Cited2 protein level in cumulus cells is a biomarker for human embryo quality and pregnancy outcome in one in vitro fertilization cycle

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Objective: To determine whether the levels of CBP/p300 interacting transactivator with ED-rich tail 2 (Cited2) protein in cumulus cells (CCs) derived from patients undergoing IVF related to infertility factors, embryo quality, and clinical outcomes in one IVF cycle.
Design: Retrospective analysis of human CCs.
Setting: Public hospital and university.
Patient(s): A total of 103 (conventional) IVF patients and 32 intracytoplasmic sperm injection patients.
Intervention(s): All CCs from each patient's oocytes were considered as one sample. The patients were divided into two groups according to whether the Cited2/β-actin levels in their CCs were above or below the mean level detected for all patients.
Main Outcome Measure(s): Embryo quality and clinical outcomes of IVF patients.
Result(s): The oocytes derived from the group of patients whose CCs showed lower Cited2 levels displayed higher fertilization, transferable embryo, and implantation rates. Moreover, the patients in this group were more likely to have a successful pregnancy outcome. Among different infertility factors, a total of 78.6% of patients with polycystic ovary syndrome had a higher Cited2 level in CCs. Additionally, patients with a lower basal FSH level belonged to the higher Cited2 levels group. The expression of two genes (phosphoenolpyruvate carboxykinase 1 [*PCK1*] and progesterone receptor [*PR*]) and the glucose content in CCs were also markedly increased in CCs derived from patients with higher Cited2 levels.
Conclusion(s): The present findings imply that Cited2 level in CCs is associated with polycystic ovary syndrome, embryo quality, and pregnancy outcome of IVF patients. (Fertil Steril® 2016;105:1351–9. ©2016 by American Society for Reproductive Medicine.)
Key Words: Cited2, cumulus cells, embryo, human, pregnancy

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The development of the cumulus–oocyte complex (COC) is a key event in the female reproductive cycle. Because cumulus cells (CCs) are in close proximity to the oocyte, they play an important role in regulating oocyte development (1, 2), and bidirectional exchanges between the oocyte and CCs are crucial for the acquisition of oocyte competence (3, 4). In addition, CCs also facilitate fertilization and the acquisition of full embryonic developmental competence (5). Many cumulus-expressed genes critical to oocyte growth and later embryo development are under the control of both maternal- and oocyte-derived signals (6). Thus, the expression patterns of these genes can be used for molecular markers to assess the developmental potential of the oocyte and embryo (7).

Cited2 is a non–DNA-binding protein that functions as a context-dependent transcriptional co-activator or repressor (8, 9). Cited2 gene reportedly involves oocyte development because gene expression profiling revealed that *Cited2* was markedly increased at the initiation of

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oocyte growth in mouse primordial follicles (10). *Cited2* is also expressed in mouse and human CCs (11, 12). In bovine ovarian follicles, *Cited2* is up-regulated in large relative to small follicles (13). Thus, *Cited2* seems to play an important role in the development of the COC by affecting both the oocyte and CCs. In the present report, the expression of *Cited2* is higher in CCs surrounding developmentally incompetent mouse oocytes compared with competent mouse oocytes (12). However, the relationship between Cited2 levels in mammalian CCs and embryo quality remains unknown.

Several studies had demonstrated that some infertile diseases might damage oocytes and embryos qualities. For example, the CCs from patients with endometriosis displayed serious apoptosis, which lowered the embryo quality and pregnancy outcome (14–16). Moreover, the percentages of high-quality oocytes and fertilization were significantly decreased in the patients with polycystic ovaries (16), who had an increased risk of early pregnancy loss (15). Cited2 is also essential for normal mouse development: lacking Cited2 resulted in abnormal heart, adrenal, and gonadal development (17–19). In *Cited2*$^{-/-}$ female gonads, pro-ovarian genes (*Foxl2*, *Rspo1*, and *Wnt4*) were decreased, and ectopic cell migration delayed female gonadal development (20, 21). Such inhibition of ovarian-promoting genes can be sufficient to impair female gonadal development, one of the main causes of infertility (21). This was also evidenced by later research showing that mutations in Cited2 might be involved in human premature ovarian failure pathogenesis (22). In human CCs, an increase in Cited2 gene expression has been linked to diminished ovarian reserve pathogenesis and high E_2 level (11). However, the relationship between Cited2 levels in human CCs harvested from one IVF cycle, human embryo quality, and infertile diseases has not been reported.

Therefore, we first studied the relationships between the Cited2 protein levels in human CCs harvested after insemination in conventional IVF and clinical outcomes (fertilization, embryo morphologic grade, successful implantation, and pregnancy). Simultaneously, the same study on 32 intracytoplasmic sperm injection (ICSI) patients (CCs collected before insemination) was used to eliminate the influence of sperms on Cited2 levels. Then the relationships between Cited2 levels in human CCs during IVF cycle and the pathologies that caused infertility were investigated. Last, we analyzed the expressions of some genes relevant to Cited2 to pursue possible underlying molecular mechanisms reducing oocyte quality and embryo developmental potential.

