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Droplet Digital PCR Analysis of miR-191-3p in the Spent Blastocyst Culture Media Might Reflect the Reproductive Competence of the 3rd Day Human Embryo

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Abstract

Background: Droplet digital PCR analysis of human embryonic culture media collected in a proof of con-cept study on IVF pregnancy outcome. miR-191-3p was analysed in spent human embryonic culture media of morphologically similar, good quality embryos which after later transfer developed either to reproductively competent embryos and healthy neonates or of those, where miscarriage occurred in early stage of the pregnancy.

Methods: Spent culture media at the morula-blastocyst stage (3rd day) were collected from embryos fertilised with ICSI and undergoing embryo transfer. After registered pregnancy outcome 40 samples from the group of reproductively competent embryos, 40 samples from miscarriage and their parallel blank culture media samples were enrolled in miRNA analysis. Isolation and quantitative detection of miRNA from embryonic culture media was carried out on automated droplet digital polymerase chain reaction platform.

Results: Quantitative analysis and ANOVA evaluation confirmed miR-191-3p to be present in significantly higher concentration in the 3rd day culture media of reproductively competent human embryos (mean concentration difference=20,478, $p=1 \times 10^{-4}$) than the miscarriage. Control blank culturing media were negative for miR-191-3p.

Conclusion: miR-191-3p present in human blastocyst culture media indicates actual embryonic origin and changed in expression patterns depending on clinical outcome after transfer. It might be a candidate molecular marker for pre-implantation assessment of reproductive embryo competence in a non-invasive way.

Keywords: Non-invasive pre-implantation embryo assessment; Embryonic culture media; *In vitro* fertilisation; miRNA; Droplet digital polymerase chain reaction

Introduction

MicroRNAs (miRNAs), in their mature form, represent the class of 19-23 nucleotide size endogenous non-coding, but functional repressors of the molecular mechanism of mRNA translation. They influence the output of regulatory and protein coding genes in target-specific manner through partial sequence complementation on the 3' untranslated regions of their mRNA transcript and inhibit the protein synthesis on the ribosome mRNA complex [1]. Studies confirmed differential expression of miRNA during embryo development from morula to blastocyst stages [2], and expressional differences between the euploid and aneuploid embryos [3]. Cell free miRNAs also known as secretory miRNAs are an emerging class of miRNAs that are released by cells to the extracellular space via vesicular secretion. Small vesicles-that are called exosomes-protect their nucleic acid content from enzymatic degradation securing their high stability in laboratory detection procedures. Cell-free miRNA have been found to correlate with a wide variety of diseases including cancer and other chronic non-infectious diseases, infections, tissue injury. They have been detected in various body fluids such as blood, serum, saliva, urine, tear, cerebrospinal fluid, peritoneal-fluid, breast milk, semen, amniotic fluid [4]. There is a great interest in identifying miRNAs in the culture media of developing embryos. Spent blastocyst culture media was found to be appropriate to represent cell-free miRNA fraction that is originating from embryonic secretion [5]. Some miRNAs of the culture media correlated with embryonic aneuploidy and IVF failure enhancing the possibility of a non-invasive embryo-quality assessment [6]. All of the studies published in the literature applied quantitative real-time PCR and high-density oligonucleotide array-based methods combined with RNA pre-amplification prior to detection have already been used to quantify the miRNA fraction secreted by the trophectoderm cells of the embryo on the 4th-5th days at late blastocyst stage of the embryos. Amplification of the isolated total cell-free RNA content of the media can easily influence the miRNA quantity detected downstream and distort the original miRNA distribution. To exclude the pre-amplification step in the non-invasive miRNA detection from embryonic culture media, we turned toward a recently emerging quantitative technology that have higher sensitivity and require lower template nucleic acid input.

Our main goal was to analyse cell-free miRNA that is in silico correlated with early morula-blastocyst development by using the spent embryonic culture media to avoid any disturbance of the embryo and ddPCR method to be able to exclude the pre-amplification step from the detection workflow.

