 2-cell, 4-cell, morula, and blastocyst stages at approximately 18, 41, 70, and 87 h after insemination, respectively. Compaction, in which all blastomeres flatten against each other eliminating the spaces between the cells, of embryos was initiated at an average of 59 h 44 min after insemination. Subsequently, the compaction partially regressed with cleavage of embryos, in other words, with an increase in the number of blastomeres (Fig. 2) [38]. Cell division gives rise to two daughter cells that localize as one internal and one external cell or remain as two external cells with regards to the embryo. Therefore, when the daughter cells are localized externally to the embryo, compaction appears to loosen and be incomplete. However, depending on the timing of observation, when the daughter cells are localized as one internal and one external cell, compaction appears to be complete. Although the embryos formed morula with full compaction at an average of 70 h 28 min after insemination (Fig. 2), approximately 39% of embryos had not fully compacted at 72 h after insemination (Fig. 1). Therefore, it is likely that a conventional 24-h observation interval misjudges the developmental rate to the morula. When the embryonic development of IVF mouse embryos was observed at 24-h intervals, the developmental rates to the 2-cell stage at 24 h after insemination, the 4-cell stage at 48 h after insemination, the morula at 72 h after insemination, and the blastocyst at 96 and 120 h after insemination were used as an index. However, the determination of embryonic development at 72 h after insemination might be assessing compacting and compacted morula rather than compacted morula in mice. Although previous studies have observed the compaction and subsequent embryonic development of human embryos using time-lapse technology, these focused on vacuolization in blastomeres [24], the timing of morula formation [22, 23, 25, 26], and the patterns of compaction with or without extruded blastomeres [22, 27]. There have been, to our knowledge, no previous studies in which a temporary

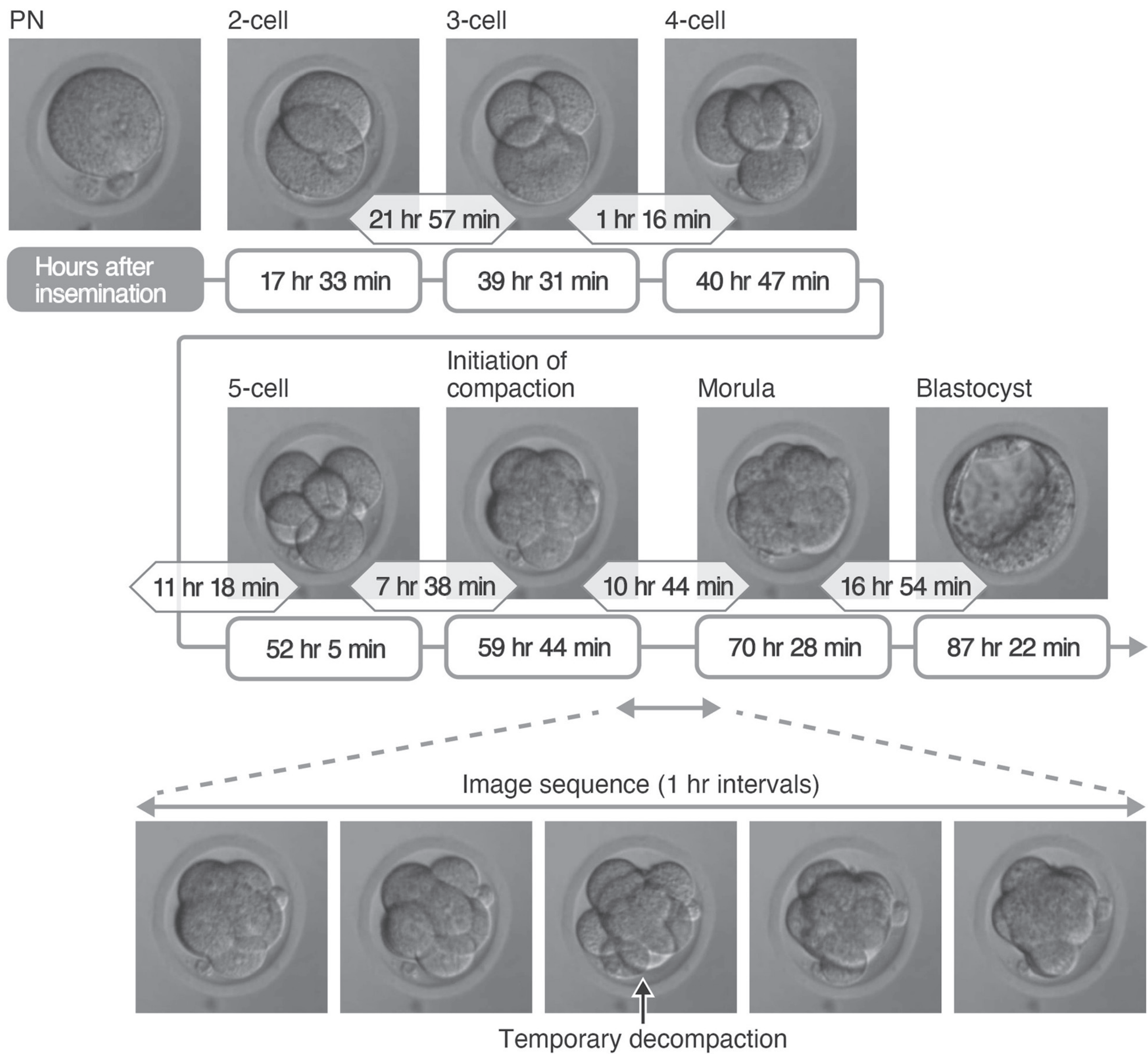

Fig. 2. Timing of cleavage of *in vitro* fertilized mouse embryos during preimplantation development in culture at 37 °C under 5% CO_2 in air in a non-humidifying incubator with time-lapse cinematography.

loosening of compaction during the formation of morula after the initiation of compaction in human embryos has been observed [39].

The procedures of oocyte collection and insemination, culture medium, and culture conditions are key to the success or failure of IVF and subsequent embryo transfer. However, the accurate monitoring of embryonic development *in vitro* is also an important element. Over time, improvements have been made to the IVF method [1–3] and culture medium [3, 5, 8–13] making it possible to obtain stable and highly reproducible results. In addition, the advent of culture methods, such as the non-humidifying incubator with time-lapse cinematography [15], has enabled the accurate monitoring of embryonic development *in vitro*. In particular, incubators with time-lapse cinematography are especially useful, as a relatively long period of monitoring is required to accurately determine development to the morula stage.

Disclosures

Conflicts of interest: Hiroyuki Watanabe, and Hiroshi

Suzuki declare that they have no conflicting interests.

Human and animal rights: This article does not describe any experiments involving human participants. All of the institutional and national guidelines for the care and use of laboratory animals were followed. The protocol for the research project was approved by a suitably constituted ethics committee.

Data availability: The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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