Journal of Assisted Reproduction and Genetics (2021) 38:1737–1743
https://doi.org/10.1007/s10815-021-02162-9

EMBRYO BIOLOGY

Embryo morphokinetic score is associated with biomarkers of developmental competence and implantation

Giovanni Coticchio[1] · Francesca Pennetta[1,2] · Roberta Rizzo[3] · Nicoletta Tarozzi[1] · Marco Nadalini[1] · Giovanna Orlando[4] · Chiara Centonze[4] · Giorgia Gioacchini[5] · Andrea Borini[1]

Received: 15 December 2020 / Accepted: 18 March 2021 / Published online: 5 April 2021

Abstract

Purpose To study embryo morphokinetics in relation to release in spent media of molecules with possible roles in development and implantation (miR-20a, miR-30c, and sHLA-G).

Methods Data were obtained from embryos generated in standard IVF and ICSI cycles. The Eeva system was used for embryo assessment, based on early morphokinetic parameters and producing a score (1–5, best–worst) corresponding to higher/medium/lower chances of development to blastocyst. miRNAs — mm miR-20a-5p and miR-30c-5p — and sHLA-G were quantified in 25 μl of spent blastocyst media (SBM) collected before vitrification or transfer. Statistical analyses were performed applying Kolmogorov-Smirnov, Shapiro-Wilk, and Spearman's correlation coefficient tests, where appropriate.

Results SBM were collected from a total of 172 viable blastocysts. Their analysis showed that concentration of miR-20a was progressively lower as Eeva score increased and probability of development to blastocyst decreased ($P = 0.016$). The opposite trend was observed in the case of miR-30c, i.e., concentration was higher as score increased and chances of development to blastocyst decreased ($P = 0.004$). Analysis of sHLA-G revealed a negative correlation with Eeva score, i.e., levels were progressively lower as Eeva score increased and probability of development to blastocyst decreased ($R = -0.388$, $N = 141$, $P = 0.001$).

Conclusion Our data suggest that morphokinetic algorithms that predict development to blastocyst stage, in fact, also identify embryos with molecular and cellular profiles more consistent with developmental functions.

Keywords Embryo · In vitro fertilization · Blastocyst · miRNA · sHLA-G · Implantation

Introduction

Since 2011 [1], time-lapse technology (TLT) has been specifically developed and extensively used to identify morphokinetic algorithms able to predict embryo pre- and postimplantation development, non-invasively and with higher precision. Among hundreds of published studies, some have raised the hope that indeed specific morphokinetics behaviors, discernible by TLT, can be used to rank embryos according to their viability [2–5]. However, such findings have been difficult to reproduce independently or confirm universally [6], irrespective of possible biases derived from patient typology, culture media, and other process-related factors [7]. Therefore, regardless of its vast potential, the impact of TLT on the efficiency of ART treatments awaits final demonstration. Despite such uncertainties, the expectations on TLT remain intact, both in clinical and research contexts. Indeed, dynamic morphological changes of the embryo, at

Giovanna Orlando is no longer an employee of Merck Serono S.p.A., Rome, Italy.

Merck Serono S.p.A. is an affiliate of Merck KGaA, Darmstadt, Germany.

✉ Giovanni Coticchio
giovanni.coticchio@nove.baby

[1] 9.baby Family and Fertility Center, Via Dante, 15, 40125 Bologna, Italy

[2] Simple Departmental Operative Unit, Reproductive Pathophysiology, Anastasia Guerriero Hospital, Marcianise, Caserta, Italy

[3] Section of Microbiology and Medical Genetics, Department of Medical Sciences, University of Ferrara, Ferrara, Italy

