Journal of Assisted Reproduction and Genetics (2022) 39:117–125
https://doi.org/10.1007/s10815-021-02368-x

EMBRYO BIOLOGY

GDF9 concentration in embryo culture medium is linked to human embryo quality and viability

Jingyu Li[1,2] · Chong Li[1,2] · Xuemei Liu[1,2] · Jingwei Yang[1,3] · Qi Zhang[1,3] · Wei Han[1,2] · Guoning Huang[1,2,3]

Received: 30 August 2021 / Accepted: 24 November 2021 / Published online: 29 November 2021

Abstract

Purpose We aimed to evaluate the link between the GDF9 concentration in day 3 human embryo culture medium and embryo quality and viability.

Methods Two independent, prospective, observational studies were conducted. In study 1, a total of 280 embryos from 70 patients who obtained at least 4 embryos with 6–10 blastomeres (2 transferable and 2 non-transferable embryos) at day 3 were enrolled. In study 2, a total of 119 embryos from 61 patients (29 fully implanted and 32 non-implanted patients) were enrolled. The corresponding GDF9 concentrations in spent culture medium of embryos were quantified by ELISA assay. The expression pattern of GDF9 in human embryos was investigated using Q-PCR and immunofluorescence.

Results GDF9 mRNA and protein were detected from human oocytes to eight-cell embryos and displayed a slow decreasing trend. In study 1, GDF9 concentration in culture medium is lower for transferable embryos compared with non-transferable embryos (331 pg/mL (quartiles: 442, 664 pg/mL) vs. 518 pg/mL (quartiles: 328, 1086 pg/mL), $P < 0.001$), and increased commensurate with the diminution of the embryo quality ($P < 0.001$). In study 2, significantly lower GDF9 concentration was detected for implanted embryos than non-implanted embryos (331 pg/mL (quartiles: 156, 665 pg/mL) vs. 518 pg/mL (quartiles: 328, 1086 pg/mL), $P < 0.001$). The same trend was found between the embryos that led to live birth and those that failed.

Conclusion The GDF9 concentration in culture medium is linked to embryo quality and viability, and exhibited the potential to be a non-invasive biomarker for embryo selection.

Keywords GDF9 · Culture medium · Human embryos · Embryo quality · Implantation · Live birth

Introduction

Infertility has become a major public health issue worldwide, and the need for assisted reproductive technology (ART) has therefore increased. Since the birth of the first baby from in vitro fertilization (IVF) [1], ART has become increasingly widespread. Despite improvements in assisted reproduction technologies and embryo culture conditions [2–5], current success rates remain unsatisfactory [6].

Improving the accuracy of selecting embryos with the greatest developmental potential may be expected to increase the chances of healthy delivery. Embryo selection for transfer is currently primarily based on morphologic parameters, including the degree of fragmentation, blastomere size, and the rate of embryonic cleavage [7]. However, morphologic assessment does not reflect the true status of the embryo [8], e.g., it is well known that even embryos that are scored as "good quality" may

The authors consider that Jingyu Li and Chong Li should be regarded as joint first authors.

✉ Jingyu Li
cqtnljy@gmail.com

✉ Guoning Huang
gnhuang217@sina.com

[1] Chongqing Reproduction and Genetics Institute, Chongqing Health Center for Women and Children, No.64 Jin Tang Street, Yu Zhong District, Chongqing 400013, China

[2] Chongqing Key Laboratory of Human Embryo Engineering, Chongqing, China

[3] Chongqing Clinical Research Center for Reproductive Medicine, Chongqing, China

not result in a successful pregnancy [9]. Non-invasive approaches of embryo assessment have recently been proposed, especially with respect to metabolomic analysis of the embryonic culture medium [10–13]. Such analyses are principally achieved by the measurement of glucose [14], pyruvate [15], and amino acids in the spent culture medium [16]. However, these biomarkers have not yet been proven to be of true clinical significance. Therefore, there is a need to identify novel non-invasive biomarkers for improving the accuracy of embryo selection.

Growth differentiation factor 9 (GDF9) is a key oocyte-secreted factor and a member of the transforming growth factor beta (TGF-β) superfamily [17] that is essential for the initial development of ovarian follicles [18]. Knockout of GDF9 in mice led to attenuation of granulosa cell proliferation, abnormal oocyte growth, and obstructed folliculogenesis at the primary stage [19]. Investigators have previously demonstrated that higher GDF9 levels in ovarian follicular fluid were significantly correlated with oocyte maturation and embryo quality [20]. In addition, Li, Li [21] reported that the expression levels of GDF9 in cumulus granulosa cells can be used as a new biomarker for predicting oocyte developmental potential. However, the procedure of using biomarkers from follicular fluid or cumulus granulosa cells for embryonic assessment presents limitations to clinical application, as it is difficult in an actual clinical setting to collect the respective follicular fluid samples or cumulus granulosa cells corresponding to every oocyte retrieved.

As the expression of GDF9 is also detected during preimplantation embryonic development [22, 23], our objective in the present study was to investigate whether the GDF9 concentration in day 3 culture medium was linked to embryo quality and viability.

Materials and methods

Patient characteristics

All patients participating in the study were recruited from the ART center of Chongqing Maternal and Child Health Care Hospital between March 2019 and May 2020. The inclusion criteria included normal range of follicle-stimulating hormone (FSH), luteinizing hormone (LH), and estradiol (E2), age ≤ 40 years, and BMI 18–30 kg/m^2. Patients with polycystic ovarian syndrome (PCOS), endometriosis, or a history of endocrine disorders were excluded. The long protocol was performed in all patients as described previously [24].

In study 1, we mainly focus on the relationship between GDF9 concentrations in culture medium and embryo quality defined by morphologic criteria; thus, only the patients who obtained at least 4 embryos with 6–10 blastomeres each (2 transferable and 2 non-transferable embryos) on day 3 were enrolled. The flow chart of patient recruitment for the study 1 is displayed in Fig. 1; and general information of patients and clinical outcome is presented in Table 1. In study 2, initially, a total of 269 culture mediums from 137 patients were collected. Then, only patients with fully implanted and non-implanted, and the corresponding embryos were analyzed.