MATERIALS AND METHODS

Patient Stimulation and Cumulus Collection

In this study 135 patients (number of oocytes retrieved more than five) underwent treatment in the PLA Naval General Hospital in Beijing, People's Republic of China. The people included 103 (conventional) IVF patients and 32 ICSI patients. A total of 81 IVF patients were used for embryo quality and clinical outcome study, and the remaining 22 IVF patients were used for gene expression and glucose tests after the patients were grouped by Cited2 levels. All patients gave informed consent, and this study was approved by the Ethics Committee of China at the PLA Naval General Hospital. Details of patient stimulation have been provided elsewhere (23).

The CCs from IVF patients were harvested from the oocytes (metaphase I and II [MII]) at 4–6 hours after insemination, avoiding too many spermatozoa (24). After incubating COCs in hyaluronidase solution (80 IU, K-SIHY-1-5(G26773); Cook Medical) for 10–20 seconds, the CCs were collected from ICSI patients by pipetting before ICSI procedures. The CCs were stored at −80°C until used for protein, RNA extraction, and glucose assays. All CCs from each patient's oocytes were considered as one sample.

Assessment of Embryo Quality

The MII oocytes with their corresponding polar bodies and fertilized oocytes with pronuclei were observed at 4–6 hours and 16–18 hours after insemination, respectively. On days 2 and 3 after insemination, the parameters indicating the quality of individually cultured embryos were evaluated using the number of blastomeres and the degree of fragmentation as criteria: grade 1–2, equally sized blastomeres and 0%–20% fragmentation; grade 3–4, blastomeres of unequal sizes and 20% fragmentation. The highest-quality embryo was defined on day 3 and transferred.

Clinical Data Collection

The research team was blind to experimental data until all clinical data had been collected from all patients. The clinical staff obtained the clinical data, including transferable rate (percentage of embryos belonging to grade 1–2 on day 3), implantation rate (ratio of the number of fetuses to the number of embryos transferred), pregnancy outcome (gestational sac present with heartbeat as determined using ultrasound; transplant times less than three), and so on. The details of other clinical data are given in Supplemental Materials (available online).

Western Immunoblot

All CCs from each patient's oocytes were considered as one sample. Whole cells were lysed in different volumes of Laemmli sample buffer (Bio-Rad) and used for two or three experiments, depending on the number of cells obtained from the patients. The relative intensities of the Cited2 bands were determined using the β-actin band as an internal reference. After all samples were analyzed (one to two times), the Cited2/β-actin levels were ordered using the positive standard sample. More details of Western immunoblot are provided in Supplemental Materials.

Quantitative Real-time Polymerase Chain Reaction

Quantitative real-time polymerase chain reaction was performed using the Bio-Rad CFX96 Real Time PCR System with Power SYBR Green PCR Master Mix (Promega) for gene expression relative to the internal constant level of transcription of the housekeeping genes (*β-actin* and *GAPDH*) (only the data of *β-actin* which corrected by *GAPDH* was showed). More details of quantitative real-time polymerase chain reaction are given in Supplemental Materials.

Glucose Assay

After washing cells in phosphate-buffered saline three times, the glucose (glycogen) content in the CCs was measured after lysing CCs (three to four individual) in RIPA lysis buffer (Applygen Technologies) with protease inhibitors for 45 minutes. The cell lysates were centrifuged at 12,000 × *g* at 4°C for 10 minutes, and the supernatants were collected. Half of the supernatant was used to determine the glucose concentration (mg/μL) with a glucose oxidase assay (Trinder, Sigma) (similar to a previous study in liver extract [25]), and another portion was used to determine the total protein concentration (mg/μL). The glucose content was determined as the ratio of the glucose value to the total protein value (mg/mg protein), which was shown in a previous report (25).

Statistical Analysis

Statistical analysis was performed with the Statistical Analysis System (SAS Institute). A comparison in the outcomes of implantation and pregnancy rates in different groups was performed using the χ^2 test. Differences of Cited2 levels among infertility factors groups were determined with Duncan's multiple-range test. Statistical comparisons of the other data were performed by using unpaired Student's *t* test and analysis of variance. Apart from implantation and pregnancy data, other data are expressed as the mean ± SEM. Differences were considered significant when the calculated *P* value was $<.05$.

