Time of morulation and trophectoderm quality are predictors of a live birth after euploid blastocyst transfer: a multicenter study

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Objective: To investigate whether the morphodynamic characterization of a euploid blastocyst's development allows a higher prediction of a live birth after single-embryo-transfer (SET).
Design: Observational cohort study conducted in two phases: training and validation.
Setting: Private in vitro fertilization centers.
Patient(s): Euploid blastocysts: 511 and 319 first vitrified-warmed SETs from 868 and 546 patients undergoing preimplantation genetic testing for aneuploidies (PGT-A) in the training and validation phase, respectively.
Intervention(s): Data collected from time of polar body extrusion to time of starting blastulation, and trophectoderm and inner-cell-mass static morphology in all embryos cultured in a specific time-lapse incubator with a continuous medium. Logistic regressions conducted to outline the variables showing a statistically significant association with live birth. In the validation phase, these variables were tested in an independent data set.
Main Outcome Measure(s): Live births per SET.
Result(s): The average live birth rate (LBR) in the training set was 40% (N = 207/511). Only time of morulation (tM) and trophectoderm quality were outlined as putative predictors of live birth at two IVF centers. In the validation set, the euploid blastocysts characterized by tM <80 hours and high-quality trophectoderm resulted in a LBR of 55.2% (n = 37/67), while those with tM ≥ 80 hours and a low-quality trophectoderm resulted in a LBR of 25.5% (N = 13/51).
Conclusion(s): Time of morulation and trophectoderm quality are better predictors of a euploid blastocyst's reproductive competence. Our evidence was reproducible across different centers under specific culture conditions. These data support the crucial role of morulation for embryo development, a stage that involves massive morphologic, cellular, and molecular changes and deserves more investigation. (Fertil Steril® 2019;112:1080–93. ©2019 by American Society for Reproductive Medicine.)
El resumen está disponible en Español al final del artículo.

Key Words: Blastocyst, embryo selection, euploid, morula, time-lapse microscopy

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In in vitro fertilization (IVF) today, achieving a high chance of live birth with concurrent low reproductive hazards per transfer (such as multiple pregnancy or miscarriage) is a task of utmost importance. To this end, some scientific societies have supported the implementation of a single-embryo transfer (SET) policy (1, 2) to limit the impact of multiple pregnancies (3). However, SET requires efficient embryo selection to be clinically sustainable (4).

Numerous studies have investigated the predictive value of parameters such as number of cells, their size and symmetry, and fragmentation for use in adopting a cleavage-stage embryo transfer policy (5). However, the results have been disappointing, probably because of the high impact of chromosomal aneuploidies in day 3 of preimplantation development (6), which indirectly hinders any other embryonic feature. Furthermore, natural selection during the first 3 days of culture is limited, so the cohort of cleavage-stage embryos produced in an IVF cycle is very similar to the initial cohort of fertilized oocytes (7, 8). The extension of embryo culture to the blastocyst stage generally reduces the cohort of embryos by 40% to 50% (7, 8). Thus, working in standard, controlled, constantly monitored culture conditions (9) might result in efficient selection in blastocyst culture. In fact, the data inspected by Glujovsky and Farquhar in 2016 in their Cochrane review (10, 11) showed improved implantation rates per embryo transfer when a blastocyst rather than a cleavage-stage embryo was transferred, with a similar cumulative live birth rate (LBR) (where the cumulative LBR is the overall live births achieved from consecutive fresh and frozen transfers of all the embryos produced in an IVF cycle). In other words, higher efficiency can be achieved without impacting the overall chance of success even when a single blastocyst is transferred, with a goal of limiting multiple gestations (3). Even so, a (single) blastocyst transfer policy does not reduce the rate of spontaneous miscarriages (11).

For an embryo cohort produced by a couple during an IVF cycle, an advanced strategy for selection involves retrieving about 5 to 10 trophectoderm (TE) cells from each blastocyst and analyzing them via comprehensive chromosome testing (CCT) techniques to identify the euploid and aneuploid embryos. The aim of this approach—known as preimplantation genetic testing for aneuploidies (PGT-A) (12, 13)—is to prevent the transfer of aneuploid embryos. Dahdouh et al. (14) and Chen et al. (15) in two meta-analyses published in 2015 reported that when CCT is adopted to identify euploid embryos in good-prognosis patients, higher implantation rates per embryo transfer (~50–60%) and lower miscarriage rates per clinical pregnancy (~10%) can be achieved. This evidence encouraged more confident implementation of a blastocyst SET policy in women with a good prognosis and those of advanced maternal age (16, 17). In the latter population, higher efficiency with no impact on IVF efficacy (i.e., fewer embryo transfers, more implantations per embryo transfer, and lower miscarriage rates with similar cumulative LBR per patient with respect to non-PGT-A cycles) was reported from both the randomized controlled trials conducted by Rubio and colleagues and the ESHRE Study into the Evaluation of Oocyte Euploidy by Microarray Analysis (ESTEEM). This was true of euploidy diagnosed by CCT on blastomeres and polar bodies (18, 19), which were less reliable specimens than TE for PGT-A purposes (20, 21).