Materials and Methods

We aimed to profile miRNA expression patterns of 40 spent culture media samples of human embryos derived from patients undergoing intracytoplasmic sperm injection (ICSI) in the Assisted Reproduction Unit, Department of Obstetrics and Gynaecology, University of Pecs, Hungary during June/2016-June/2017. The work described here was approved by the Committee of Human Reproduction, National Science Council of Hungary: 5273-3-2012/HER, later superset by Public Health Officer Hungarian Government Office in Baranya County: BAR/006/58-2/2014.) The research related to human use has been complied with all the relevant national regulations, institutional policies and in accordance the tenets of the Helsinki Declaration.

IVF procedure and sample collection

The oocytes selected for ICSI were denuded with hyaluronidase and were assessed for maturity. Complete denudation of the oocytes was performed during this preparation procedure. Only metaphase II oocytes, identified by the presence of the first polar body, were chosen for fertilisation. Intracytoplasmic sperm injection was performed 3-6 hours after oocyte recovery in a bicarbonate-buffered medium (G-IVF, Vitrolife). Fertilisation was assessed 24 hours later in G-1 v5 medium (Vitrolife) supplemented with human serum albumin (HSA; Vitrolife) in a 5 mg/mL concentration. Fertilised oocytes were sequentially cultured in separate media droplets of 40 µl volume for each embryo. Embryos were moved to fresh medium droplets on day 3 and 20 µl of the spent medium were collected and stored at -80°C for miRNA analysis. Blank media control drops were collected from the same LOT of medium and HSA Embryo transfer procedures were performed after sample collection on day 3. All embryos were found to fulfil good or fair composite score on the "optimized scoring system" morphological evaluation [7]. Samples were split into two groups according to pregnancy outcome: reproductively competent embryos that developed during complication-free pregnancies into healthy neonates or miscarriage. One or two embryos were transferred on patient's request. We enrolled individual culture media samples from embryos undergoing double embryo transfer if the pregnancy outcome was consequent: if both embryos developed to healthy neonates or miscarriage occurred. Study workflow is presented in Figure 1.

RNA isolation and reverse transcription

Cell free total RNA was isolated from 40 µl of each spent media sample and the blank controls collected using the miRNeasy serum/plasma kit (Qiagen, Hilden, Denmark) Isolated RNA quality was checked on Thermo Scientific NanoDropTM 2000 (Thermo Fisher Scientific, Grand Island, NY) 5 ng total RNA from each sample was reverse transcribed with miRCURY LNA Universal RT microRNA PCR Kit (Qiagen).

Droplet digital PCR analysis

For quantitative PCR analysis we purchased Qiagen miRCury LNA miRNA PCR assays (Qi-agen, Hilden, Denmark) according hsa-miR-191-3p. Master mixes were prepared to contain 1 µl of miRCury LNA miRNA assay containing the target-miRNA specific forward and reverse primer pair, 12 µl of QX200 ddPCR EvaGreen Super mix (Bio-Rad) and 9 µl PCR grade water and 2 µl (~100 pg) of cDNA sample. For positive control we used reverse transcript of total RNA fraction isolated from healthy adult serum. On the plate we used an approx. 10 ng/µl and a 10 pg/µl template concentration of the positive control. For negative controls we pre-pared a master mix where template cDNA was substituted with PCR grade water (no template control=NTC and a master mix where template cDNA and primer were both substituted with water (no primer control=NTC+NPC). From each master mix droplets were generated in the automated droplet generator unit of QX200 automated ddPCR system using Droplet Generation Oil for EvaGreen (Bio-Rad). PCR amplification was carried out in the QX200 Thermo cycler with the following thermic conditions (enzyme activation 1 cycle: 95°C for 5 min; amplification 40 cycles {96°C for 30s and 58-60°C primer dependently for 1 min}, signal stabilization 1 cycle {4°C for 5 min and 90°C for 5 min} and hold on 4°C. Droplets were immediately analysed after the PCR reaction in the QX200 Droplet Reader. Fluorescence data were converted into concentrations according to the Poisson distribution statistical analysis used by the QuantaSoft® analysis Pro software version 1.0.596 (Bio-Rad, CA, USA).