RESULTS

Detection of Cited2 Protein

The relative expression of Cited2 protein from different patients (81 IVF patients, 32 ICSI patients) was determined by normalization to the internal control, β-actin, which varied in CCs from different patients (Supplemental Fig. 1). The IVF patients were divided into two groups according to whether the Cited2 expression level in their CCs was above (n = 40) or below (n = 41) the mean value for all patients. The ICSI patients were also grouped by the mean value of Cited2 level from all ICSI patients (above, n = 15; below, n = 17).

Relationship between Cited2 Level and Embryo Development

Compared with those in the (IVF) patients with the higher Cited2 levels, the fertilization and transferable rates (day-3 embryo of grade 1–2) in the patients with low Cited2 levels were significantly higher (77.11% ± 9.09% vs. 69.18% ± 12.72%; 75.59% ± 15.19% vs. 66.08% ± 17.08%, $P<.05$) (Fig. 1A). The transferable rate was also remarkable higher (65.02% ± 5.22% vs. 51.67% ± 8.00%, $P<.05$) (Fig. 1B) in the ICSI patients with the lower Cited2 protein levels. However, there was no difference in oocyte maturation (MII) rate between the higher or lower groups in IVF and ICSI patients (IVF: 93.14% ± 5.85 % vs. 89.32% ± 8.53%; ICSI: 94.07% ± 1.72% vs. 90.27% ± 4.07%, $P>.05$) (Fig. 1A, B). The rates of fertilization and transferable in IVF patients correlated negatively with the Cited2 level in CCs (fertilization rate: correlation coefficient = −0.415, $R^2 = 0.172$; transferable rate: correlation coefficient = −0.374, $R^2 = 0.140$).

Relationship between Cited2 Level and Pregnancy Outcome

As shown in Figure 1C and D, low Cited2 levels in CCs were positively associated with a higher implantation rate (IVF: 50.94 % vs. 26.42%; ICSI: 31.25 % vs. 18.18%, $P<.05$) and a higher pregnancy rate (IVF: 71.80% vs. 40.00%; ICSI: 50.00% vs. 28.57%, $P<.05$) in IVF or ICSI patients. Correspondingly, the mean of the relative Cited2 levels was significantly lower in the CCs obtained from the patients who became pregnant (IVF: 0.38 ± 0.17 vs. 0.64 ± 0.17; ICSI: 0.29 ± 0.08 vs. 0.68 ± 0.24, $P<.05$) (Fig. 1E, F).

Effects of Various Infertility Factors on the Levels of Cited2 in CCs

Various infertility factors, including unexplained, male, ovarian, tubal, and uterine factors, were analyzed in the patients with different Cited2 levels. The results demonstrated that the percentage of patients with high Cited2 level in the polycystic ovary syndrome (PCOS) group was significantly higher than others (78.6% vs 21.4%, $P<.05$), where the percentage of patients with high Cited2 level in the tubal factor (TF) group was significantly lower (63.63% vs 36.37%, $P<.05$) (Fig. 2A). Consistently, the patients with PCOS displayed higher Cited2 levels, and the patients with TF showed lower Cited2 levels (Fig. 2B).

Characteristics of the Patients with Different Cited2 Levels

Because hormone levels, follicle count, and patient age are important factors for clinical outcome in IVF treatment, we determined these data in patients in both groups of Cited2 levels. The results in Table 1 show that the basal FSH levels were significantly different between the patients with high and low Cited2 levels.