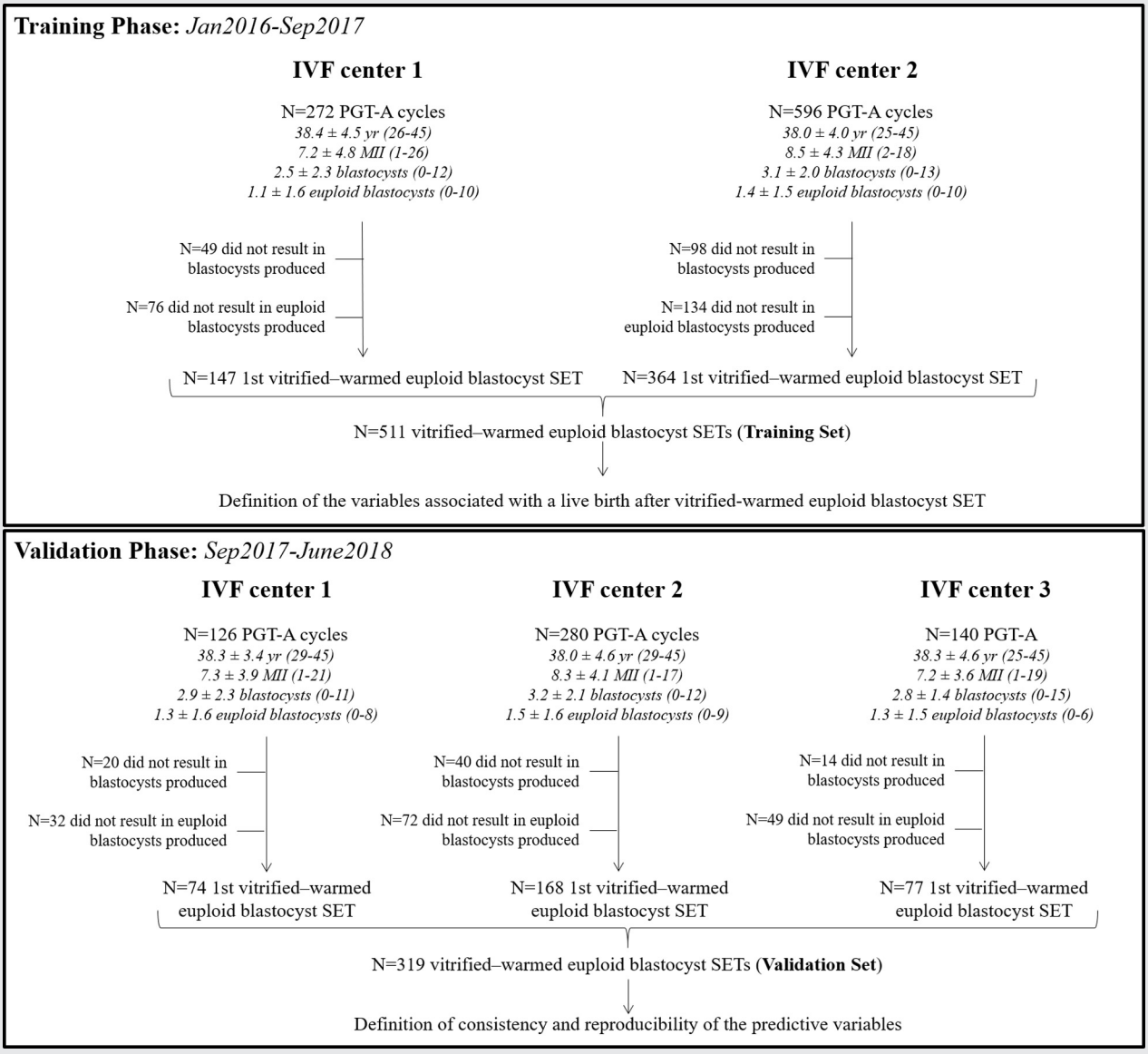
Yet even if a normal chromosomal constitution is critical in predicting embryo implantation outcomes, it does not represent the only feature determining embryo competence—there is still room for improvement in embryo selection. Moreover, TE biopsy, albeit reported safe in the hands of experienced operators (21–25), still represents an invasive procedure that involves embryo manipulation and requires proper training and expertise. Therefore, the quest for noninvasive criteria is ongoing. Unfortunately, although several intriguing metabolomic/proteomic approaches have been adopted to analyze the byproducts of IVF (e.g., follicular fluid, cumulus cells, spent culture media), the clinical efficiency of these approaches has yet to be determined. Indeed, more extensive research and validation are needed before any consensus can be achieved or a clinical application developed (4, 26, 27).

The most commonly used method for embryo selection worldwide still is static morphology. At the blastocyst stage, numeric or alphabetic scales have been proposed (28), but all are based on the scoring system proposed by Gardner and Schoolcraft in 1999 (29), which consisted in the grading of three different variables: expansion, cohesiveness of the TE, and dimension and form of the inner cell mass (ICM). Several studies have attempted to identify the individual contribution of these parameters to implantation potential or live births, but different conclusions were reported. Some investigators emphasized the crucial role of the TE over the ICM (30–36); others instead emphasized the latter parameter as more predictive of a live birth (37–39).

The assessment of static morphology remains highly subjective (40), and most importantly static morphology is only mildly associated with chromosomal aneuploidies, which so far still represent the main cause of implantation failure and miscarriage in humans (41–45). Static morphologic evaluation has been improved with the introduction of time-lapse microscopy (TLM), which allows the assessment of embryo morphodynamic patterns throughout preimplantation development. The benefit of time-lapse incubators in providing an undisturbed culture environment is generally recognized, but TLM parameters have produced contrasting results when adopted to foresee embryo euploid constitution or reproductive competence. Their predictive value has not been consistent across studies and is still subject to debate (46–50). Importantly, morphokinetics appears to be affected by several parameters, such as infertility factor, maternal age, stimulation protocol, and culture strategies (51), which clearly limits the wide reproducibility of the algorithms used to date (52–55).

We propose that even if morphology (static and dynamic) only in part allows envisaging the chromosomal constitution

FIGURE 1



Flowchart of study phase 1 (training set to outline the variables associated with live birth after vitrified-warmed euploid blastocyst single embryo transfer) and phase 2 (validation set to define the consistency and reproducibility of those predictive variables). MII = metaphase II oocyte; PGT-A = preimplantation genetic testing of aneuploidies; SET = single-embryo transfer.

Rienzi. Euploid blastocysts' morphodynamic analysis. Fertil Steril 2019.

or the implantation competence of untested embryos, it still can be investigated for its potential to predict the latter in blastocysts diagnosed as euploid after PGT-A. To this end, we assessed the main variables associated with live birth after euploid blastocyst transfer in a data set from two IVF centers (training set). These variables were then tested in an independent data set that included a third IVF center (validation set). All the IVF centers involved in this study are reference laboratories for blastocyst culture, PGT, and vitrified-warmed SET. Our overarching aim was to address the consistency and reproducibility of any morphologic/morphodynamic parameter associated with live birth and identified in a clinical setting, subject to the lowest number of putative sources of bias: undisturbed culture with a continuous media and euploid blastocyst SET.