Embryo culture and transfer

Normal fertilized zygotes were individually placed into pre-equilibrated culture dishes (Thermo Scientific) in 25-μL of culture droplets (Vitrolife Sweden AB, Sweden), then cultured in a SANYO incubator (MCO-5 M, Japan) at 37 °C with 5% O_2 and 6% CO_2 until embryo transfer or cryopreservation on day 3.

Generally, two embryos were transferred per patient, and the transfers that led to full implantation or no

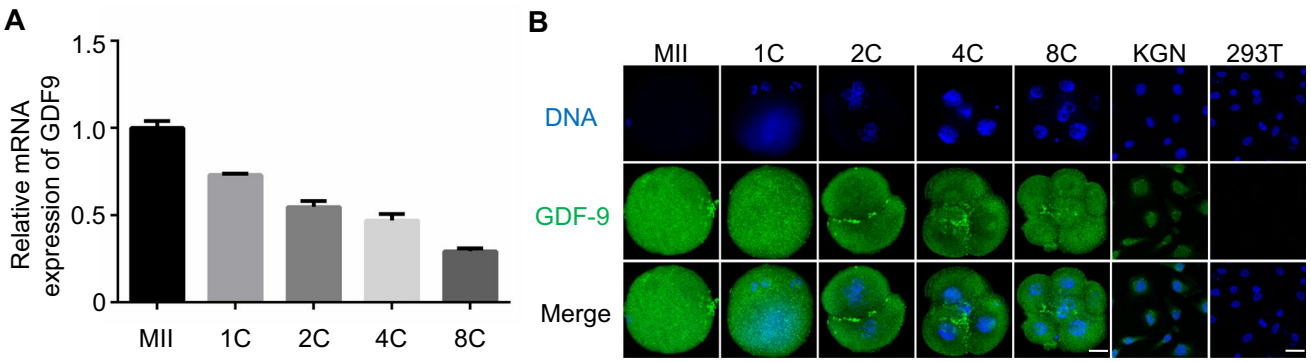

Fig. 1 The expression pattern of GDF9 in human oocytes and embryos. **A** mRNA expressions of GDF9 detected by Q-PCR. **B** Protein expressions of GDF9 detected by immunofluorescence. MII, MII oocytes; 1C, one-cell embryos; 2C, two-cell embryos; 4C, four-cell embryos; 8C, eight-cell embryos. Scale bar: 50 um, 25um

Table 1 General information for all the patients of study 1

Variable	Value
Cases	70
Age (years)	31.46 ± 3.68
BMI (kg/m^2)	22.42 ± 3.04
Basal FSH (mIU/mL)	5.52 ± 1.71
Basal estradiol (pg/mL)	33.97 ± 13.71
AMH (ng/mL)	3.16 ± 1.73
Total gonadotropin dose (IU)	2152.78 ± 734.21
No. of oocytes retrieved per patient	13.34 ± 5.23
No. of 2PN oocytes	9.46 ± 3.75
Fertilization rate (%)	662/934 (70.88%)
No. of cleaved embryos	9.11 ± 3.64
Cleavage rate (%)	638/662 (96.37%)
No. of transferable embryos	3.65 ± 1.86

Categorical variables are presented as proportion (%). Continuous variables are presented as mean ± SD

BMI, body mass index; *FSH*, follicle-stimulating hormone; *AMH*, anti-Mullerian hormone

implantation of the two embryos were retained. The cases in which only one good quality embryo was used for transfer were also retained. However, cases in which two embryos were transferred but only one embryo was implanted were also excluded because it was not possible to distinguish which embryo was implanted and which was not. Biochemical pregnancy and early miscarriages were excluded. Finally, a total of 119 embryos from 61 patients (29 fully implanted and 32 non-implanted patients) were enrolled in the study 2. The corresponding GDF9 concentrations in culture medium were compared between fully implanted and non-implanted embryos, and also between embryos that led to twin live birth and embryos with no live birth.

Embryo scoring

Day 3 embryos were morphologically scored according to consensus guidelines of the European Society of Human Reproduction and Embryology/Alpha [7]. Embryos were scored according to blastomere number, symmetry, and degree of fragmentation. Briefly, an embryo was defined as "good" when blastomere shape was even and showed ≤ 10% fragmentation, embryos with uneven blastomeres and 10–25% fragmentation were defined as "fair," and embryos with uneven blastomeres and ≥ 25% fragmentation were defined as "poor." The embryos defined as "good" and "fair" were transferable, and the "poor" ones were non-transferable.

Culture medium collection

Spent embryo culture media were collected after fresh embryo transfer or cryopreservation on day 3. The media were then immediately frozen in sterile polymerase chain reaction (PCR) tubes free from DNA, DNase, RNase, and PCR inhibitors (Eppendorf, Germany), and stored at − 80 °C until evaluation with ELISA.