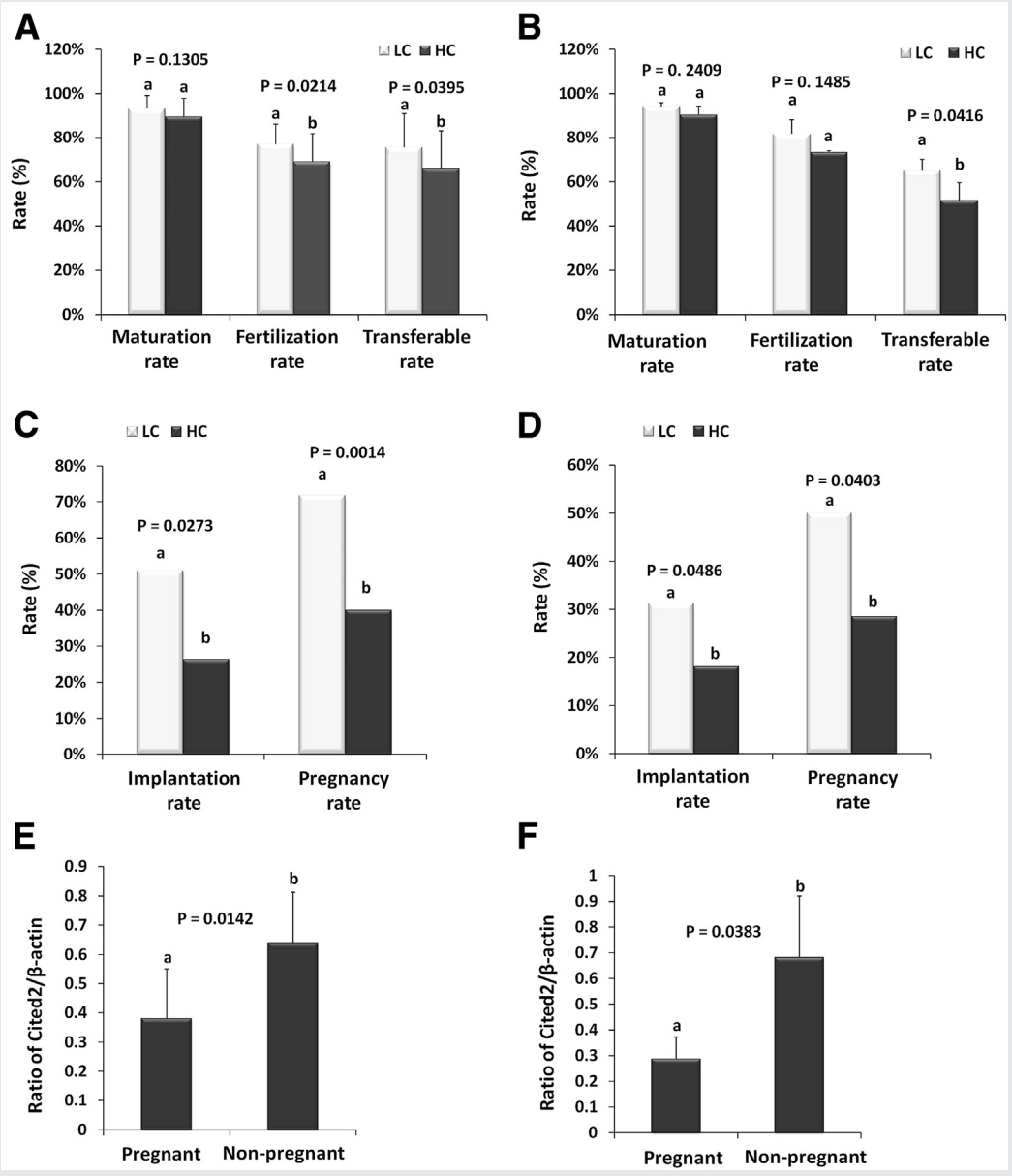
Relationship between Cited2 Levels and CC Apoptosis

Western blot analysis revealed that the cleaved (active) caspase-3 (CL-caspase-3) level was up-regulated in the patients with higher Cited2 protein levels compared with lower Cited2 levels (Fig. 3A, C). However, the p53 protein level was not correlated with the expression of Cited2 (Fig. 3B, D).

Relationship between Cited2 Levels and Expressions of its Relative Genes in CCs

The expression profiles of three genes related to steroidogenesis (*StAR*, *CYP11A1*, *CYP19A1*) and three genes related to glucose metabolism (*PPARγ*, *PGC-1α*, *GLUT1*) were not significantly different between the patients with low and high Cited2 levels (Supplemental Fig. 2). However, messenger RNA (mRNA) expressions of the *progesterone receptor* (*PR*) and *PCK1* genes were markedly increased in patients with higher Cited2 protein levels in CCs ($P<.05$) (Fig. 3E).

FIGURE 1



Association of Cited2 levels in CCs with embryo development potential. HC = high Cited2 group; LC = low Cited2 group. Association of Cited2 levels in CCs with embryo development potential from (**A**) IVF and (**B**) ICSI patients. Embryos that could be used for transplantation (grades 1 and 2) were estimated on day 3 after insemination according to the grading system described in Materials and Methods (grades 1–4). Association of the Cited2 levels in CCs with pregnancy outcome in (**C**) IVF and (**D**) ICSI patients. A comparison of the outcome of implantation and pregnancy rates in both the study and control groups was performed using the χ^2 test. (**E**) Mean of Cited2/β-actin levels in pregnant and nonpregnant IVF patients. (**F**) Mean of Cited2/β-actin levels in pregnant and nonpregnant ICSI patients. *Bars with different letters* (a, b) are significantly different ($P<.05$).

Fang. Cited2 level reflects embryo quality of patient. Fertil Steril 2016.