MATERIALS AND METHODS

Study Design

The study design, mean maternal age, and main cycle data are shown in Figure 1. In the training phase, an observational cohort study of PGT-A cycles was performed between January 2016 and September 2017 at two European IVF centers: G.EN.E.R.A. Center for Reproductive Medicine (Rome, Italy; IVF center 1) and IVI (Valencia, Spain; IVF center 2). This phase was aimed at identifying the variables associated

with a live birth after the first vitrified-warmed euploid blastocyst SETs at both IVF centers.

In the validation phase, the variables outlined in the training set were tested for consistency and reproducibility on an independent data set. The latter set encompassed the first vitrified-warmed euploid blastocyst SETs performed after PGT-A between September 2017 and June 2018 at IVF centers 1 and 2 and at a third clinic (Center for Reproductive Medicine, European Hospital, Rome, Italy: IVF center 3).

Only the first SETs of euploid vitrified-warmed blastocysts were included in both study phases to avoid a putative cohort effect upon the LBR achieved in this population of patients. All PGT-A cycles entailing embryo culture conducted with a sequential media were not included for the sake of consistency within and between the centers, and because a lower blastulation and a slower development with respect to a continuous medium was previously reported at IVF center 1 (56).

IVF Procedures

Controlled ovarian stimulation was performed as previously described elsewhere (57). Thirty-five hours after ovulation trigger, the oocytes were collected by transvaginal ultrasound-guided aspiration. Fertilization was conducted only by intracytoplasmic sperm injection (ICSI) after denudation, as previously described elsewhere (58). Embryo culture was conducted individually in 25 μL of continuous single-culture medium (CSCM; Irvine Scientific) covered by preequilibrated mineral oil in a microwell of the EmbryoSlide (Vitrolife). No media changeover in day 3 was performed.

Only an EmbryoScope time-lapse incubator (Vitrolife) was used at IVF centers 1, 2, and 3. Images of each embryo were acquired every 15 minutes on seven different focal planes. Embryos were cultured up to the blastocyst stage in 6% CO_2 and 5% O_2. In IVF centers 1 and 3, a simultaneous zona opening and TE biopsy approach (41) was used; IVF center 2 adopted a day-3 hatching-based TE biopsy approach (59). We conducted PGT-A by comprehensive chromosome testing techniques (60–64). The transferred blastocysts were selected independent from their morphodynamic parameters and based only on an euploid chromosomal constitution.

All euploid SETs were performed in a subsequent prepared cycle (57). The luteal phase was supported by vaginal micronized progesterone, 400 mg/day (Progeffik; Effik). We did not perform SET in cases of [1] an endometrium $<$7 mm and/or not trilaminar, [2] endometrial fluid in the uterine cavity, [3] hydrosalpinx, and/or [4] high levels of thyroid-stimulating hormone ($\geq$2.5 IU/L). Vitrification (conducted within 30 minutes of the TE biopsy) and warming were performed according to previous publications (23, 45, 65).

Training phase. Between January 2016 and September 2017, 868 PGT-A cycles performed at IVF centers 1 and 2 were submitted to continuous embryo culture in an EmbryoScope incubator. The first vitrified-warmed euploid blastocyst SETs meeting the inclusion criteria were 147 and 364 from IVF centers 1 and 2, respectively (see Fig. 1). The time-lapse parameters adopted in this study were previously defined by Ciray et al. (66): timing of second polar body appearance (tPB2); timing of completion of cleavage to 2, 3, 4, 5, and 8 cells (t2, t3, t4, t5, and t8, respectively); timing of morula formation (tM, the stage where visualization of individual blastomeres was no longer possible and full compaction was achieved); timing of starting blastocyst formation (tSB); length of the first (cc1 = t2 − tPB2), second (cc2 = t4 − t2), and third cell cycle (cc3 = t8 − t4); length of synchronization of cell divisions (s2 = t4 − t3); and synchronization of cleavage pattern (s3 = t8 − t5).

The time of blastocyst formation (tB) and blastocyst full expansion (tEB) were not included among the parameters under investigation because a different blastocyst biopsy approach was conducted at IVF center 2. Specifically, zona drilling in this center was performed on day 3, a procedure that induces a precocious hatching of the blastocyst (tHB) before the achievement of full expansion status (tEB) so that the TE biopsy and blastocyst grading are conducted significantly before with respect to IVF center 1, where both grading and zona drilling are instead routinely performed at the tEB.

Therefore, to reach a consensus between the different IVF centers involved in the study, the grading of the ICM and TE quality was here performed at the tB. The ICM and TE were graded according to the parameters defined in the Istanbul consensus (28, 67). Class 1 and class 2 were clustered together and defined as "high quality," whereas class 3 was defined "low quality." All the morphodynamic and static morphologic criteria were tested through logistic regression analyses adjusted for confounders.