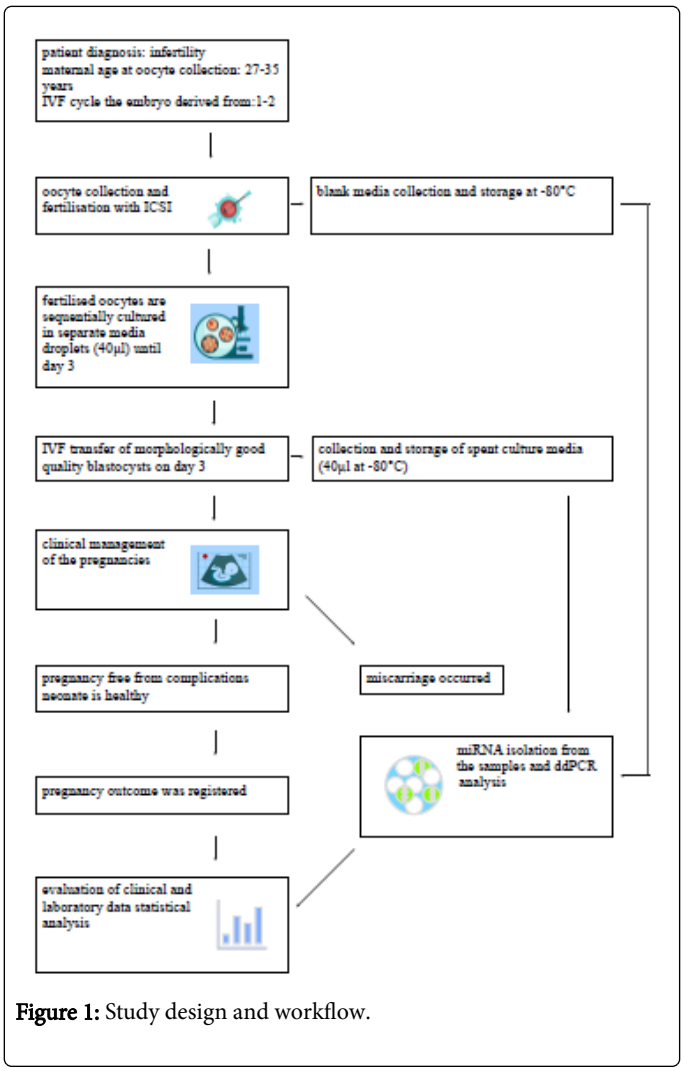

Figure 1: Study design and workflow.

Statistical analysis

Concentration data from QuantaSoft software were exported and analysed for quantitative differences between miscarriage [0], reproductively competent embryos [1] and blank media [2] study groups on IBM SPSS Statistics 21.0 using ANOVA test (with $p \leq 0.01$)with the LSD post hoc analysis.

(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	p value	95% Confidence Interval	
					Lower Bound	Upper Bound
miscarriage	competent embryo	-20.478	1.041	0.0001	-22.546	-18.41
	blank media	3.733	1.127	0.0013	1.495	5.972
competent embryo	miscarriage	20.478	1.041	0.0001	18.41	22.547
	blank media	24.212	1.16	1×10^{-16}	21.907	26.517
blank media	miscarriage	-3.733	1.127	0.0013	-5.972	-1.496
	competent embryo	-24.212	1.16	1×10^{-16}	-26.517	-21.908

Table 1: Statistical analysis. Results of ANOVA and LSD posthoc analysis of miR-191-3p concentrations measured in the culture media of reproductively competent embryos, embryos who failed to induce pregnancy (miscarriage) and control blank media samples. The mean difference was considered to be significant at the 0.005 ≥ level.

Results

We were able to detect miR-191-3p in the spent culture media samples of 3rd day human embryos cultured *in vitro*, while it was not was not detected in the similarly kept blank media samples (Figure 2). We found significant differences of the miR-191-3p concentrations between the study groups on the ANOVA test.