[4] Merck Serono S.p.A., Rome, Italy

[5] Department of Life and Environmental Sciences, Polytechnic University of Marche, Ancona, Italy

cellular and sub-cellular levels, can underpin critical developmental events [8]. For example, direct division of the normally fertilized egg into three blastomere, without an intervening 2-cell stage, represents the morphological manifestation of a mitotic division mediated by a tripolar spindle [9]. Likewise, exclusion of blastomeres from the compaction of the morula can be associated with the elimination of chromosomically abnormal cells from the rest of the embryo [10]. The above studies inspirated us to test the hypothesis that morphokinetic patterns may be associated with important manifestations of embryo viability. In this proof-of-concept study, we assessed possible relationships between embryo morphokinetics, as revealed by automatic morphokinetic annotation and prediction of development to blastocyst stage, with putative biomarkers of embryo quality, such as regulators and embryo growth and implantation. The Eeva system was chosen as a TLT device [11], while embryo score derived by such tool was compared with levels of miRNAs (miR-20a/miR-30c) and soluble human leukocyte antigen-G (sHLA-G) in spent culture media. miR-20a/miR-30c regulates functions with possible implication for embryo development and represent emerging indicators of embryo implantation competence [12]. sHLA-G has been studied for decades in relation to embryo development, with particular reference to the regulation of the maternal immune response during implantation [13–15]. sHLA-G has been previously reported to accumulate in embryo culture spent media, correlating with embryo quality and pregnancy rate [15, 16]. The purpose of our study is to assess whether embryo morphokinetics underpin biochemical and molecular regulatory patterns of development.

Materials and methods

This proof-of-principle study included embryos generated in standard IVF and ICSI cycles carried out between October 2016 and May 2018. Approval for the study was obtained from the local Institutional Review Board (document ref. R69/PA21 Rev. 0; data appl. 29 June 2016). Laboratory non-clinical data were used for research purposes only.

Diagnosis of infertility included various causes, including male factor, tubal factor, and polycystic ovary (PCO, but not PCOS) with or without chronic anovulation. Ovarian stimulation was carried out as previously described by Zacà et al. [17]. Major criterion for inclusion was at least 4 normally fertilized oocytes. Successful fertilization was assessed at 16–18 h postinsemination based on digital images acquired with Eeva system for ICSI or by conventional microscope for IVF (in the latter case, after fertilization check, embryos were placed into Eeva system). The Eeva system assesses the embryo according to early morphokinetic parameters, producing a score (1–5, best–worst) corresponding to higher/medium/lower chances of development to blastocyst. Embryo morphology was evaluated on day 3 (64–72 h postinsemination), based on the acquired digital images to generated Eeva scores. After day 3 of development, embryos were cultured up to blastocyst stage. SBM was collected from drops of media where blastocysts suitable for embryo transfer or cryopreservation were cultured, i.e., with a lower morphological grade limit of 2BC or 2CB and herein referred to as "viable".

MicroRNA isolation, retrotranscription, and amplification

Twenty-five microliters of SBM were used for column-based microRNA purification of miR-20a-5p and miR-30c-5p, followed by SYBR green based quantitative polymerase chain reaction (qPCR) with no need of preamplification. MicroRNA isolation from SBMs was conducted with miRCURY RNA Isolation Kit for Biofluids (EXIQON) according to manufacturer's instructions; elution was performed in 20 μl. Retrotranscription (RT) was conducted according to miRCURY LNA Universal RT assay (EXIQON) modifying only the sample input volume. In particular, 2 μl of 5X reaction buffer and 1 μl of enzyme mix were added to 6.5 μl of sample. During miRNA isolation and RT assay synthetic RNA spike-in have been added to provide a control for the quality of RNA isolation and cDNA synthesis. In particular, the UniSp2, UniSp4, and UniSp5 spike-in mix have been used during RNA isolation and UniSp6 during RT experiments. Finally, qPCR reaction was performed according to the individual protocol miRCURY LNA *SYBR® Green PCR* assay (EXIQON). The data obtained were analyzed using the iQ5 optical system software version 2.0 (Bio-Rad) including Genex Macro iQ5 Conversion and Genex Macro iQ5 files. Data were normalized using Genex Macro iQ5 file.