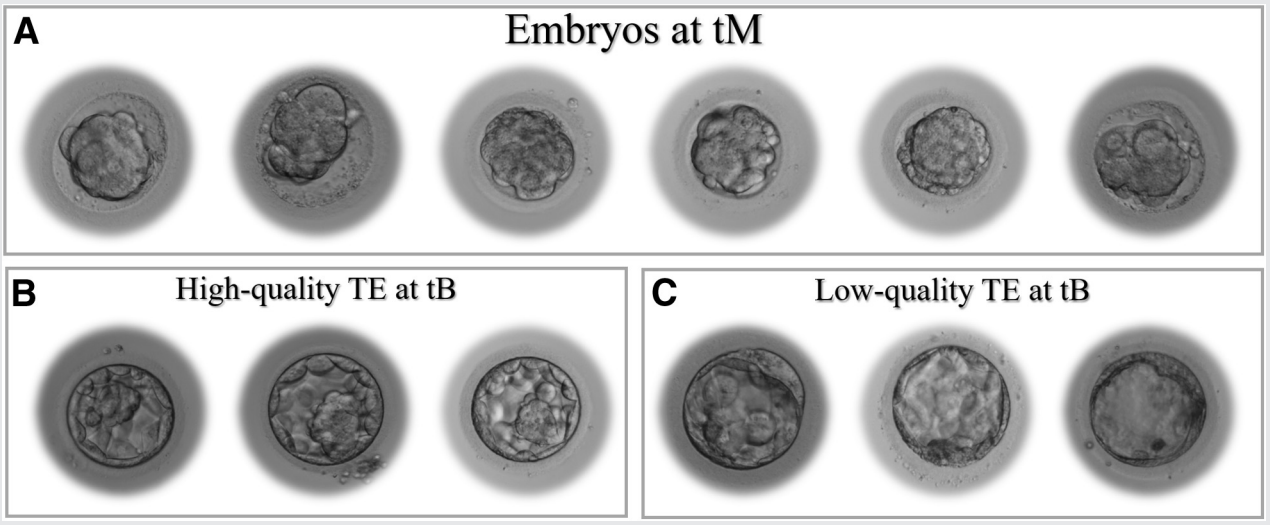
Validation phase. Between September 2017 and June 2018, 546 PGT-A cycles performed at IVF centers 1, 2, and 3 were submitted to continuous embryo culture in an EmbryoScope incubator. The variables found to be statistically significantly associated with a live birth after euploid blastocyst SETs were tested for consistency and reproducibility on a further 319 vitrified-warmed euploid blastocyst SETs conducted at IVF centers 1 (N = 74) and 2 (N = 168) as well as in a third clinic (IVF center 3, N = 77) (see Fig. 1). The study was approved by the institutional review boards of the contributing clinics.

All the evaluations were performed by a single expert operator at each center. In a preliminary phase, the chosen operators were compared for their consistency in the definition of tM and in TE grading at tB on a set of 40 randomly chosen consecutive pictures (some examples can be found in Fig. 2). They showed an interclass correlation coefficient of 0.9 and 0.8 for the two variables, respectively. Both these values indicate an excellent reproducibility according to the thresholds defined by Koo and Li in 2016 (68).

Statistical Analysis

The investigated euploid blastocysts were divided into two groups based on whether they resulted in a live birth. Continuous variables are shown as mean ± standard deviation (SD) and range. Shapiro-Wilk tests were conducted to investigate whether the data followed a normal (Gaussian) distribution. Student's *t*-tests or Mann-Whitney *U* tests were conducted to assess statistically significant differences. Categorical variables are shown as rate with 95% confidence interval (CI).

FIGURE 2



(A) Examples of embryos at the time of morulation (tM). **(B)** Examples of high-quality trophectoderm (TE) and the time of blastulation (tB). **(C)** Examples of low quality TE at tB.

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Fisher's exact or chi-square tests were conducted to assess statistically significant differences.

We investigated the morphodynamic and static criteria from univariate and multivariate regression analyses by defining the binary response parameter as a live birth achieved ("1") or not ("0"). If required, the morphokinetic timings were converted from continuous variables into categorical by defining the cutoff value corresponding to the 50th percentile of prediction of a live birth. $P<.05$ was considered statistically significant. Statistical analyses were performed using the Statistical Package for the Social Sciences 19 (SPSS; IBM, Inc.). Power calculations were performed using G*Power v3.1 (Universität Düsseldorf).

RESULTS

Training Phase: Definition of the Variables Associated with a Live Birth after Vitrified-Warmed Euploid Blastocyst Transfer

In the training phase, 272 and 596 PGT-A cycles were conducted at IVF centers 1 and 2, respectively. The mean maternal age and main cycle data are shown in Figure 1. The cohort of metaphase 2 (MII) oocytes collected at IVF center 2 was larger than at IVF center 1 ($P<.01$), but then no difference was shown in terms of blastulation or euploidy rates. At IVF center 1, 49 (18%) and 76 (28%) PGT-A cycles resulted in no blastocyst and no euploid blastocyst produced, respectively. At IVF center 2, 98 (16%) and 134 (22.5%) PGT-A cycles resulted in no blastocyst and no euploid blastocyst produced, respectively. Therefore, 147 and 364 first vitrified-warmed euploid blastocyst SETs were included from IVF centers 1 and 2, respectively (see Fig. 1). The overall LBR per first vitrified-warmed euploid blastocyst SET was 40.5% (N = 207/511; 95% CI, 36.2–44.9). There was no difference between the two IVF centers (43.5%; 95% CI, 35.5–52.0; N = 64/147 vs. 39.3%; 95% CI, 34.3–44.5; N = 143/364, respectively; P=not statistically significant [NS]).

Overall, the euploid embryos cultured at IVF center 2 rather than at IVF center 1 were consistently slower from tPB2 up to t5 (on average 1–2 hours). Statistically significant differences were indeed reported for tPB2, cc1, s2, cc2, and t5. However, after the eight-cell stage was reached, they could manage to decrease their delay; thus, starting from t8 onward no statistically significant difference was reported thereafter between the two centers (Supplemental Table 1, available online).

Of note, the tM was the first moment of preimplantation development where the timings at the two centers were almost perfectly overlapping (87.4 ± 9.2, 66.9–116.0 hours vs. 87.4 ± 10.0, 58.2–115.6 hours at centers 1 and 2, respectively). When the data in the two centers were clustered according to clinical outcome after first vitrified-warmed euploid blastocyst SETs (live birth vs. implantation failure/miscarriage), again different behaviors were reported during the stages before t8. Specifically, statistically significant differences were reported among reproductively competent and incompetent euploid blastocysts at center 2 for t2, t3, and t5, which were not confirmed at center 1 (Supplemental Table 2, available online). Conversely, the two centers were concordant for the differences reported for t8, cc3, s3, and tM, all statistically significantly longer in nonimplanted/miscarried euploid blastocysts (see Supplemental Table 2).

The tSB was also statistically significantly longer in nonimplanted/miscarried euploid blastocysts only at center 2 (see Supplemental Table 2). We did not include the blastulation timings any further (tB and tEB) due to the different blastocyst biopsy policies adopted at the two centers (i.e., a simultaneous zona opening and TE cell retrieval policy versus a day-3 zona opening-based approach at centers 1 and 2, respectively).