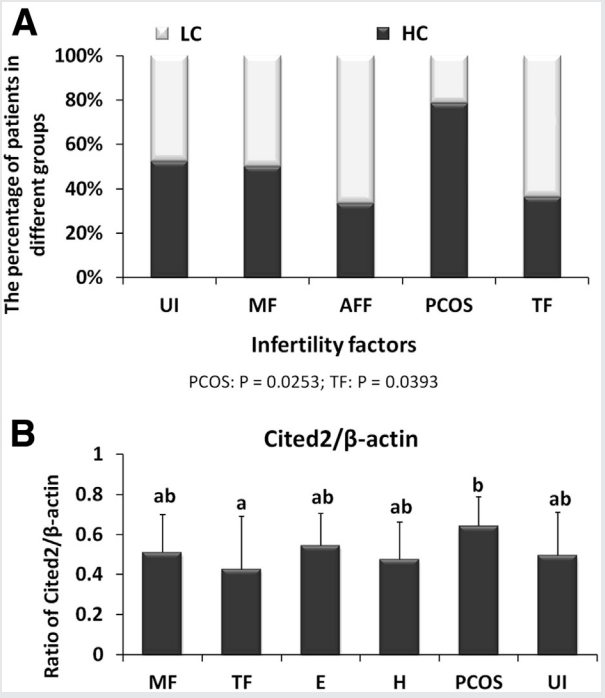
Relationship between Cited2 Levels and glucose Metabolism in CCs

Because PCK1 plays an important role in gluconeogenesis, we determined the glucose content in the CCs. Compared with patients with low Cited2 levels, the glucose contents in the CCs from the patients with higher Cited2 were markedly higher (1.05 ± 0.07 vs. 0.60 ± 0.05, $P<.05$) (Fig. 3F).

Relationship between Pregnancy Outcomes and the Related Factors of Cited2 (*PCK1*, CL-Caspase-3 and glucose Level)

The CL-caspase-3 levels in CCs from nonpregnant patients were higher than from pregnant patients (2.33± 0.81 vs. 1.45 ± 0.25, $P<.05$) (Supplemental Fig. 3A). The CCs from pregnant patients displayed higher mRNA expressions of *PCK1* than the ones from nonpregnant patients, but the difference was not

FIGURE 2



Association of infertility factors with the expression of Cited2 protein in human CCs. A total of 72 patients were examined. HC = high Cited2 group; LC = low Cited2 group. UI = unexplained infertility (unexplained causes of infertility from male or female except the following factors; n = 19): TF (n = 22); male factor (MF) (n = 11); endometriosis (E) (n = 2); hypo-ovarianism (H) (n = 4); PCOS (n = 14). The rest of the patients (n = 9) had various known causes of infertility and therefore did not participate in statistics. The proportions of PCOS (and TF) in the high and low Cited2 patients were statistically compared by χ^2 test. (**A**) Effect of different infertility factors on the distribution of patients in the high and low Cited2 level groups. AFF = all female factors except PCOS and TF (n = 9). (**B**) Effects of different infertility factors on the levels of Cited2 in human CCs. *Bars with different letters* (a, b) are significantly different ($P<.05$).

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significant ($P=.193$; Supplemental Fig. 3B). However, the patients whose CCs contained low glucose had higher pregnancy rate than the patients whose CCs contained high glucose (71.43 % vs 47.82%, $P<.05$) (Supplemental Fig. 3C).

DISCUSSION

Previous studies have suggested that detection of cumulus gene products is currently considered one of the most promising approaches for assessing oocyte and embryo quality (26), which may improve the clinical outcomes during IVF cycle. The results of the present study demonstrated a close relationship between the Cited2 levels in CCs, embryo quality, and the pathologic conditions of infertility in patients.

In this study we showed that Cited2 protein was expressed in CCs, and its level varied among patients. These results were consistent with those of other reports that detected *Cited2* mRNA expression in mouse and human CCs (11, 12). In our study the patients with lower Cited2 levels had higher rates of fertilization, transferable embryos, implantation, and pregnancy. However, the Cited2 level in CCs did not correlate with oocyte maturation. Moreover, the similar results in ICSI patients suggested that the effects of Cited2 levels on the embryo quality were not influenced by insemination. Recent studies also showed that Cited2 played an important role in female/male gonadal development, and abnormal expression of Cited2 results in infertility (11, 12, 21, 22, 27). Although silencing *Cited2* gene expression contributes to embryonic lethality and abnormal gonadal development, over-expression of *Cited2* may cause other problems. A higher *Cited2* mRNA level was also detected in nonsurrounded nucleolus mouse oocytes, which displayed more widespread chromatins and easily ceased development at the two-cell stage (12). These data suggest that the high expression of Cited2 was involved in poor embryo quality.