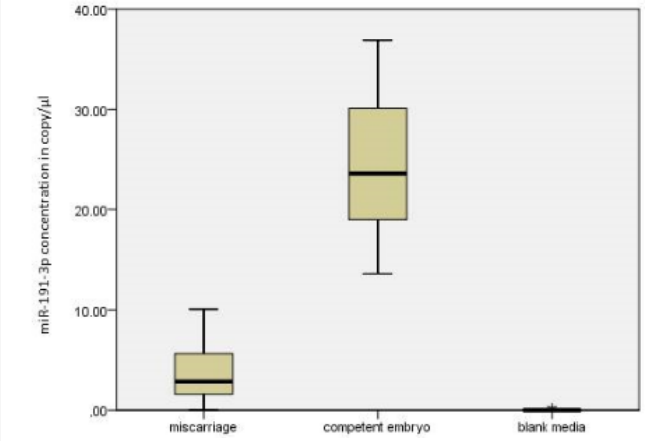

Figure 2: Automated droplet digital analysis of miR-191-3p. Samples A01-F01: culture media samples from the miscarriage group, G01-H01: blank media droplets of the miscarriage group. A06-F06: culture media samples from the reproductively competent embryo group G06-H06: blank culture media droplets of the reproductively competent embryo group. A08: positive control In 10 pg/µl concentrations, B8: positive control In 10 ng/µl concentrations B11: negative control (NTC+NPC). Y axis show miR-191-3p expression on the fluorescent detection channel (530 nm) amplitude with the positive (target miRNA containing) droplet counts (colored blue) and negative droplets (colored grey).

The blank media was negative for miR-191-3p and differed significantly from the media samples of the miscarriage group, which had mean concentration value of 3,733 copy/µl ($p=1,3 \times 10^{-3}$). The media samples from reproductively competent embryo group showed significantly higher miR-191-3p absolute quantity in the group compared to both the miscarriage group (mean concentration difference=20,487 copy/µl $p=1 \times 10^{-4}$) and the blank media samples (mean concentration difference=24,212 copy/µl; $p=1 \times 10^{-16}$) (Figures 2 and 3, Table 1).

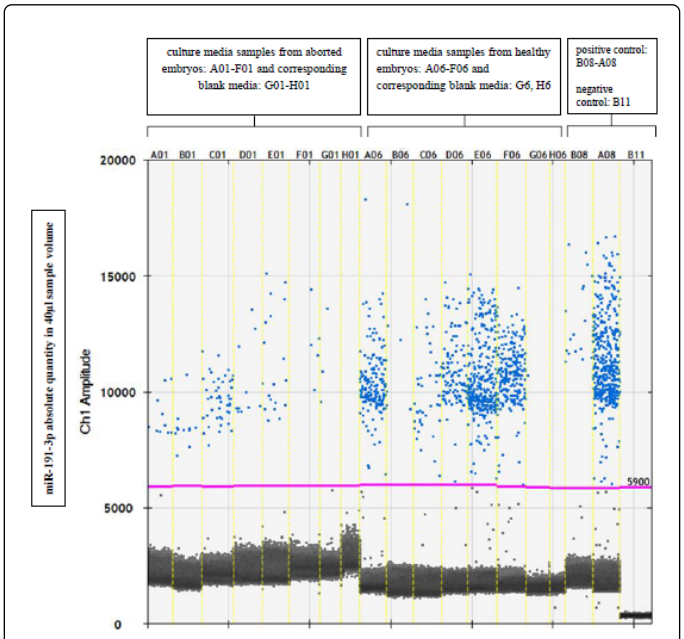

Figure 3: Comparison of miR-191-3p expression values in the blastocyst culture media of the different study groups. Boxplots represent miR-191-3p concentrations in copy/µl measured in the culture media of reproductively competent embryos, embryos who failed to induce pregnancy (miscarriage) and control blank media samples.