TABLE 1

Logistic regression analysis of the parameters associated with a live birth after euploid vitrified-warmed single-blastocyst transfer.

	Univariate			Multivariate		
Parameter	**OR**	**95% CI**	***P* value**	**OR**	**95% CI**	***P* value**
Maternal age	0.99	0.94–1.04	NS	—	—	—
IVF center						
1	—	—	—	—	—	—
2	0.84	0.57–1.23	NS	—	—	—
tPB2	0.86	0.74–0.99	.04	0.89	0.76–1.04	NS
tM	0.96	0.94–0.98	<.01	0.97	0.94–0.99	.04
tSB	0.97	0.95–0.99	<.01	1.03	0.99–1.07	NS
t2	0.95	0.89–1.00	NS	—	—	—
t3	0.96	0.93–1.00	NS	—	—	—
t4	0.95	0.92–0.99	.01	0.96	0.86–1.09	NS
t5	0.97	0.95–1.0	.05	1.05	0.95–1.17	NS
t8	0.95	0.93–0.97	<.01	0.97	0.93–1.02	NS
cc1	0.97	0.91–1.04	NS	—	—	—
cc2	0.96	0.91–1.01	NS	—	—	—
cc3	0.95	0.92–0.97	<.01	0.96	0.87–1.05	NS
s2	0.96	0.91–1.01	NS	—	—	—
s3	0.94	0.92–0.97	<.01	1.03	0.93–1.14	NS
ICM at tB						
High quality	—	—	—	—	—	—
Low quality	0.41	0.26–0.67	<.01	0.77	0.43–1.37	NS
TE at tB						
High quality	—	—	—	—	—	—
Low quality	0.30	0.19–0.45	<.01	0.38	0.23–0.63	<.01

Note: All embryos were cultured with the same continuous culture media and in the same time-lapse incubator in two different IVF centers. Static morphologic scoring was performed at tB (time of blastulation) in both centers. The Istanbul consensus criteria were adopted and class 1 and 2 were defined as high-quality, and class 3 was considered as low quality. Timing of second polar body appearance (tPB2); timing of cleavage to 2, 3, 4, 5, and 8 cells (t2, t3, t4, t5, and t8); timing of morula formation (tM); timing of starting blastocyst formation (tSB); timing of blastocyst formation (tB); length of the first, second and third cell cycle (cc1, cc2, and cc3); length of synchronization of cell divisions (s2) and synchronization of cleavage pattern (s3). CI = confidence interval; ICM = inner cell mass; NS = not statistically significant; OR = odds ratio; TE = trophectoderm.

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At IVF center 1 a shorter period of time also was required to reach full expansion from euploid blastocysts, resulting in a live birth with respect to nonimplanted/miscarried ones (tEB − tSB: 18.0 ± 5.6 hours, 7.7–34.6 vs. 20.9 ± 7.5 hours, 4.5–43.3, respectively; $P=.01$; Supplemental Fig. 1, available online). In future studies, only centers adopting a simultaneous zona opening and trophectoderm biopsy approach would better assess these dynamics.

Table 1 shows the logistic regression analysis. Neither maternal age nor IVF center showed any association with live birth. Among the morphodynamic parameters under investigation, a statistically significant association was reported from the univariate analyses for tPB2, t4, t5, s3, cc3, t8, tM, and tSB. However, the tM was the only parameter found to be statistically significant from the multivariate analysis (odds ratio [OR] 0.97; 95% CI, 0.94–0.99; $P=.04$). Among the static morphologic features at tB, both the ICM and TE were statistically significantly associated with a live birth after euploid blastocyst vitrified-warmed SET from the univariate analyses. However, only the TE was still statistically significant from the multivariate analysis (OR 0.38; 95% CI, 0.23–0.63; $P<.01$).

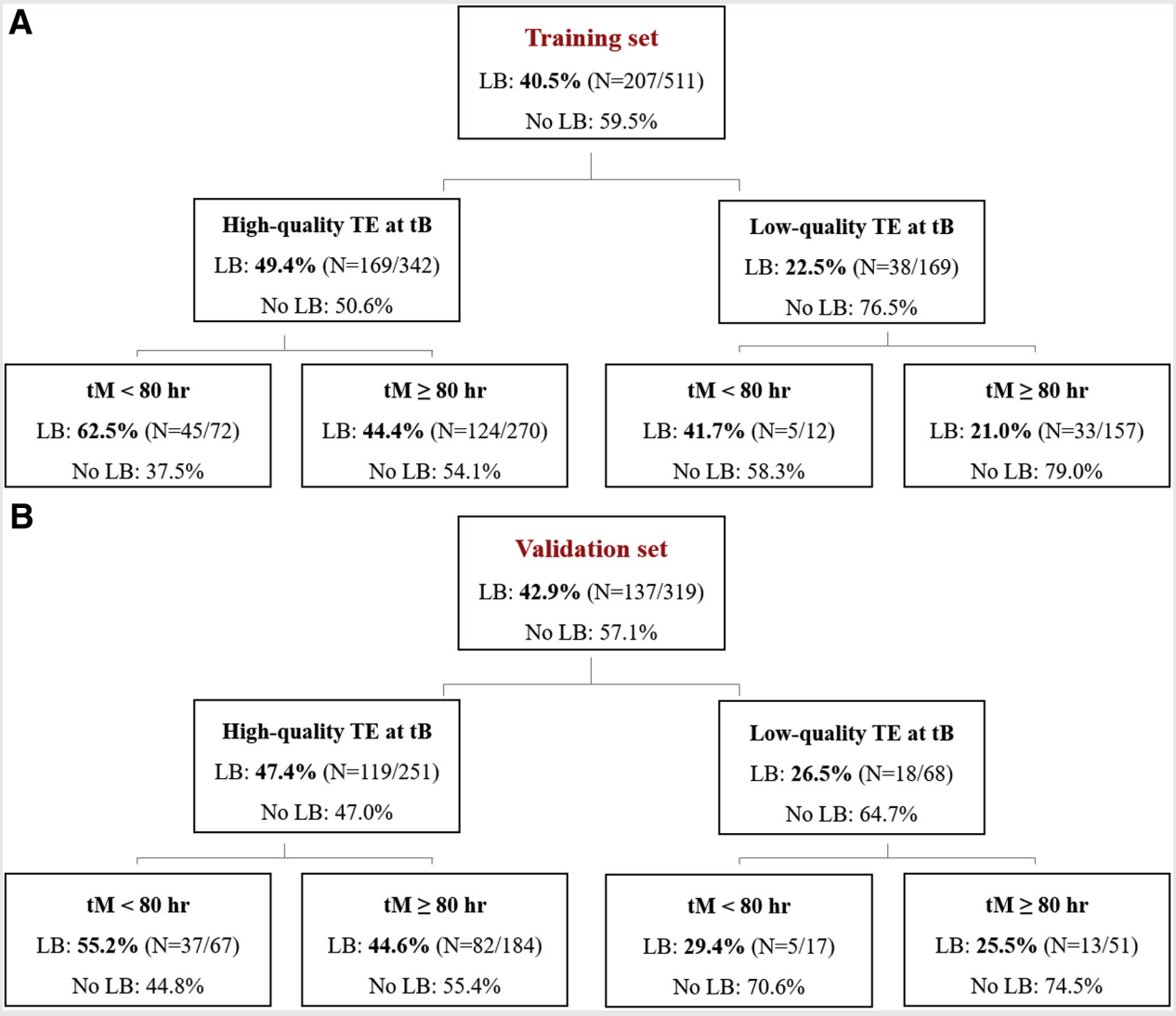
A cutoff value for tM was then calculated based on the logistic regression analysis. In detail, a tM of 80 hours during the preimplantation development of an euploid blastocyst corresponded to the 50th percentile of prediction of a live birth after vitrified-warmed SET in the training set. Therefore, we clustered the results in four groups according to TE morphologic quality evaluated at tB (high versus low) and tM (<80 hours versus ≥80 hours) (Fig. 3A). Interestingly, the euploid blastocysts characterized by a high-quality TE at tB and a tM <80 hours resulted in a LBR of 62.5% (N = 45/72; 95% CI, 50.3–73.4) after vitrified-warmed SET while the euploid blastocysts characterized by a low-quality TE at tB and a tM >80 hours resulted in a LBR of 21.0% (N = 33/157; 95% CI, 15.0–28.4) (see Fig. 3A).