HLA-G Bio-Plex assay

Ten microliters of SBM were collected and subsequently assayed for sHLA-G using a bead array system Bio-Plex (Bio-Rad) according to the manufacturer's instructions. Briefly, 50 μl of sHLA-G standards (prepared in the same fresh culture medium and assayed in duplicate) or samples (embryo culture supernatants in duplicate) incubated with 50 μl of anti-sHLA-G conjugated beads (5000 beads/well) in 96-well filter plates for 60 min at room temperature with shaking. Plates were washed by vacuum filtration three times with 100 μl of Bio-Plex wash buffer, 25 μl of biotinylated antibody W6/32 (10 μg/ml) (Dako) was added, and plates were incubated for 30 min at room temperature with shaking. After three filter washes, 50 μl of streptavidin-phycoerythrin was added, and the plates were incubated for 15 min at room temperature with shaking. Finally, plates were washed by vacuum filtration three times, beads were suspended in 125 μl of Bio-Plex assay buffer, and samples were analyzed on the

Instrument Bio-Plex system in combination with the Bio-Plex Manager software. We calculated the mean of the fluorescence value obtained in negative control wells, present in triplicate on each plate, and SD, with the value of the lower limit of detection being 3.29 SD added to the mean FI. The specificity of this assay was validated with an isotype control (Mouse IgG1 Isotype control, code 1B-457-C100 Biotin; Exbio) used in the place W6/32 Biotin Moab. The background observed was lower than the selected detection limit. We obtained the limit of sensitivity at 1.0 pg/ml.

Statistical analysis

We verified the normal distribution of data using the Kolmogorov-Smirnov test with the Lilliefors' amendment and the Shapiro-Wilk test. Then, to explore correlation between selected miRNA/sHLA-G expression and Eeva scores, data were analyzed by Spearman's correlation coefficient (R). The differential expression analysis of miRNA and sHLA-G among the five categories according to Eeva scores was assessed by non-parametric Kruskall-Wallis test followed by post hoc tests. Statistical analysis was performed with SPSS v19.0 and the differences were considered statistically significant when P-value was less than 0.05.

Results

Forty-six individual cycles were included in the study. Average female age was 36.0 years (95% CI 35.07–37.49), while number of retrieved, inseminated and fertilized oocytes were 10.7 (95% CI 10.33–11.19), 9.5 (95% CI 9.16–10.8), and 7.0 (95% CI 6.64–7.46), respectively. Spent blastocyst culture media (SBM) was collected from a total of 172 viable blastocysts. Actual sample size was 136 and 141 for assessment of correlation between Eeva scores and miR-20a/miR-30c and sHLA-G, respectively.

Eeva scores and miR-20a/miR-30c levels in spent blastocyst media

A non-normal distribution of expression levels of miR-20a and miR-30c was observed (Supplementary Table 1 and Supplementary Figure 1; Supplementary Table 2 and Supplementary Figure 2; respectively). Therefore, non-parametric tests were performed in the following analysis.

Correlation of miR-20a-5p/miR-30c-5p expression profile and Eeva scores

To test the hypothesis of correlation between miRNA profiles and Eeva scores, Spearman's showed that concentration of miR-20a was progressively lower as Eeva score increased and probability of development to blastocyst decreased ($R = -0.209$, $N = 136$, $P = 0.016$, Table 1). The opposite trend was observed in the case of miR-30c, i.e., concentration was higher as score increased and chances of development to blastocyst decreased ($R = 0.248$, $N = 136$, $P = 0.004$, Table 1).

Comparison of miRNA expression profiles across Eeva categories

The distribution of miR-20a expression profiles did not show significant differences across categories of Eeva scores, as proved by Kruskal-Wallis test (Table 2). Therefore, multiple comparisons were not carried out.

Statistical analysis comparing miR-30c profiles across Eeva categories revealed differential expression levels (Kruskal-Wallis test, $P < 0.01$; Table 3). As Kruskal-Wallis test could not indicate which specific groups of our data were significantly different from each other, we performed pairwise comparisons as a post hoc analysis. The results indicated statistically significant differences, in terms of miR-30c expression levels, occurring between score 2 and score 5, score 4, and score 5 and finally between score 1 and score 5 (Table 4 and Supplementary Figure 4).