Assay of embryo culture medium for GDF9

GDF9 concentrations in culture medium were assayed using a commercial GDF9 enzyme-linked immunosorbent assay (ELISA) kit (AL-176, Ansh Labs, USA). Briefly, medium samples (10-µL embryo culture medium diluted with 40-µL calibrator A) were first added to GDF9 antibody-coated microtiter wells and incubated for 3 h at room temperature. Then, the plates were washed 5 times, and this was followed by incubation with biotinylated GDF9 antibody for 1 h at room temperature. Then, we incubated the wells with streptavidin horseradish peroxidase conjugate (SHRP) for half an hour at room temperature. Then, 100 µL of tetramethylbenzidine (TMB) substrate was added and incubated for 17 min at room temperature. We added 100 µL of an acid solution to stop the colorimetric reaction, and detected the antibody-antigen-biotin conjugate-SHRP complex bounding by the enzyme–substrate reaction. The degree of enzymatic turnover of the substrate was determined by dual-wavelength absorbance measurement at 450 nm. Concentrations of GDF9 in the culture medium were determined using a calibration curve of solutions with known concentrations of GDF9 (from 0 to 5800 pg/mL). For each experiment, two positive controls (control I, GDF9 concentration is 320 pg/mL; control II, 3585 pg/mL) and negative control (blank embryo culture medium) were tested. Furthermore, GDF9 concentrations of the positive controls fall within established confidence limits indicated the reagent non-pollution and stability. The GDF9 concentrations of 16 replicates of calibrator A (0 pg/mL) and calibrator B (48 pg/mL) were assayed for analyzing the specificity and sensitivity, and all the true positive and true negative were detected, indicating the accuracy of this assay ($n = 3$ tests).

Quantitative reverse transcription PCR

RNA was extracted from 20 oocytes or 3PN embryos using QIAGEN RNeasy Mini Kit, and cDNA was made by using PrimeScript™ RT Master Mix (Takara, Japan). Quantitative reverse transcription PCR (Q-PCR) was performed by using a Bio-rad CFX96 Real-Time System and TB Green Real-Time PCR Master Mix (Takara, Japan). The primers for GDF9 and β-actin were as follows: GDF9: forward 5′-GGC AAATTCTACCACCATTTGGA-3′, reverse 5′-GAGGAC

ACCGCCTTTAACCT-3′; β-actin: forward 5′-CTCCATCCT GGCCTCGCTGT-3′, reverse 5′-GCTGTCACCT TCACCG TTCC-3′. The $2^{-\Delta\Delta}$Ct algorithm was used to calculate the GDF9 mRNA levels relative to the β-actin level. Experiments were performed at least three times.

Immunofluorescence

Oocytes and embryos were fixed with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS, pH 7.4) for 30 min and then permeabilized for 20 min with 0.5% Triton X-100 in PBS at room temperature. Then, samples were blocked with 3% BSA in PBS for 1 h and incubated with anti-GDF9 antibody (1:100, Sangon Biotech, China) at 4 °C overnight. After washing 3 times with 0.1% Tween-20 in PBS, samples were incubated with Alexa Fluor 488-conjugated goat anti-rabbit IgG (Invitrogen, USA) for 1 h at room temperature. Then samples were counterstained with Hoechst for 10 min. Finally, samples were mounted on glass slides and observed under a laser scanning confocal microscope (SP8, Laica, Germany).

Statistical analyses

We performed statistical analysis using SPSS software version 22.0, 2013 (SPSS, Chicago, IL, USA). Continuous variables are presented as the mean ± standard deviation, and categorical variables are presented as *n* (%). Data of GDF9 concentrations are presented as the median and quartiles. For continuous variables, we used a paired Student's *t* test to assess differences between 2 groups, and Duncan's multiple-range test to assess the differences among 3 groups. A chi-square test was used for dichotomous variables. The correlations between factors and the clinical outcome were analyzed using a binary logistic regression model, and the odds ratio (OR) and 95% confidence interval were calculated. $P < 0.05$ was considered to be statistically significant.

Results

Expression pattern of GDF9

The expression pattern of GDF9 in human in vitro matured MII oocytes and 3PN embryos was investigated by Q-PCR and immunofluorescence. As expect, the high expression level of GDF9 mRNA and protein was detected in oocytes. After fertilization, the GDF9 mRNA and protein were also present from zygote to eight-cell embryo, with a slow decreasing trend, which in line with previous studies and the data from published single cell RNA-seq [22, 23] (Fig. 1 and Supplementary Fig. 1). The KGN and 293 T cells were used as positive and negative control, respectively.

GDF9 concentrations and embryo quality

The flow chart of patient recruitment for the study 1 is displayed in Fig. 2, and general information of patients and clinical outcome is presented in Table 1. A total of 70 patients were enrolled, and 66 patients (94%) showed a lower GDF9 concentration in the spent medium from transferable embryos compared with medium from non-transferable embryos (Supplementary Fig. 2). The medium from the transferable embryos exhibited a significantly lower GDF9 concentration relative to non-transferable embryos on the whole (331 pg/mL (quartiles: 442, 664 pg/mL) vs. 518 pg/mL (quartiles: 328, 1086 pg/mL), $P < 0.001$, Fig. 3A). The ROC curve for predicting transferable embryos using GDF9 concentration had an area under the curve (AUC) of 0.785, with a sensitivity of 61.31%, and a specificity of 89.47% (Fig. 3B). Moreover, GDF9 concentration in the spent culture medium increased significantly with the decreasing of the embryo quality (good, 336 pg/mL (quartiles: 181, 530 pg/mL); fair, 540 pg/mL (quartiles: 313, 902 pg/mL); and poor, 956 pg/mL (quartiles: 478, 1509 pg/mL); $P < 0.001$; Fig. 3C).

GDF9 concentrations and implantation

The flow chart of patient recruitment for the study 2 is displayed in Fig. 4, and patient information is presented in Table 2. Only the age showed significantly higher in the non-implanted group ($P = 0.022$). However, the GDF9 concentration was not different between young group (< 35 years) and old group (≥ 35 years) (469 pg/mL (quartiles: 188,