Validation Phase: Definition of the Reproducibility of the Predictive Variables in an Independent Multicenter Data Set

The predictive power of the variables was tested on an independent data set from IVF centers 1 and 2 as well as from IVF center 3. This design allowed us to test the consistency and reproducibility on this validation set. In this study phase, 126, 280, and 140 PGT-A cycles were conducted at IVF center 1, 2, and 3, respectively. The mean maternal age and main cycle data are shown in Figure 1. Also in this case, the cohort of MII oocytes collected at IVF center 2 was larger than at IVF center 1 and 3 ($P=.02$), but then no difference was shown in terms of blastulation or euploidy rates.

After blastocyst culture and aneuploidy testing, 74, 168, and 77 first vitrified-warmed euploid blastocyst SETs could be included from IVF centers 1, 2, and 3, respectively. The rate of cycles where no blastocysts (16%, 14%, and 10%) and no euploid blastocysts (25%, 26%, and 35%) were

FIGURE 3



(A) Definition of the variables associated with a live birth (LB) after 511 vitrified-warmed euploid blastocyst single-embryo transfers (SET) outlined in two IVF centers in study phase 1. **(B)** Validation of the predictive variables on 319 vitrified-warmed euploid blastocyst SET performed at three IVF centers in study phase 2. *Note:* tB = time of blastocyst formation; TE = trophectoderm; tM = time of morula formation.

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obtained were similar across the three clinics. The overall LBR per first vitrified-warmed euploid blastocyst SET was 42.9% (N = 137/319), consistent among the three IVF centers (45.9%, 40.5%, and 45.5% at IVF centers 1, 2, and 3, respectively).

Figure 3B shows the LBR in each specific group. For group A, we found the high-quality TE and tM <80 hours (N = 37/67, 55.2%; 95% CI, 42.6–67.2). For group B, we found the high-quality TE and tM ≥80 hours (N = 82/184, 44.6%; 95% CI, 37.3–52.1). For group C, we found the low-quality TE and tM <80 hours (N = 5/17, 29.4%; 95% CI, 11.4–55.9). For group D, we found the low-quality TE and tM ≥80 hours (N = 13/51, 25.5%; 95% CI, 14.8–39.9) (chi-squared=0.01; Fisher's exact test group A versus D, $P<.01$, and power = 0.9, group B versus D, $P=.02$ and power = 0.7). The results were consistent among the three IVF centers (e.g., group A: 61.5%, 55.0%, and 50.0% at IVF centers 1, 2, and 3, respectively; group D: 30.0%, 25.0%, and 23.1% at IVF centers 1, 2, and 3, respectively).

DISCUSSION

Over the past decade, morphokinetic analysis of embryo development has become an exciting area of human assisted reproduction. Expectations for it were high because of the amount of previously inaccessible information that could be finally collected. Even more importantly, the majority of data was either intrinsically quantitative (including time of well-defined events) or could be quantified (for example, some degenerative processes), signifying a considerable

opportunity to measure important features related to embryo competence.

Time-lapse technology has been therefore tested over these years to discern the relationship between morphokinetics and embryo developmental competence up to the blastocyst stage (55, 69–75) with different proposed algorithms (55, 76, 77), or between morphokinetics and implantation potential (53, 71, 76, 78–83) with the implementation of other algorithms (76, 79, 81). Also further associations were reported between some peculiar morphodynamic features (intercellular contact number or blastocyst collapse) and implantation potential (77, 84). Also specific dysmorphisms such as multinucleation, reverse cleavage, direct unequal cleavage, and irregular chaotic division have been revealed in human preimplantation embryos as related to embryo developmental or reproductive competence (85–89). Moreover, different trials were conducted to combine morphokinetics with proteomic and metabolomic investigations as well as with cumulus cells' gene expression patterns (90–92), aiming at improved predictivity. Future studies are needed to confirm the value such attempts in this field.