Eeva scores and sHLA-G levels in spent blastocyst medium

As for miRNA expression profiles, a non-normal distribution of expression levels of sHLA-G was observed (Supplementary table 3 and Supplementary figure 3). As a result, non-parametric tests were performed in the following analysis. To test the hypothesis of a correlation between sHLA-G expression levels and Eeva scores, a Spearman's correlation test was performed. Analysis revealed a negative correlation with Eeva score, i.e., levels were progressively lower as Eeva score increased and probability of development to blastocyst decreased ($R = -0.388$, $N = 141$, $P = 0.001$, Table 1).

Table 1 Spearman's Rho correlation coefficient with the associated p-value

Variables	Spearman's Rho correlation coefficient	Sig. (2-tailed)	N
miR-20a – Eeva SCORE	-.209(*)	.016	136
miR-30c – Eeva SCORE	.248(**)	.004	136
sHLA-G – Eeva SCORE	-.388(**)	.001	141

*Correlation is significant at the 0.05 level (2-tailed)

**Correlation is significant at the 0.01 level (2-tailed)

N sample size, *Sig. (2-tailed)* two-tailed p-value

Table 2 Kruskal-Wallis test of miR-20a expression profiles

	miR-20a
Kruskal-Wallis H - Chi-Square	6.341
df	4
Asymp. Sig.	.175

df degrees of freedom, *Asymp. Sig*. asymptotic significance

Comparison of sHLA-G expression levels among Eeva categories

The distribution of sHLA-G expression levels across Eeva categories was compared by Kruskal-Wallis test. A statistically significant difference was found (Table 5). Therefore, it was assumed that the distribution of sHLA-G was not the same across categories of Eeva score.

Pairwise comparisons, as a post hoc analysis demonstrated that statistically significant differences occurred only between score 1 and score 5 (Table 6 and Supplementary Figure 5).

Discussion

In order to better appreciate the significance of human embryo morphokinetics, in this study, we tested the hypothesis that embryo morphokinetic patterns reflect inherent developmental functions. Indeed, we found that morphokinetic scoring, based on algorithms that predict development to blastocyst stage, are associated with the amount of molecules, miR-20a/miR-30c, and sHLA-G in spent media, which are putative regulators of embryo growth and/or endometrial receptivity. Only very limited data are available on the involvement of miR-20a/miR-30c in the regulation of human preimplantation development. Evidence on sHLA-G is more copious, but relevant embryo morphokinetics data — as revealed by time-lapse microscopy — are lacking. Both factors support the design of this investigation.

Introduced in the IVF environment approximately a decade ago, TLT has changed observation of embryo morphology from static at isolated time points (usually once a day) to continued. This has allowed more precise recording of previously recognized developmental landmarks [1, 8] (e.g., the first cleavage) and revealed a plethora of other morphological changes, until recently unknown or only presumed [4, 10, 18, 19]. On this basis, several morphokinetic algorithms have been developed in the attempt to make the prediction of clinical or laboratory outcomes more accurate [5]. Implantation is certainly the clinical endpoint more intensely investigated. Algorithms were also developed to predict the risk of aneuploidy, as assessed by preimplantation genetic testing for aneuploidy (PGT-A) performed at the blastocyst stage on trophectoderm cells [20, 21]. Development to the blastocyst stage is another essential endpoint tested to assess the predictive power of embryo morphokinetics. In particular, the Eeva system assesses the embryo on the basis of early morphokinetic parameters, producing a score corresponding to higher/medium/lower chances of development to the blastocyst stage [11]. Uncertainties, however, remain also in such a case.

The lack of consensus surrounding TLT induces therefore to rethink this technology, in the attempt to better understand its biological significance and ultimately enhance the value of information derived from embryo morphokinetics. An exemplification of this concept may be found in the study of Tejera et al. [22]. They showed that too slow or to fast developmental paces were characterized by lower levels of oxygen consumption, while sustained oxygen rates were associated with optimal morphokinetic pattern and implantation: a clear example of association between morphokinetics and metabolism.