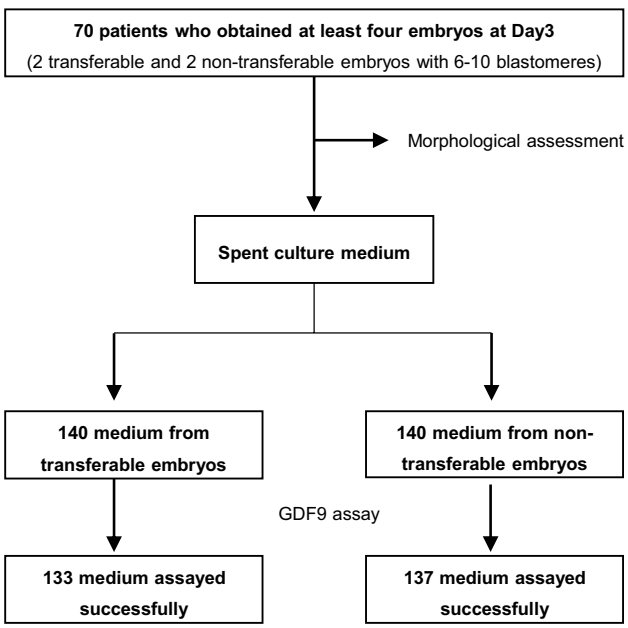

Fig. 2 Flow chart of culture medium collection in study 1

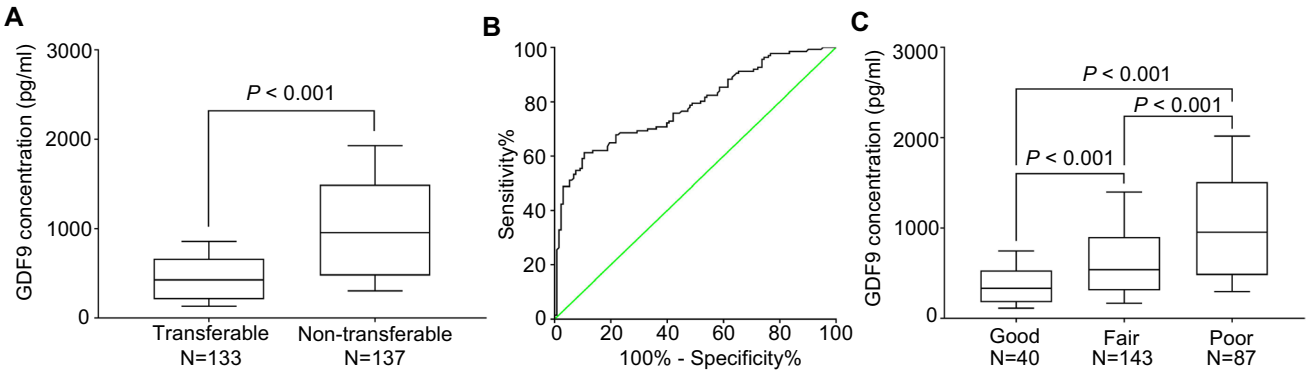

Fig. 3 The relationship between GDF9 concentration in culture medium and embryo quality. **A** Boxplot of GDF9 concentration between all transferable and non-transferable embryos, using Student's *t* test was performed. **B** ROC analysis for obtaining transferable embryos predicted by GDF9 concentrations in spent medium. **C** Boxplot comparing GDF9 concentration among spent media of embryos graded as good, fair, or poor, using Duncan's multiple-range test

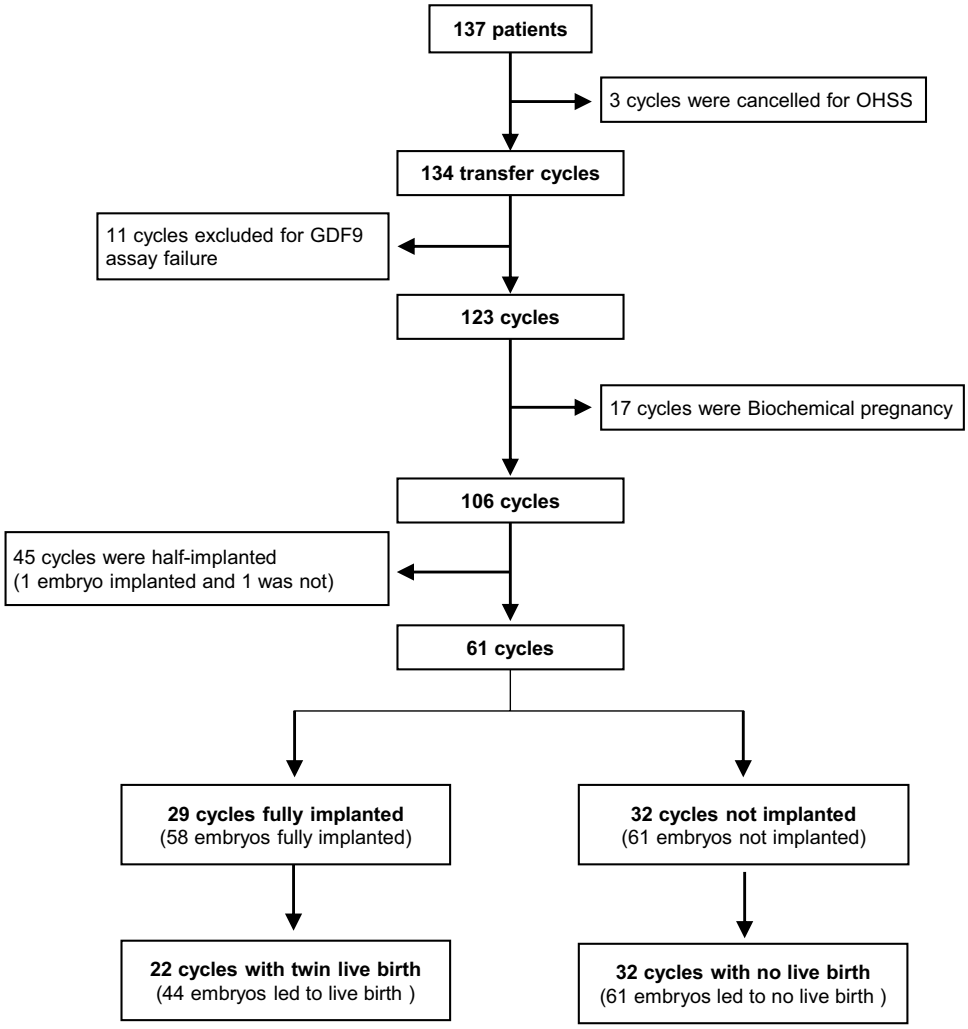

Fig. 4 Flow chart summarizing the patients and the embryos enrolled in the study 2