Thus, the controversial results for morphokinetics in predicting embryo implantation may be explained by various external and internal factors—differences in terminologies and parameters investigated, various culture conditions, or time-lapse systems used. However, far more uncertainty remains with aneuploidies. Studies dealing with the predictive value of morphokinetics for aneuploidy have contradicted each other, and they do not seem to have provided robust results. Apparently, morphodynamic behavior is in fact similar, especially between euploid embryos and embryos affected from a single chromosomal impairment. Multiple aneuploidies might instead have a greater impact on the timing of preimplantation development, but their incidence in viable blastocysts might also be lower, thereby limiting their predictive power (45, 47, 52, 93–97).

In summary, despite an enormous amount of studies and a meta-analysis reporting moderate evidence in favor of time-lapse microscopy (98), the superiority of morphokinetic assessment over traditional microscopic evaluation of morphology or PGT-A cannot be supported at present (99). Moreover, euploidy status does not exceed 50% to 60% of positive predictive value upon blastocyst reproductive competence after SET (14). Therefore, the purpose of our study was to combine blastocyst morphologic static and dynamic evaluation with euploidy.

A previous group had designed a similar study (100) and adopted morphokinetic parameters to visualize competent embryos among euploid ones. Their results were encouraging but were based on a limited sample size. Our study instead accounts for a large sample size from a multicenter perspective in both the training and validation set. This strategy allowed us to ensure the consistency and reproducibility of our evidence. Of note, the embryos cultured in sequential media were excluded from this data set because a significantly lower blastulation and slower development with respect to the continuous media was reported at IVF center 1 in a previous study (56). Therefore, both the training and validation phases were conducted at two to three IVF centers but using the same time-lapse incubator, in the same conditions, and only with a continuous media.

The TE quality at tB was the only parameter deriving from a static morphologic evaluation and concordant among the two IVF centers as associated with euploid blastocysts' reproductive competence in the training set. This finding is consistent with the many previous studies that claimed its importance in both fresh (30, 32–34) and vitrified-warmed cycles (31, 35, 36). Also, blastocyst expansion and hatching status have been previously reported as predictive of implantation (39), but here they could not be assessed in the absence of bias at both centers. In fact, the practice of zona drilling on day 3 at IVF center 2 limited the reliability of such evaluation and required embryo morphologic grading to be conducted at tB (and not at tEB) also at IVF center 1 for the sake of homogeneity between the two data sets.

Nonetheless, TE quality and blastocyst expansion might be directly associated, as some investigators proposed previously. Specifically, a high-quality TE might involve a more efficient pumping of the ions, in turn prompting the accumulation of fluid in the blastocoel cavity (30). Therefore, the main contrasting results derive only from those studies claiming a higher importance of the ICM in the establishment of a pregnancy (37, 38). In our study, the ICM quality—once corrected for the other factors associated with euploid blastocyst implantation (including TE quality)—lost its significance. This higher importance of the TE in the process of implantation might be ascribed to its roles in this crucial phase: [1] it is the origin of the placenta and its annexes, [2] it promotes hatching and invasion of the endometrium (30, 101), and [3] it establishes the communication with the maternal immune system by secreting human chorionic gonadotropin (hCG) and inducing immunological tolerance (102, 103). In this regard, in the 1990s, a better quality a TE was reported to secrete higher levels of hCG at an earlier time (104, 105). Likewise, more recently, gene expression profiled from TE cells outlines the overexpression of gene families involved in cell adhesion and cell communication to be associated with positive pregnancy outcomes (106, 107). All of this is evidence that supports our findings.

The tM was the only parameter derived from a morphodynamic evaluation of embryo preimplantation development that was concordant among the two IVF centers in the training set as associated with euploid blastocysts' reproductive competence. Consistent delayed development was reported for nonimplanted euploid blastocysts already at t8, which then accumulated (longer cc3 and s3) to culminate in an average 4 hours longer tM. Indeed, this last parameter in itself was sufficient to result in a statistically significant association with the live birth.

It is important to recognize that, despite morulation being a crucial step in preimplantation development (108), little attention has been paid to this stage in the literature. Compaction and subsequent morula development involve the flattening of the blastomeres and the consequent formation of junctions between them, followed by a massive redistribution of surface microvilli and other components of the plasma membrane (109). The embryo shows also a concurrent

increase in transcriptional and translational processes, possibly resulting from the full activation of the embryonic genome (which in humans normally is initiated at the 4- to 8-cell transition) as well as a marked change in the patterns of phospholipid synthesis (110). The cells lose their totipotency and get polarized radially. Across this axis, a differential division occurs, which generates two populations of cells with unequal distribution of organelles: outer polar cells with surface microvilli that will originate the TE and inner apolar cells with tight junctions containing basal nuclei, which will originate the ICM (111). Although the exact timings of the onset of such clusterization are unknown, it might be regulated via posttranslational modifications of specific proteins such as E-cadherins, the putative key effectors enabling a tighter adhesion between the cells (112–116).

When investigating clinical studies, Kramer et al. (117) reported in 2014 the importance of tM. Specifically, they found a significant association between the duration of compaction and an euploid chromosomal constitution in human embryos—yet this parameter was insufficient to provide results comparable to the actual CCT. In addition, both delayed (118) and precocious (109) compaction have been reported to be detrimental for embryo developmental potential and quality from other groups. A study by Mizobe et al. (119) outlined the timing of compaction to be associated with eventual pregnancy outcome, although this was not confirmed by other studies (73, 120).

Finally, also some studies conducted with conventional incubation and static morphologic grading systems highlighted the importance of morula assessment to foresee blastocyst quality and/or competence (121–125). All these findings together with our data should prompt future investigation of the morulation stage of development. More data focused on this issue will indeed be pivotal in understanding how euploid embryos acquire, maintain, or lose their reproductive competence during morulation.