Discussion

Assisted reproductive techniques (ARTs) are used worldwide. In Europe, the prevalence of children, who have born from ARTs ranges from 1.4-5.9%, depending on the country, and it increases by 0.1-0.2% each year [8]. Current selection of the best embryo for transfer during an IVF cycle is relying on morphologic criteria. At the early blastocyst

stage embryos with better morphology are more likely to have normal chromosomal content and more likely to implant and result pregnancy. It is known that many well-developed, morphologically good quality blastocyst may still be chromosomally abnormal, will not implant or will not develop to produce pregnancy [9-12]. Thereby an intensive research interest is focusing on finding a non-invasive biomarker that will allow better selection of the best embryo beyond morphologic criteria. Such a biomarker could lead to higher pregnancy rates per transfer, and reduce the rate of multiple gestations. Several potential embryonic biomarkers have been recently investigated: secreted proteins, metabolomics profiles have been identified in embryonic culture media [13,14]. There is mounting evidence that miRNA can potentially regulate every aspect of cellular function including growth, proliferation, differentiation, development, metabolism, infection, immunity, cell death, organellar biogenesis, messenger signaling, DNA repair and self-renewal [15]. Rapidly growing undifferentiated cells, such as embryonic stem cells or dedifferentiated cells, like cancer cells, highly express miRNA. Intracellular miRNA expression patterns are very specific for certain genomic malfunction including some aneuploid states [16]. Previous studies which aimed to assess chromosomal aneuploidy and implantation failure from spent culture media in non-invasive way chose miRNA structures to use as viability biomarkers due to their high resistance to environmental degradation and reproducibility. They were successful in the identification of miRNA candidates that are strongly correlated with either aneuploidy status or pregnancy outcome [5,6,16,17]. However, as a matter of fact, minimal or no overlap can be seen among the reported biomarkers. This lack of overlap is mainly attributable to the different study protocols (different insemination technique of the oocyte, media collection in different embryonic developmental phase, wide variety of culture media used), that no stable diagnostic protocol is available yet. There is evidence in the literature, that these circumstances can substantially affect miRNA expression and vesicular secretion [18,19]. Although early-cleavage embryos are able to grow in a huge variety of culture conditions, these conditions do matter and are known to have an effect on gene expression [20]. The potential impact on different culture conditions or different components of embryo culture media on pre-implantation embryos has been the subject of intense discussion [21]. Droplet digital PCR (ddPCR) represents a highly sensitive and reproducible method that can serve as an appropriate tool for miRNA quantification. In contrast to real-time quantitative PCR performed in one single reaction volume, ddPCR allows for the partitioning of the sample into 1-2 × 10^4 Nano liter droplets, which all constitute as individual nanoreactors. Each droplet contains either zero or one copy of the target nucleic acid (DNA or cDNA molecule) following Poisson distribution [22]. After primer specific PCR reaction in presence of double strain saturating fluorescein, the counting of positive and negative events reveals the absolute quantity of targets initially present in the tested sample. In this work we hypothesised that by using ddPCR we can detect expression pattern differences of a set of embryonic cell-specific miRNAs in the morula-blastocyst developmental phase from the 3rd day spent human blasto-cyst culture medium according to pregnancy outcome. We performed early blastocyst culture media analysis, because if the time point for embryo viability assessments could be shifted to the 3rd day, a non-invasive complex embryo selection algorithm (including morphologic and genomic assessment) could be carried out within 4-8 hours (depending on sample number and number of analysed targets) and the blastocyst transfer would be feasible in the subsequent natural cycle planned between day 3 and 5 and cryopreservation of the blastocysts could be minimized or omitted. Compared to quantitative real-time PCR, ddPCR technique allowed precise quantification of rare events thereby enabled us to identify even extremely low amounts of miRNAs present in the culture media. Running cost was a bit higher than cost of a quantitative real-time PCR patforms but there is no difference in pre-analytical requirements from those in routine clinical workflow. We designed our study to focus on miR-191-3p based on in silico selection (miRBASE) for reliable embryo specific miRNA that is found to have key role in previous human and murine embryonic cells.