823 pg/mL) vs. 555 pg/mL (quartiles: 306, 731 pg/mL), $P = 0.353$; Fig. 5A). In addition, correlation analysis also demonstrated that the GDF9 concentration is not associated with age, BMI, and AMH, and the coefficients of correlation were 0.041 ($P = 0.655$), 0.096 ($P = 0.300$), and 0.087 ($P = 0.345$), respectively (Supplementary Fig. 3). The GDF9

Table 2 General information for all the patients of study 2

	Implanted embryos ($n=58$)	Non-implanted embryos ($n=61$)	P-value
Age (years)	29.72 ± 3.83	31.97 ± 3.62	0.02
BMI (kg/m^2)	21.67 ± 2.99	22.14 ± 3.21	0.56
Basal FSH (mIU/mL)	8.42 ± 5.41	5.67 ± 1.62	0.06
Basal estradiol (pg/mL)	46.80 ± 6.97	34.45 ± 14.30	0.24
AMH (ng/mL)	2.21 ± 0.87	2.96 ± 2.39	0.60
Total gonadotropin dose (IU)	4.64 ± 1.75	3.09 ± 1.11	0.08
Endometrial thickness (cm)	1.04 ± 0.17	0.98 ± 0.15	0.95
No. of oocytes retrieved per patient	10.45 ± 3.82	10.28 ± 4.03	0.86
Quality of transferred embryo			0.26
Good (%)	30 (51.72)	25 (40.98)	
Fair (%)	28 (48.28)	36 (59.02)	

Categorical variables are presented as proportion (%). Continuous variables are presented as mean ± SD

BMI, body mass index; *FSH*, follicle-stimulating hormone; *AMH*, anti-Mullerian hormone

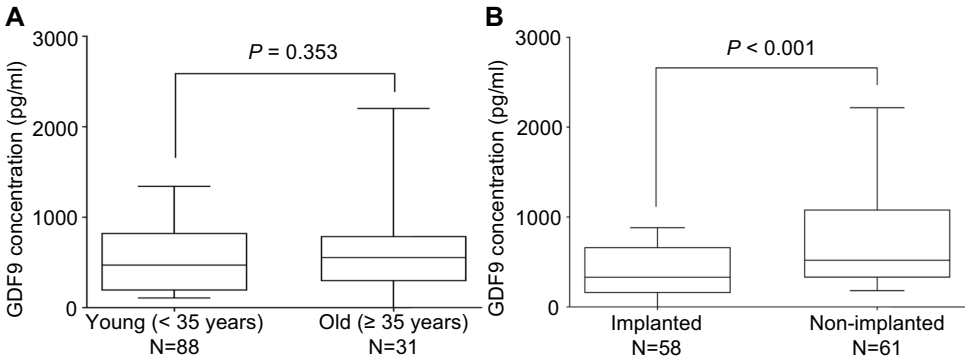

Fig. 5 Analysis of GDF9 concentration of culture medium in relation to implantation. **A** The GDF9 concentration of culture medium at different maternal ages. **B** The relationship between GDF9 concentration in culture medium and embryo implantation

concentration in the spent medium from implanted embryos was significantly lower than that for the non-implanted embryos (331 pg/mL (quartiles: 156, 665 pg/mL) vs. 518 pg/mL (quartiles: 328, 1086 pg/mL), $P<0.001$; Fig. 5B). After adjusting for the age, BMI, and AMH factors, a high GDF9 concentration ($P=0.002$, OR = 0.533, CI (0.359, 0.790)) was a statistically significant risk factor for the probability of embryo implantation.

GDF9 concentrations and live birth

As described in Table 2, a total of 58 embryos from 29 patients were fully implanted in study 2. Of these, 44 embryos from 22 patients led to twin live birth. Then, only the patients with twin live birth were included in the subsequently analysis of the correlation between the GDF9 concentration and live birth. No significant differences were detected between the two groups. The GDF9 concentration was significantly lower in the spent medium from the embryos that led to live birth respect to those that failed (320 pg/mL (quartiles: 156, 593 pg/mL) vs. 518 pg/mL (quartiles: 328, 1086 pg/mL), $P<0.005$; Fig. 6A). To predict live birth, the ROC analysis was performed. The AUC was 0.680, with a sensitivity of 88.52% (Fig. 6B). After adjusting for the age, BMI, and AMH factors, a high GDF9 concentration ($P=0.001$, OR = 0.451, CI (0.287, 0.707)) was a statistically significant risk factor for the probability of live birth.

Discussion

Although embryo selection currently primarily relies on morphological criteria, however, morphologic assessment does not reflect the true status of the embryo. Elucidation of novel non-invasive biomarkers that correlate with developmental potential of embryo is helpful in increasing the accuracy of embryo selection. In the present study, we demonstrated for the first time that GDF9 concentrations in human embryo culture medium were associated with embryo quality and clinical outcome.