To our knowledge, only one study has compared day-3 zona opening versus simultaneous zona opening and TE fragment retrieval blastocyst biopsy methods (126). In a patient-based randomized controlled trial by Zhao et al. (126), no difference was reported between the two groups, apart from a higher number of blastocysts cryopreserved when the latter biopsy approach was used. Nevertheless, it might be argued that artificial zona opening on day 3 could affect the developmental timing between the cleavage and the blastocyst stage. Here, by comparing three centers using two different biopsy approaches, we could instead indirectly report that the presence of a hole in the zona pellucida probably does not impair the timing up to morulation. Thus, if a difference in embryo morphokinetic development does result from the presence of an opening in the zona pellucida from day 3, it might affect the timings after blastulation rather than before (i.e., blastocyst expansion and hatching).

In the training phase of this study the interval of time between starting blastulation and blastocyst full expansion (tEB − tSB) at IVF center 1 was also different between euploid blastocysts resulting in a live birth rather than an implantation failure/miscarriage, but this timing could not be verified at IVF center 2. Future single-center or multicenter studies conducted solely in IVF laboratories adopting a simultaneous zona opening and trophectoderm biopsy approach are highly recommended to better address these dynamics and their predictive power upon euploid blastocyst implantation.

The main strengths of our study are [1] the definition of two variables statistically significantly associated with euploid blastocyst reproductive competence, whose consistency and reproducibility was independently validated in three IVF centers (euploid blastocysts with high-quality TE and tM <80 hours resulted in 62% and 55% LBR in the training and validation sets versus 21% and 25% for euploid blastocysts with low-quality TE and tM ≥ 80 hours); and [2] the applicability of these predictive variables regardless of the TE biopsy protocol adopted in each laboratory. Nevertheless, our study was conducted under specific culture conditions at the three centers (continuous media, same time lapse incubator and low oxygen tension atmosphere). Further investigations are needed to understand the strength of this association in different conditions.

Ideal future studies would also entail [1] a powered randomized controlled trial comparing a study arm where the first euploid vitrified-warmed blastocyst to transfer is chosen based on both TE quality and tM versus a control arm where no selection scheme is adopted, and [2] an investigation into the predictive power of these two variables during standard ICSI cycles in the absence of aneuploidy testing. Clearly the same conditions as in this report should be adopted for both these studies.

From a clinical perspective, this study highlights the importance of a rigorous validation and of analogous culture conditions when static and morphodynamic parameters are tested as selection criteria. In this regard, artificial intelligence and machine learning represent a concrete future perspective to further increase the standardization and objectivity of each evaluation performed by different operators in different clinics (127, 128).

From a biological perspective, the morula stage emerged as a putative crucial step for the acquisition/maintenance of reproductive competence across human embryo preimplantation development. Future studies will better unravel the cellular and molecular events characterizing the process of morulation.

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El tiempo de morulación y la calidad del trofoectodermo son predictores de nacido vivo tras la transferencia de un blastocisto euploide: un estudio multicéntrico

Objetivo: Investigar si la caracterización morfodinámica del desarrollo de un blastocisto euploide permite una mayor predicción de un nacido vivo después de la transferencia de un solo embrión (SET).

Diseño: Estudio de cohorte observacional realizado en dos fases: entrenamiento y validación.

Entorno: Centros privados de fertilización in vitro.

Paciente(s): Blastocistos euploides: 511 y 319 primeros SET vitrificados y descongelados de 868 y 546 pacientes sometidos a cribado genético preimplantacional de aneuploidías (PGT-A) en la fase de entrenamiento y validación, respectivamente.

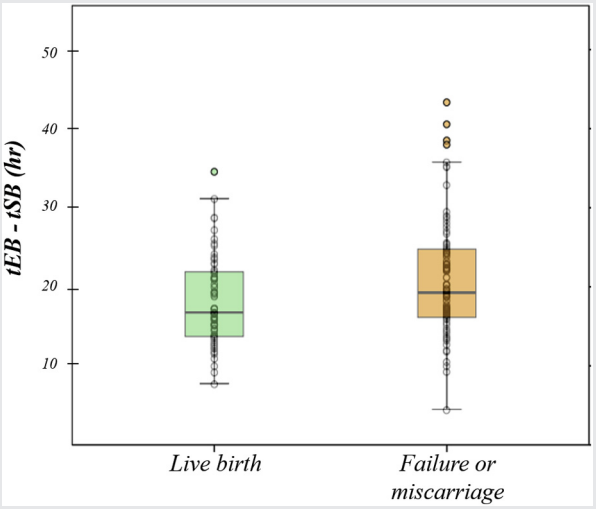
Intervención(es): Recopilación de datos desde el momento de la extrusión del corpúsculo polar hasta el momento de comenzar la blastulación, y la morfología estática del trofoectodermo y la masa celular interna en embriones en una incubadora *time-lapse* con un medio continuo. Realización de regresiones logísticas para mostrar la asociación estadísticamente significativa con el nacido vivo. En la fase de validación, estas variables se probaron en un conjunto de datos independiente.

Principales medidas de resultados: Nacimientos vivos por SET

Resultados: La tasa promedio de nacido vivo (LBR) en el grupo de entrenamiento fue del 40% (N = 207/511). Solo el tiempo de morulación (tM) y la calidad del trofectodermo se mostraron como supuestos predictores en dos de los centros de FIV. En el grupo de validación, los blastocistos euploides caracterizados por tM <80 horas y trofectodermo de alta calidad resultaron en un LBR de 55.2% (n = 37/67), mientras que aquellos con tM ≥ 80 horas y un trofoectodermo de baja calidad resultaron en un LBR de 25.5% (N = 13/51).

Conclusión(es): El tiempo de morulación y la calidad del trofoectodermo son mejores predictores de la competencia reproductiva del blastocisto euploide. Nuestra evidencia ha sido reproducible en diferentes centros bajo condiciones de cultivo específicas. Estos datos respaldan el papel crucial de la morulación para el desarrollo embrionario, una etapa que implementa cambios morfológicos, celulares y moleculares masivos y merece más investigación.

SUPPLEMENTAL FIGURE 1



Box plots showing the time to reach blastocyst full expansion (tEB − tSB) versus the clinical outcome after the first vitrified-warmed euploid blastocyst single-embryo transfers conducted at IVF center 1 in the training set. *Note:* tEB = time of blastocyst full expansion; tSB = time of starting blastulation.

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