In this study, we intended to verify the relationships between the Eeva score that assigns a degree of probability of blastocyst formation and markers of embryo viability, i.e., levels of miR-20a, miR-30c, and sHLA-G in spent medium.

miRNas are short-length RNA molecules acting as gene expression regulators by silencing or influencing post-transcriptional utilization of previously synthesized RNAs [23]. Clearly, they are found intracellularly but can also be intercepted in extracellular compartments, where they are released in various packaging forms to mediate cell-to-cell interactions. Notably, among many physiological and pathological conditions, miRNAs are considered putative regulators of gamete/embryo function and implantation [24, 25].

We found miR-20a correlated with the Eeva scores, i.e., higher levels were found in spent media of embryos scored as 1 (corresponding to a higher probability of blastocyst development), with progressively lower levels associated with embryos scoring 2 to 5. In previous studies, experimental analysis of this miRNA indicated its involvement in the regulation of five genes (CCND1, MAPK1, PTEN, MYC, and NRAS), while in silico prediction suggested the regulation of two mRNAs (TCF7L1 and SOS1) playing a role in the proliferation and growth of endometrial cells [26]. In preimplantation embryos, miR-20a was ubiquitously detected in cells of mouse blastocyst [27] and found at higher concentrations in

Table 3 Kruskal-Wallis test of miR-30c expression profiles

	miR-30c
Kruskal-Wallis H-Chi-square	20.623
df	4
Asymp. Sig.	< 0.01

df degrees of freedom, *Asymp.Sig*. asymptotic significance

Table 4 Post hoc tests (pairwise comparisons of Eeva score). Each row tests the null hypothesis that the sample 1 and sample 2 distributions are the same. Asymptotic significances (2-sided tests) are displayed. The significance level is .05

Sample 1–sample 2	Test statistic	Std. error	Std. test statistic	Sig.	Adj. Sig.a
2–4	− 13.980	11.630	− 1.202	.229	1.000
2–1	16.814	9.773	1.720	.085	.853
2–3	− 22.715	10.642	− 2.134	.033	.328
2–5	− 47.698	10.754	− 4.435	< 0.01	< 0.01
4–1	2.834	11.121	.255	.799	1.000
4–3	8.735	11.893	.734	.463	1.000
4–5	− 33.718	11.993	− 2.812	.005	.049
1–3	− 5.901	10.084	− .585	.558	1.000
1–5	− 30.884	10.202	− 3.027	.002	.025
3–5	− 24.983	11.037	− 2.263	.024	.236

[a] Significance values have been adjusted by the Bonferroni correction for multiple tests

spent media of euploid human blastocyst with implantation ability [26]. Therefore, our data confirm that this molecule is extracellularly released during preimplantation development and are consistent with the characterization of miR-20a as a putative positive biomarker of developmental ability. In the present study, unlike miR-20a, we observed an inverse correlation between the probability of an embryo to develop to blastocyst stage, as predicted by the Eeva algorithm, and levels of miR-30c in spent media. This would indicate that miR-30c is a biomarker of reduced developmental competence. Such indication is in apparent contradiction with a previous study, in which this miRNA, in combination with miR-20a, was more associated with blastocyst with implantation ability [26]. It should be noted however, that the study of Capalbo et al. [26] had a much smaller sample size and, above all, a considerably different design, based on data derived from only blastocyst stage euploid embryos. In addition, in that study, samples were collected from spent media of embryos developed at different stages: cleavage, morula, and blastocyst. Our data are consistent with a study carried out in the bovine model [28]. That investigation revealed that miR-30c was more abundant in spent media of slow-cleaving embryos, which had reduced ability to develop to the blastocyst stage. Interestingly, following uptake of miR-30c mimics experimentally added to culture medium, bovine embryos showed enhanced blastomere apoptosis, down-regulation of a positive regulator of the cell cycle (cycling-dependent kinase 12, CDK12) and repression of several genes involved in the DNA damage response (DDR). Although comparable human data are not available, this evidence is in agreement with the proposition that miR-30c is as a negative regulation of cell viability, cell cycle progression, and DNA integrity; all functions predicted to be affected in embryos unable or less competent to develop to the blastocyst stage.