Several studies have confirmed that embryos secrete a large variety of proteins into the culture medium [25], and the sources of these proteins can then regulate pre-implantation embryonic development [26, 27]. We found herein

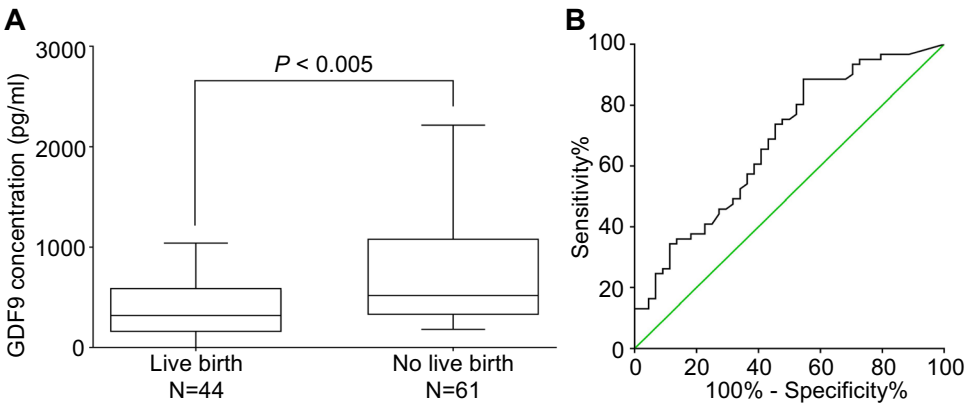

Fig. 6 Analysis of GDF9 concentration of culture medium in relation to live birth. **A** The relationship between GDF9 concentration in culture medium and live birth. **B** ROC analysis to calculate the diagnostic capacity of GDF9 in spent medium to predict live birth

that 96% of culture-medium samples contained GDF9 protein, indicating its release by human embryos. A series of new non-invasive approaches for assessing embryo has been developed over the past decade using proteomics, metabolomics, and small non-coding RNA [28, 29] to target analysis of Alpha-1 [30], SOD [31], mitochondrial DNA [32, 33], hCG [34], HLA-G [35, 36], soluble CD146 [37], or sets of proteins. LC–MS and MALDI-TOF–MS have also been used to identify new peptides, proteins, or mass ranges as biomarkers for embryonic assessment [38, 39].

In earlier reports it was shown that GDF9 and BMP15, members of the TGF-beta family, play an important role in the folliculogenesis and oocyte development [40]. Previous studies also showed that GDF9 and BMP15 promoted proliferation and differentiation of granulosa cells and oocyte maturation through paracrine and autocrine signaling pathways [40, 41]. In addition, the supplement of GDF9 in oocyte maturation medium can enhance the embryonic development and fetal viability in mice [41]. And several recent studies indicated that GDF9 has the potential to be a novel biomarker for predicting embryo quality. Gode, Gulekli [20] reported that increased levels of GDF9 in follicular fluid were significantly correlated with oocyte maturation and embryo quality using western immunoblotting, and Li, Li [21] found that the expression levels of GDF9 mRNA in cumulus granulosa cells were closely associated with oocyte maturation, embryo quality, and pregnancy outcome. However, relative to these aforementioned studies, the measurement of GDF9 levels in culture medium from day 3 embryos is simpler, rapid, and non-invasive. Furthermore, a non-invasive approach using ELISA is more practical in eventual clinical assessment compared with western blotting, qPCR, or mass spectrometry.

During the measurement of GDF9, no GDF9 was detected in the negative control spent medium (without embryos), suggesting that the GDF9 was secreted by the embryo. Generally, the fertilization was checked at 17 ± 1 h post-insemination, according to the Istanbul consensus [7]. Then, the normal fertilized embryos were moved into the new culture medium from IVF medium following the cumulus cells removal. Therefore, the GDF9 detected in culture medium in this study was secreted during the developmental process from late stage of zygote to day 3 embryo. In addition, we also detected the persistent expression of GDF9 mRNA and protein in early human embryos, in line with previous studies [22, 23] and that data from other high-throughput sequencing of human embryos [42], which confirming the ability of secreting GDF9 by human embryos.

In study 1, we explored the correlation between GDF9 concentrations in culture medium and embryo quality. Only four culture medium samples that corresponded to two transferable and two non-transferable embryos per patient were used, which obviated any negative effects from patient heterogeneity. In fact, 94% couples displayed higher GDF9 concentrations in spent medium from non-transferable embryos compared with transferable embryos. Another very important aspect of our work was that all the culture media were collected from day 3 embryos, and that the embryos were at their expected cell stage of 6–10 blastomeres. Thus, we posit that our work is more suitable to the actual clinical setting, in which we generally select the embryo with a normal cell number for transfer on day 3.

After confirming the association between the GDF9 concentrations in culture medium and embryo quality defined by morphologic criteria, we further examine the question of whether GDF9 concentrations are linked to implantation and live birth potential. In study 2, we showed a significantly lower GDF9 concentrations in culture medium from embryos that led to successfully implantation and live birth. Moreover, the analysis of binary logistic regression taking into account the factors identified in the study as being linked with the clinical outcome (age and GDF9 concentrations in culture medium) indicated a close independent relationship between the GDF9 concentrations in culture medium and implantation and live birth potential. No significant difference between the live birth and the no live birth group in terms of embryo

quality, suggesting that the GDF9 concentrations in culture medium may be used as a biomarker of embryo viability.

Maternal mRNA clearance is an essential process that occurs during maternal-to-zygotic transition (MZT) [43, 44]. We postulated that GDF9 is one of the maternal genes that needed to be decayed to some extent, then activate the zygotic genome. The mRNA level of GDF9 in eight-cell embryos has been decreased by 70%, compared with oocytes, which verified our hypothesis. Therefore, the inadequate degradation of GDF9 mRNA leading to the high level of GDF9 might be one reason of poor developmental potential. In addition, the high GDF9 concentrations in culture medium might be due to the high biological activity in poor embryos, including the process of releasing the extracellular vesicle containing GDF9. Our finding supports the "quiet embryo" hypothesis, which proposes that the better viable embryos exhibit relatively lower metabolic activity [45, 46]. The reason of the high GDF9 concentrations in culture medium of poor developmental embryos needs further exploration.

Despite the small size of our population, these results shown in the present paper suggested that the detection of GDF9 concentrations in culture medium may have application prospects in embryo selection. Larger and prospective studies are needed for confirming these preliminary results before considering any use in clinical application.

Conclusions

In the present study, we demonstrated that GDF9 concentration in spent culture medium is linked to embryo quality and viability. Thus, GDF9 in culture medium has the potential to constitute a non-invasive biomarker for embryo selection.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10815-021-02368-x.