In our recent work we identified miR-191-3p to be present in in the culture media of 3rd day human embryos and the concentration was found to be significantly higher in case of the re-productive competence of the embryo. Our recent results support the importance of miR-191-3p for early stage blastocyst assessments on highly sensitive quantitative analytical platforms. The exact role of miR-191-3p has not been completely clarified for early human blastocysts. It was found to be significantly changed in concentration in the human spent embryonic culture media by a study from Rosenbluth et al. In 2014, they cultured cryopreserved embryos and subsequently fresh oocytes for same cycle single embryo transfer in three different experimental series. During sequential culturing they used different culture media and higher con-centration of serum protein substitute. In miRNA quantification they used RNA pre-amplification step, different primer design and qPCR analysis. In the results they confirmed miR-191 to reach higher concentration in aneuploidy embryos and failed IVF (non ICSI) cycles, which can suggest just the opposite role of miR-191 that we conclude upon our higher miR-191-3p concentration results in the reproductively competent embryo group. For the multiple differences that can harshly influence embryonic gene expression regulatory mechanisms (cryopreservation, fertilization method, culture media contents, and miRNA quantification technique) it is difficult to compare their results with our findings. The only similarity, that neither they, nor our group detected miR-191 in the control media droplets, which can confirm embryonic origin of this molecule. In 2015 Mahdipour et al. searched for reliable miRNA normalizers in bovine and porcine embryonic culture media. Different embryo species, continuous culturing method, different workflow and quantification method was used than in our current project. Their quantitative real-time PCR analysis of bovine and porcine two-to-eight cell embryos, morulae and blastocysts confirmed a stable expression of miR-191 as a marker ready to be a miRNA expression normalizer [23]. In 2016 Capalbo et al. conducted a study in which they sequentially cultured fertilized oocytes and analysed cleavage stage and matured blastocysts and their culture medium. After day 3 they used cryopreservation, vitrification and warming procedures and chromosomal aneuploidy screening prior to implantation. They gained miRNA quantitative results by using RNA pre-amplification and TaqMan low density arrays including 381 miRNA assays. They could find miR-191 in the culture media of matured blastocysts (day 5) but not at the cleavage stage and miR-191 was not correlated with aneuploidy. In all of the aforementioned studies, culturing protocols, techniques and embryo sample numbers are different from ours. We avoided cryopreservation and PGT and pre-amplification during our experiment, we used different culture media and extremely low-load quantification platform and we were able to detect miR-191-3p from culture media at the cleavage stage. Comparing all these evidences, the role of miR-191 in human blastocyst development is still hard to define. In mouse embryonic stem cells (ESCs) high expression of miR-191 repressed mesendoderm differentiation through direct targeting of Smad2 and subsequently

had post-transcriptional control on Activin/Nodal signaling, which resulted con-trolled cell proliferation [24,25].

Conclusion

Based on our own data and data from the literature it is quite clear that miRNAs which appear in the embryonic culture media might serve as indicators for embryo viability, but cannot be limited to a fix panel of sequences until environmental culturing conditions (different culture media and protein supplement products are available on the market) and interventional procedures (insemination procedures, cryopreservation, etc.) are differing so much. Early blastocysts are physiological singularities seem to be highly adaptive to their environment, and might establish their genomic responses to their niche. To be able to define dynamic embryonic adaptation capacity molecular characterization of embryos is needed to be involved adjacently to morphological evaluation algorithm. To support this aim we can assume that precisely selected miRNA predictors should be developed accordingly to IVF techniques and culturing conditions. Beside the multiple experimental differences between our and the previous studies, and the fact, that our low sample size is limiting the interpretation of our data, this is the first study to underlie the potential significance of miR-191-3p in 3rd day *in vitro* embryo culturing excluding cryopreservation, vitrification and all invasive pre-implantation genetic testing methods. Using ddPCR analysis we can confirm miR-191-3p to be appropriate candidate for embryonic culture media-based non-invasive blastocyst assessment.

Conflict of Interest and Author Contributions

None of the authors have any financial, personal or professional conflict of interests in connection with the content of the manuscript. All the authors have accepted responsibility for the entire content of the submitted manuscript and approved submission.

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