As mentioned before, the higher Cited2 level in CCs together with E_2 increase may contribute to female sterility (11). For searching the relationship between the Cited2 levels and infertile factors, which may be the main original reasons for the lower oocyte and embryo quality (14, 16), we later studied effects of pathologic conditions on the levels of Cited2 in CCs. Interestingly, a total of 78.6% of the patients with polycystic ovaries (PCOS) in our study displayed high Cited2 protein levels, and the Cited2 levels in PCOS patients were higher than that in other patients (but with no statistical differences). Moreover, Chen et al. (28) reported that the patients with PCOS tended to have higher peak E_2 levels, lower percentage of high-quality oocytes,

TABLE 1

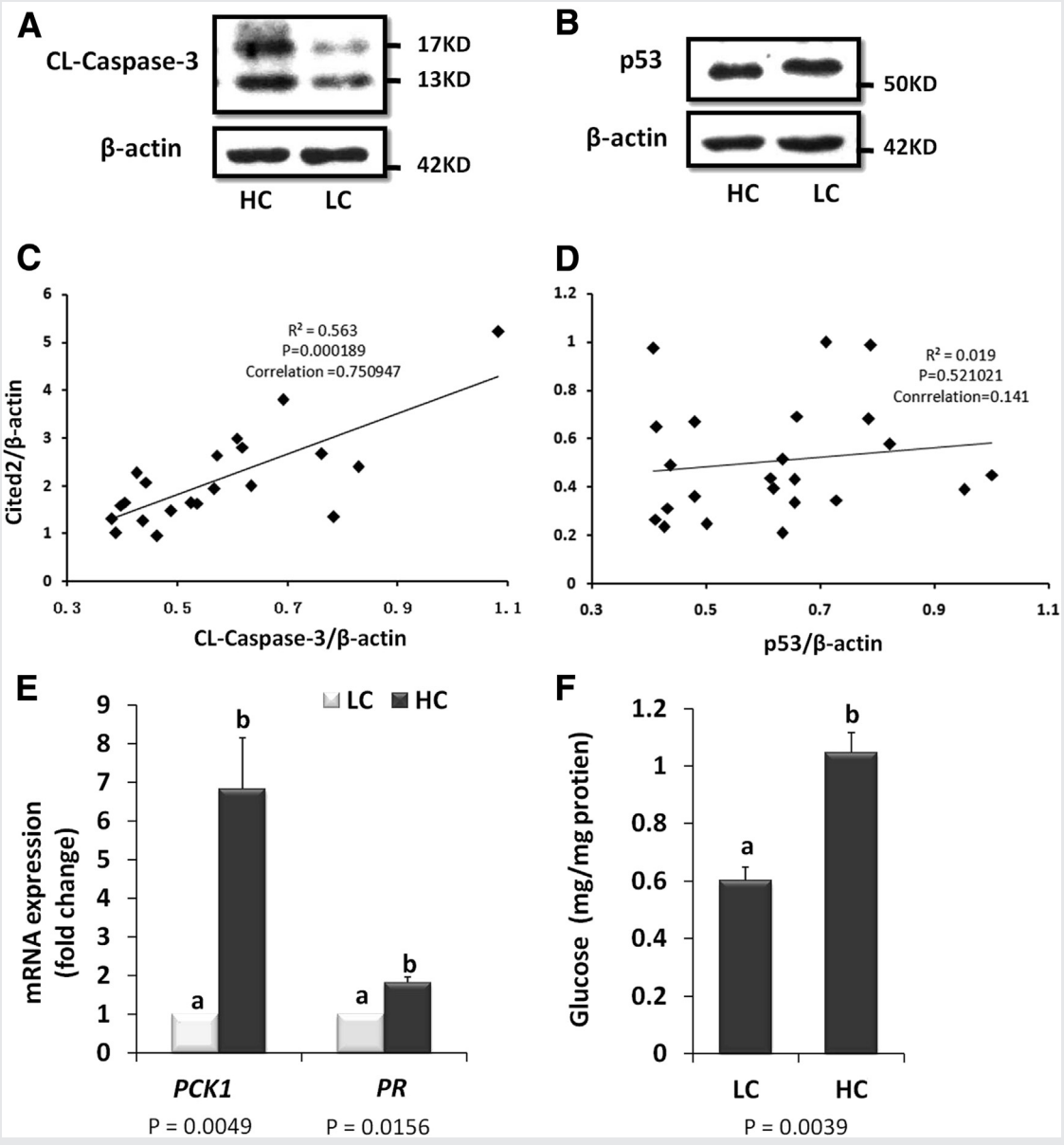
Characteristics of patients grouped according to their Cited2 protein levels in CCs.

Variable	High Cited2 group	Low Cited2 group	*P* value
Age (y)	33.12 ± 4.801	33.87 ± 4.326	NS (.481)
Basal follicle count	27.92 ± 18.68	21.8 ± 14.72	NS (.410)
Basal FSH (IU/L)	6.84 ± 1.45	7.99 ± 3.00	.042[a]
Basal E_2 (pg/ml)	35.00 ± 28.05	35.29 ± 26.63	NS (.965)
Basal LH (IU/L)	4.34 ± 2.97	4.49 ± 2.56	NS (.845)
Duration of stimulation (d)	11.74 ± 2.36	12.31 ± 4.06	NS (.461)
Total FSH administered (IU)	2,299.92 ± 836.265	2,512.82 ± 867.33	NS (.294)
Antral follicle count	13.43 ± 6.58	12.07 ± 7.29	NS (.428)
E2 on hCG day (pg/mL)	3,403.37 ± 2,152.23	2,444.3 ± 1,482.42	NS (.067)
Oocytes retrieved (n)	14.32 ± 7.73	14.89 ± 10.43	NS (.586)

[a] Data are significantly different ($P<.05$).