Author contribution J.L. conceived and designed the study. J.L. and C.L. performed the experiments. J.L., W.H., and F.X. collected the samples. C.L., J.Y., and Q.Z. performed the data statistics. J.L., X.L., and G.H. contributed to manuscript drafting with the help from all authors. All authors approved the final manuscript.

Funding This study was supported by the Chongqing Science and Health Joint Project (2021MSXM072); Special Research Project of Chongqing Health Center for Women and Children (2019YJMS01).

Availability of data and material The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Code availability Not applicable.

Declarations

Ethics approval This research was approved by the Institutional Review Board (IRB) of Chongqing Health Center for Women and Children (2019-RGI-05). All patients gave their written informed consent for culture medium analysis.

Conflict of interest The authors declare no competing interests.

References

1. Steptoe PC, Edwards RG. Birth after the reimplantation of a human embryo. Lancet. 1978;2(8085):366.
2. Ebner T, Moser M, Sommergruber M, Tews G. Selection based on morphological assessment of oocytes and embryos at different stages of preimplantation development: a review. Hum Reprod Update. 2003;9(3):251–62.
3. Racowsky C, Combelles CM, Nureddin A, Pan Y, Finn A, Miles L, et al. Day 3 and day 5 morphological predictors of embryo viability. Reprod Biomed Online. 2003;6(3):323–31.
4. Mantikou E, Youssef MA, van Wely M, van der Veen F, Al-Inany HG, Repping S, et al. Embryo culture media and IVF/ICSI success rates: a systematic review. Hum Reprod Update. 2013;19(3):210–20.
5. Kirkegaard K, Hindkjaer JJ, Ingerslev HJ. Effect of oxygen concentration on human embryo development evaluated by time-lapse monitoring. Fertil Steril. 2013;99(3):738-44 e4.
6. Lane M, Gardner DK. Embryo culture medium: which is the best? Best Pract Res Clin Obstet Gynaecol. 2007;21(1):83–100.
7. Alpha Scientists in Reproductive M, Embryology ESIGo. The Istanbul consensus workshop on embryo assessment: proceedings of an expert meeting. Hum Reprod. 2011;26(6):1270–83.
8. Capalbo A, Rienzi L, Cimadomo D, Maggiulli R, Elliott T, Wright G, et al. Correlation between standard blastocyst morphology, euploidy and implantation: an observational study in two centers involving 956 screened blastocysts. Hum Reprod. 2014;29(6):1173–81.
9. Rhenman A, Berglund L, Brodin T, Olovsson M, Milton K, Hadzi-osmanovic N, et al. Which set of embryo variables is most predictive for live birth? A prospective study in 6252 single embryo transfers to construct an embryo score for the ranking and selection of embryos. Hum Reprod. 2015;30(1):28–36.
10. Singh R, Sinclair KD. Metabolomics: approaches to assessing oocyte and embryo quality. Theriogenology. 2007;68(Suppl 1):S56-62.
11. Bromer JG, Seli E. Assessment of embryo viability in assisted reproductive technology: shortcomings of current approaches and the emerging role of metabolomics. Curr Opin Obstet Gynecol. 2008;20(3):234–41.
12. Botros L, Sakkas D, Seli E. Metabolomics and its application for non-invasive embryo assessment in IVF. Mol Hum Reprod. 2008;14(12):679–90.
13. Katz-Jaffe MG, McReynolds S. Embryology in the era of proteomics. Fertil Steril. 2013;99(4):1073–7.
14. Jones GM, Trounson AO, Vella PJ, Thouas GA, Lolatgis N, Wood C. Glucose metabolism of human morula and blastocyst-stage embryos and its relationship to viability after transfer. Reprod Biomed Online. 2001;3(2):124–32.
15. Gardner DK, Lane M, Stevens J, Schoolcraft WB. Noninvasive assessment of human embryo nutrient consumption as a measure of developmental potential. Fertil Steril. 2001;76(6):1175–80.
16 Gada RP, Daftary GS, Walker DL, Lacey JM, Matern D, Morbeck DE. Potential of inner cell mass outgrowth and amino acid