Table 5 Kruskall-Wallis test for sHLA-G levels

	sHLA-G
Kruskal-Wallis H- Chi-Square	12.377
df	4
Asymp. Sig.	.015

df degrees of freedom, *Asymp. Sig.* asymptotic significance

We also investigated the hypothesis that embryo morphokinetics, as assessed by Eeva, is associated with release of HLA-G in the spent medium. This molecule is believed to participate in the regulation of tissue interactions by which genetically "alien" fetal tissues are tolerated in a maternal environment in the course of pregnancy [29]. Specifically, regulation by HLA-G crucially occurs during implantation to modulate cytokine action and ultimately assist trophoblast cell penetration and assure spatially restricted immunotolerance. Several lines of evidence suggest that production and extracellular release of sHLA-G during embryo culture is positively associated with embryo developmental and implantation ability [16, 30–34]. We observed that sHLA-G levels associated with the best Eeva score (1) were higher compared with levels found in the worst Eeva score (5). Significant differences were not found between other scores. Such a partial association between sHLA-G levels and Eeva scores may reflect the relatively small size of the study samples. Clearly, this represents a limitation that should be addressed in future investigations. Restriction of both miRNA and sHLA-G detection to only embryos able to develop blastocysts, although of different morphokinetic grade, represents another limitation of this study. Regardless, we confirmed that higher potential to develop to blastocyst stage, as suggested by morphokinetic analysis, is associated with increased extracellular production of sHLA-G. Once released, this molecule could then act as a regulator at the maternal-fetal interface [29].

Table 6 Post hoc tests (pairwise comparisons of Eeva score). Each row tests the null hypothesis that the sample 1 and sample 2 distributions are the same. Asymptotic significances (2-sided tests) are displayed. The significance level is .05

Sample 1–sample 2	Test statistic	Std. error	Std. test statistic	Sig.	Adj. Sig.a
5–3	14.725	11.142	1.322	.186	1.000
5–4	16.205	12.248	1.323	.186	1.000
5–2	24.414	11.142	2.191	.028	.284
5–1	35.367	10.514	3.364	.001	.008
3–4	− 1.480	11.867	− .125	.901	1.000
3–2	9.690	10.722	.904	.366	1.000
3–1	20.642	10.067	2.050	.040	.403
4–2	8.209	11.867	.692	.489	1.000
4–1	19.162	11.279	1.699	.089	.893
2–1	10.952	10.067	1.088	.277	1.000

[a] Significance values have been adjusted by the Bonferroni correction for multiple tests

Conclusions

The results of this study confirm the hypothesis that morphokinetic patterns are associated with important manifestations of embryo viability. Our data suggest that morphokinetic algorithms that predict development to blastocyst stage, in fact, also identify embryos with molecular and cellular profiles more consistent with functions required for growth and implantation, such as cell cycle/apoptosis regulation, proliferation/growth of endometrial cells, and immune-modulation of maternal-embryonic interactions. In ART, this lends credit to the approach of using morphokinetic algorithms for the selection of more viable embryo to prioritize for transfer.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10815-021-02162-9.

Acknowledgements This research was financially supported by Merck Serono S.p.A., Italy, an affiliate of Merck KGaA, Darmstadt, Germany