Fang. Cited2 level reflects embryo quality of patient. Fertil Steril 2016.

FIGURE 3



Relationship between Cited2 levels and the levels of its related gene and proteins in CCs. HC = high Cited2 group; LC: low Cited2 group. (**A, C**) Correlation analysis between CL-caspase-3 and Cited2 protein levels in CCs derived from IVF patients. (**B, D**) Correlation analysis between p53 and Cited2 protein levels in CCs derived from IVF patients. (**E**) The mRNA expression levels (relative to internal control *β-actin*; these data were also corrected by *GAPDH*) of *PR* and *PCK1* in patients with different Cited2 levels. Data are presented relative to the values in the LC groups. (**F**) Glucose levels in patients with high and low Cited2 levels are shown relative to total protein release in cell lysate. *Bars with different letters* (a, b) are significantly different ($P<.05$).

Fang. Cited2 level reflects embryo quality of patient. Fertil Steril 2016.

and lower fertilization rate during the IVF cycle when compared with other patients (15, 16). In this study, the patients with higher Cited2 level in CCs also had high peak E_2 levels during the IVF cycle. *Cited2* is an estrogen (E)-responsive gene in breast cancer, and its protein promotes breast cancer metastasis to bone (29). Interestingly, the patients with TF in our study displayed lower Cited2 protein levels than other patients. However, the reason was not clear, because we can find very few relative researches. These data suggested that the high expression of Cited2 might be involved in pathologic conditions that relate to E_2 levels.

Surprisingly, we observed that basal FSH levels were significantly lower in patients with high Cited2 levels. It is generally accepted that FSH inhibits follicle atresia by suppressing granular cell apoptosis (30–32). Cited2 promotes apoptosis by activating the p53-dependent apoptosis pathway via acetylating the p53 protein (33). Moreover, Cited2 had been shown to be upstream of caspases in the apoptosis process (33, 34). In our study, p53 protein expression was not associated

with Cited2 level. However, the levels of CL-caspase-3 protein were significantly increased in the patients with high Cited2 protein levels. It is well known that the sequential activations of caspases play vital roles in executing apoptosis (35, 36). Some studies have demonstrated that the CCs from poor embryos after insemination showed higher apoptotic rates than those from good embryos in human and cow (14,37–39). The embryos derived from oocytes surrounded by CCs with lower CL-caspase-3 level also showed increased pregnancy outcome after embryo transfer in human (40). In diabetic mice, the activation of caspase-3 in CCs was involved in the poor pregnancy outcomes (41). We also confirmed that the CL-caspase-3 level in CCs was negatively correlated to pregnancy outcome of its embryo. However, the relationship of caspase-3 activation and Cited2 level awaits deciphering.

Cited2 also generally acts as a cofactor with other transcriptional factors, such as TFAP2, PPARα/γ, PGC-1α, E receptor, and SMAD2/3, to regulate downstream gene expression (9,42–44). To find out the downstream factors of Cited2, we studied the genes that had been reported to be associated with the human embryo quality and pregnancy outcome. At the time of oocyte collection, a full reduction in *PR* mRNA levels and increase of the genes related to steroidogenesis in human CCs are associated with good morphology of human embryo (45). In a previous study, high Cited2 expression induced up-regulation of the E receptor–regulated gene trefoil factor 1 and *PR* under basal E levels in human breast cancer cells (46). In our study the *PR* mRNA level was significantly increased in CCs from patients with high Cited2 expression. However, there was no difference in expressions of the genes related to steroidogenesis (*StAR*, *CYP11A1*, *CYP19A1*) between the two groups. These results indicated that poor embryo quality in the patients with high Cited 2 levels might be attributable to high *PR* expression.