turnover as markers of quality in the in vitro fertilization laboratory. Fertil Steril. 2012;98(4):863-9 e1.
17. Aaltonen J, Laitinen MP, Vuojolainen K, Jaatinen R, Horelli-Kuitunen N, Seppa L, et al. Human growth differentiation factor 9 (GDF-9) and its novel homolog GDF-9B are expressed in oocytes during early folliculogenesis. J Clin Endocrinol Metab. 1999;84(8):2744–50.
18. Juengel JL, Bodensteiner KJ, Heath DA, Hudson NL, Moeller CL, Smith P, et al. Physiology of GDF9 and BMP15 signalling molecules. Anim Reprod Sci. 2004;82–83:447–60.
19. Dong J, Albertini DF, Nishimori K, Kumar TR, Lu N, Matzuk MM. Growth differentiation factor-9 is required during early ovarian folliculogenesis. Nature. 1996;383(6600):531–5.
20. Gode F, Gulekli B, Dogan E, Korhan P, Dogan S, Bige O, et al. Influence of follicular fluid GDF9 and BMP15 on embryo quality. Fertil Steril. 2011;95(7):2274–8.
21. Li Y, Li RQ, Ou SB, Zhang NF, Ren L, Wei LN, et al. Increased GDF9 and BMP15 mRNA levels in cumulus granulosa cells correlate with oocyte maturation, fertilization, and embryo quality in humans. Reprod Biol Endocrinol. 2014;12:81.
22. Pennetier S, Uzbekova S, Perreau C, Papillier P, Mermillod P, Dalbies-Tran R. Spatio-temporal expression of the germ cell marker genes MATER, ZAR1, GDF9, BMP15, andVASA in adult bovine tissues, oocytes, and preimplantation embryos. Biol Reprod. 2004;71(4):1359–66.
23. Palomino J, Herrera G, Dettleff P, Martinez V. Growth differentiation factor 9 and bone morphogenetic protein 15 expression in previtellogenic oocytes and during early embryonic development of Yellow-tail Kingfish Seriola lalandi. Biol Res. 2014;47:60.
24. Xiong S, Han W, Liu JX, Zhang XD, Liu WW, Liu H, et al. Effects of cumulus cells removal after 6 h co-incubation of gametes on the outcomes of human IVF. J Assist Reprod Genet. 2011;28(12):1205–11.
25. Dyrlund TF, Kirkegaard K, Poulsen ET, Sanggaard KW, Hindkjaer JJ, Kjems J, et al. Unconditioned commercial embryo culture media contain a large variety of non-declared proteins: a comprehensive proteomics analysis. Hum Reprod. 2014;29(11):2421–30.
26. Montsko G, Zrinyi Z, Janaky T, Szabo Z, Varnagy A, Kovacs GL, et al. Noninvasive embryo viability assessment by quantitation of human haptoglobin alpha-1 fragment in the in vitro fertilization culture medium: an additional tool to increase success rate. Fertil Steril. 2015;103(3):687–93.
27. Zhu J, Li M, Chen L, Liu P, Qiao J. The protein source in embryo culture media influences birthweight: a comparative study between G1 v5 and G1-PLUS v5. Hum Reprod. 2014;29(7):1387–92.
28. Rodgaard T, Heegaard PM, Callesen H. Non-invasive assessment of in-vitro embryo quality to improve transfer success. Reprod Biomed Online. 2015;31(5):585–92.
29. Katz-Jaffe MG, Schoolcraft WB, Gardner DK. Analysis of protein expression (secretome) by human and mouse preimplantation embryos. Fertil Steril. 2006;86(3):678–85.
30. Montsko G, Godony K, Herczeg R, Varnagy A, Bodis J, Kovacs GL. Alpha-1 chain of human haptoglobin as viability marker of in vitro fertilized human embryos: information beyond morphology. Syst Biol Reprod Med. 2019;65(2):174–80.
31. Combelles CM, Holick EA, Racowsky C. Release of superoxide dismutase-1 by day 3 embryos of varying quality and implantation potential. J Assist Reprod Genet. 2012;29(4):305–11.
32. Stigliani S, Anserini P, Venturini PL, Scaruffi P. Mitochondrial DNA content in embryo culture medium is significantly associated with human embryo fragmentation. Hum Reprod. 2013;28(10):2652–60.
33. Stigliani S, Persico L, Lagazio C, Anserini P, Venturini PL, Scaruffi P. Mitochondrial DNA in day 3 embryo culture medium is a novel, non-invasive biomarker of blastocyst potential and implantation outcome. Mol Hum Reprod. 2014;20(12):1238–46.
34. Ramu S, Acacio B, Adamowicz M, Parrett S, Jeyendran RS. Human chorionic gonadotropin from day 2 spent embryo culture media and its relationship to embryo development. Fertil Steril. 2011;96(3):615–7.
35. Sher G, Keskintepe L, Fisch JD, Acacio BA, Ahlering P, Batzofin J, et al. Soluble human leukocyte antigen G expression in phase I culture media at 46 hours after fertilization predicts pregnancy and implantation from day 3 embryo transfer. Fertil Steril. 2005;83(5):1410–3.
36. Fisch JD, Keskintepe L, Ginsburg M, Adamowicz M, Sher G. Graduated Embryo Score and soluble human leukocyte antigen-G expression improve assisted reproductive technology outcomes and suggest a basis for elective single-embryo transfer. Fertil Steril. 2007;87(4):757–63.
37. Bouvier S, Paulmyer-Lacroix O, Molinari N, Bertaud A, Paci M, Leroyer A, et al. Soluble CD146, an innovative and non-invasive biomarker of embryo selection for in vitro fertilization. PloS one. 2017;12(3):e0173724.
38. Iles RK, Sharara FI, Zmuidinaite R, Abdo G, Keshavarz S, Butler SA. Secretome profile selection of optimal IVF embryos by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. J Assist Reprod Genet. 2019;36(6):1153–60.
39. Ji H, Shi X, Wang J, Cao S, Ling X, Zhang J, et al. Peptidomic analysis of blastocyst culture medium and the effect of peptide derived from blastocyst culture medium on blastocyst formation and viability. Mol Reprod Dev. 2020;87(1):191–201.
40. Peng J, Li Q, Wigglesworth K, Rangarajan A, Kattamuri C, Peterson RT, et al. Growth differentiation factor 9:bone morphogenetic protein 15 heterodimers are potent regulators of ovarian functions. Proc Natl Acad Sci U S A. 2013;110(8):E776–85.
41. Yeo CX, Gilchrist RB, Thompson JG, Lane M. Exogenous growth differentiation factor 9 in oocyte maturation media enhances subsequent embryo development and fetal viability in mice. Hum Reprod. 2008;23(1):67–73.
42. Xue Z, Huang K, Cai C, Cai L, Jiang CY, Feng Y, et al. Genetic programs in human and mouse early embryos revealed by single-cell RNA sequencing. Nature. 2013;500(7464):593–7.
43. Sha QQ, Zheng W, Wu YW, Li S, Guo L, Zhang S, et al. Dynamics and clinical relevance of maternal mRNA clearance during the oocyte-to-embryo transition in humans. Nat Commun. 2020;11(1):4917.
44. Tadros W, Lipshitz HD. The maternal-to-zygotic transition: a play in two acts. Development. 2009;136(18):3033–42.
45. Baumann CG, Morris DG, Sreenan JM, Leese HJ. The quiet embryo hypothesis: molecular characteristics favoring viability. Mol Reprod Dev. 2007;74(10):1345–53.
46. Leese HJ. Quiet please, do not disturb: a hypothesis of embryo metabolism and viability. BioEssays. 2002;24(9):845–9.