References

1. Meseguer M, Herrero J, Tejera A, Hilligsøe KM, Ramsing NB, Remoh J. The use of morphokinetics as a predictor of embryo implantation. Hum Reprod. 2011;26:2658–71.
2. Basile N, Nogales MDC, Bronet F, Florensa M, Riqueiros M, Rodrigo L, et al. Increasing the probability of selecting chromosomally normal embryos by time-lapse morphokinetics analysis. Fertil Steril. 2014;101:699–704.e1.
3. Basile N, Vime P, Florensa M, Aparicio Ruiz B, García Velasco JA, Remohí J, et al. The use of morphokinetics as a predictor of implantation: a multicentric study to define and validate an algorithmfor embryo selection. Hum Reprod. 2015;30:276–83.
4. Rubio I, Kuhlmann R, Agerholm I, Kirk J, Herrero J, Escribá MJ, et al. Limited implantation success of direct-cleaved human zygotes: a time-lapse study. Fertil Steril. 2012;98:1458–63.
5. Petersen BM, Boel M, Montag M, Gardner DK. Development of a generally applicable morphokinetic algorithm capable of predicting the implantation potential of embryos transferred on Day 3. Hum Reprod. 2016;31:2231–44.
6. Kirkegaard K, Campbell A, Agerholm I, Bentin-Ley U, Gabrielsen A, Kirk J, et al. Limitations of a time-lapse blastocyst prediction model: a large multicentre outcome analysis. Reprod BioMed Online. 2014;29:156–8.
7. Apter S, Ebner T, Freour T, Guns Y, Kovacic B, Le Clef N, et al. Good practice recommendations for the use of time-lapse technology†. Hum Reprod Open. 2020;2020:1–26.
8. Coticchio G, Renzini MM, Novara PV, Lain M, De Ponti E, Turchi D, et al. Focused time-lapse analysis reveals novel aspects of human fertilization and suggests new parameters of embryo viability. Hum Reprod. 2018;33:23–31.
9. McCoy RC, Newnham LJ, Ottolini CS, Hoffmann ER, Chatzimeletiou K, Cornejo OE, et al. Tripolar chromosome segregation drives the association between maternal genotype at variants spanning PLK4 and aneuploidy in human preimplantation embryos. Hum Mol Genet. 2018;27:2573–85.
10. Lagalla C, Tarozzi N, Sciajno R, Wells D, Di Santo M, Nadalini M, et al. Embryos with morphokinetic abnormalities may develop into euploid blastocysts. Reprod Biomed Online [Internet]. Elsevier Ltd; 2017;34:137–46. Available from: https://doi.org/10.1016/j.rbmo.2016.11.008
11. Conaghan J, Chen AA, Willman SP, Ivani K, Chenette PE, Boostanfar R, et al. Improving embryo selection using a computer-automated time-lapse image analysis test plus day 3 morphology: Results from a prospective multicenter trial. Fertil Steril. 2013;100:412–419.e5.
12. Cimadomo D, Rienzi L, Giancani A, Alviggi E, Dusi L, Canipari R, et al. Definition and validation of a custom protocol to detect miRNAs in the spent media after blastocyst culture: Searching for biomarkers of implantation. Hum Reprod. 2019;34:1746–61.
13. Nowak I, Wilczyńska K, Radwan P, Wiśniewski A, Krasiński R, Radwan M, et al. Association of Soluble HLA-G Plasma Level and HLA-G Genetic Polymorphism With Pregnancy Outcome of Patients Undergoing in vitro Fertilization Embryo Transfer. Front Immunol. 2019;10:2982.
14. Rodríguez Díaz R, Blanes Zamora R, Vaca Sánchez R, González Pérez J, Alberto Bethencourt JC. Embryo sHLA-G secretion is related to pregnancy rate. Zygote. Cambridge University Press. 2019;27:78–81.
15. Rizzo R, Vercammen M, Van De Velde H, Horn PA, Rebmann V. The importance of HLA-G expression in embryos, trophoblast

cells, and embryonic stem cells. Cell Mol Life Sci. 2011;68:341–52.
16. Fuzzi B, Rizzo R, Criscuoli L, Noci I, Melchiorri L, Scarselli B, et al. HLA-G expression in early embryos is a fundamental prerequisite for the obtainment of pregnancy. Eur J Immunol. 2002;32:311–5.
17. Zacà C, Bazzocchi A, Pennetta F, Bonu MA, Coticchio G, Borini A. Cumulative live birth rate in freeze-all cycles is comparable to that of a conventional embryo transfer policy at the cleavage stage but superior at the blastocyst stage. Fertil Steril [Internet]. 2018;110:703–9. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0015028218304138.
18. Lagalla C, Coticchio G, Sciajno R, Tarozzi N, Zacà C, Borini A. Alternative patterns of partial embryo compaction: prevalence, morphokinetic history and possible implications. Reprod Biomed Online [Internet]. Elsevier Ltd; 2020;40:347–54. Available from: https://doi.org/10.1016/j.rbmo.2019.11.011
19. Liu Y, Chapple V, Roberts P, Matson P. Prevalence, consequence, and significance of reverse cleavage by human embryos viewed with the use of the embryoscope time-lapse video system. Fertil Steril. 2014;102:1295–1300.e2.
20. Campbell AJ, Fishel SB, Duffy S, Montgomery S. Embryo selection model defined using morphokinetic data from human embryos to predict implantation and live birth. Fertil Steril. 2013;100:S502.
21. Campbell A, Fishel S, Bowman N, Duffy S, Sedler M, Hickman CFL. Modelling a risk classification of aneuploidy in human embryos using non-invasive morphokinetics. Reprod Biomed Online. 2013;26:477–85.
22. Tejera A, Castelló D, de los Santos JM, Pellicer A, Remohí J, Meseguer M. Combination of metabolism measurement and a time-lapse system provides an embryo selection method based on oxygen uptake and chronology of cytokinesis timing. Fertil Steril. 2016;106:119–126.e2.
23. Gebert LFR, MacRae IJ. Regulation of microRNA function in animals. Nat Rev Mol Cell Biol. 2019;20:21–37.
24. Salas-Huetos A, James ER, Aston KI, Jenkins TG, Carrell DT, Yeste M. The Expression of miRNAs in human ovaries, oocytes, extracellular vesicles, and early embryos: a systematic review. Cells. 2019;8:1–23.
25. Yuan S, Schuster A, Tang C, Yu T, Ortogero N, Bao J, et al. Sperm-borne miRNAs and endo-siRNAs are important for fertilization and preimplantation embryonic development. Dev. 2016;143:635–47.
26. Capalbo A, Ubaldi FM, Cimadomo D, Noli L, Khalaf Y, Farcomeni A, et al. MicroRNAs in spent blastocyst culture medium are derived from trophectoderm cells and can be explored for human embryo reproductive competence assessment. Fertil Steril. 2016;105:225–235.e3.
27. Foshay KM, Gallicano GI. miR-17 family miRNAs are expressed during early mammalian development and regulate stem cell differentiation. Dev Biol [Internet]. Elsevier Inc.; 2009;326:431–43. Available from: https://doi.org/10.1016/j.ydbio.2008.11.016
28. Lin X, Beckers E, Mc Cafferty S, Gansemans Y, Joanna Szymańska K, Chaitanya Pavani K, et al. Bovine embryo-secreted microRNA-30c is a potential non-invasive biomarker for hampered preimplantation developmental competence. Front Genet [Internet]. Frontiers Media S.A.; 2019 [cited 2020 Aug 14];10:315. Available from: https://www.frontiersin.org/article/10.3389/fgene.2019.00315/full.
29. Nilsson LL, Djurisic S, Hviid TVF. Controlling the immunological crosstalk during conception and pregnancy: HLA-G in reproduction. Front Immunol. 2014;5:198.
30. Sher G, Keskintepe L, Nouriani M, Roussev R, Batzofin J. Expression of sHLA-G in supernatants of individually cultured 46-h embryos: a potentially valuable indicator of "embryo competency" and IVF outcome. Reprod BioMed Online. Reproductive Healthcare Ltd. 2004;9:74–8.
31. Yie SM, Balakier H, Motamedi G, Librach CL. Secretion of human leukocyte antigen-G by human embryos is associated with a higher in vitro fertilization pregnancy rate. Fertil Steril. 2005;83:30–6.
32. Noci I, Fuzzi B, Rizzo R, Melchiorri L, Criscuoli L, Dabizzi S, et al. Embryonic soluble HLA-G as a marker of developmental potential in embryos. Hum Reprod. Oxford University Press. 2005;20:138–46.
33. Rebmann V, Switala M, Eue I, Grosse-Wilde H. Soluble HLA-G is an independent factor for the prediction of pregnancy outcome after ART: a German multi-centre study. Hum Reprod. Oxford University Press. 2010;25:1691–8.
34. Kotze D, Kruger TF, Lombard C, Padayachee T, Keskintepe L, Sher G. The effect of the biochemical marker soluble human leukocyte antigen G on pregnancy outcome in assisted reproductive technology - a multicenter study. Fertil Steril. 2013;100:1303–9.