The *PCK1* gene was proposed as one marker in CCs to assess pregnancy outcome of human embryos (47). PCK1 is regulated by PGC-1a and Cited2, which plays an important role in gluconeogenesis in diabetic mice (48). A previous study showed that PPARγ acted as a critical mediator in Cited2-induced apoptosis (34), in which Cited2 was required for the regulation of hepatic gluconeogenesis through the PPARγ coactivator 1α (PGC-1α) (48). However, the CCs with higher Cited2 protein levels in our results showed a significant increase in *PCK1* mRNA, but no changes in *PPAR*γ or *PGC-1a* mRNA levels. As expected, the higher glucose in CCs was also found in the patients with high Cited2 levels in our study. Moreover, the content of glucose in CCs closely correlated to pregnancy outcome. The patients with poor pregnancy outcomes had more glucose in their CCs. Although there was no statistical difference in *PCK1* mRNA expressions between pregnant and nonpregnant patients, the mRNA expression in the CCs from nonpregnant patients displayed an increasing tendency. This result was consistent with a previous report (47). It is suggested that the high Cited2 level might damage oocyte quality by up-regulating *PCK1* mRNA expression to cause abnormal glucose metabolism in CCs. In rodent diabetic models, the elevated glucose concentration in embryo development environment resulted in abnormal embryo morphogenesis (49). Furthermore, the embryos derived from this environment showed increased resorptions and pregnancy loss after embryo transfer (50–52). In human, overweight glucose-tolerant women have greater adverse pregnancy outcomes than normal women (53). The exact mechanism of high intracellular glucose in CCs damaging embryo development needs further investigation.

The CCs supply the oocyte with pyruvate by taking up and metabolizing glucose through glycolysis (54–58), and FSH can stimulate glucose consumption of COCs to support oocyte maturation (59). Reduced glucose transporter 1 (GLUT1) expression and decreased glucose transport into the murine blastocyst induce an increase in apoptosis (41). However, the CCs with higher Cited2 protein levels did not show lower *GLUT1* levels in our study. A previous report showed that 27% of premenopausal women with type 2 diabetes also presented with PCOS (60). Therefore, the high glucose in the CCs with high Cited2 levels may result from abnormal gluconeogenesis, which may be partly caused by PCOS.

In conclusion, we found that high Cited2 level in CCs was associated with low embryo quality and pregnancy outcome in IVF patients. Further studies will be needed to elucidate the molecular pathway how Cited2 in CCs regulates oocyte quality and embryo development potency.

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SUPPLEMENTAL MATERIALS

Clinical Data Collection

The basal levels of E_2, FSH, and LH were detected during the menstrual period of the second and third day before the IVF procedure. The level of E_2 on the day of hCG administration is the peak E_2 level during the IVF cycle. The basal follicles were counted before the IVF procedure. The progression of follicular development was followed by daily ultrasound evaluation and serum E_2 level during IVF cycle. When at least three follicles were developed to 18 mm in diameter, a single injection of hCG (10,000 IU) was administered for oocyte retrieval, using transvaginal ultrasound guidance.

Western Immunoblot

Equal volumes (7 μL) of samples (9–10 IVF samples were randomly selected as one group and run on the same gel with a positive standard sample) were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Millipore). The membrane was blocked with 5% nonfat milk in TBS-T (10 mM Tris at pH 7.5, 150 mM NaCl, and 0.1% Tween 20) for 1 hour at room temperature. The membrane was then incubated with primary antibodies (4°C, overnight), washed in TBS-T, and incubated with horseradish peroxidase–conjugated secondary antibodies for 1 hour at room temperature. Antibodies against Cited2 (JA22) and caspase-3 were purchased from Santa Cruz Biotechnology and Cell Signaling Technology, respectively. Anti-actin antibodies were obtained from Abmart Biotechnology. The horseradish peroxidase–conjugated anti-rabbit antibody was obtained from Zhong Shan Biotechnology. The protein bands were visualized using enhanced chemiluminescence detection reagents (Applygen Technologies) and X-Omat BT film (Eastman Kodak), according to the manufacturers' instructions. The films were digitized, and densitometric analysis was performed with ImageJ version 1.44p software (National Institutes of Health).

Quantitative Real-time Polymerase Chain Reaction

Total cellular RNA from CCs derived from an individual (IVF) patient was extracted using an RNAprep pure tissue kit (TIANGEN Biotech) according to the manufacturer's instructions. This RNA was then used for complementary DNA synthesis to generate a complementary DNA template (RevertAid First Strand cDNA Synthesis Kit, Thermo Fisher Scientific) for quantitative real-time polymerase chain reaction. The quantitative real-time polymerase chain reaction was performed using the Bio-Rad CFX96 Real Time PCR System with Power SYBR Green PCR Master Mix (Promega) for gene expression relative to the internal constant level of transcription of the housekeeping gene *β-actin* and *GAPDH* (only showing the data of *β-actin* that corrected by *GAPDH*). After a 10-minute incubation at 95°C, amplification was performed for 40 cycles at 9°C for 15 seconds and 60°C for 1 minute, followed by a dissociation stage for 15 seconds at 95°C, 1 minute at 60°C, 15 seconds at 95°C, and 15 seconds at 60°C. Standard polymerase chain reaction conditions were used. Primer sequences and primers used (SUN Biotech) are shown in Supplemental Table 1.

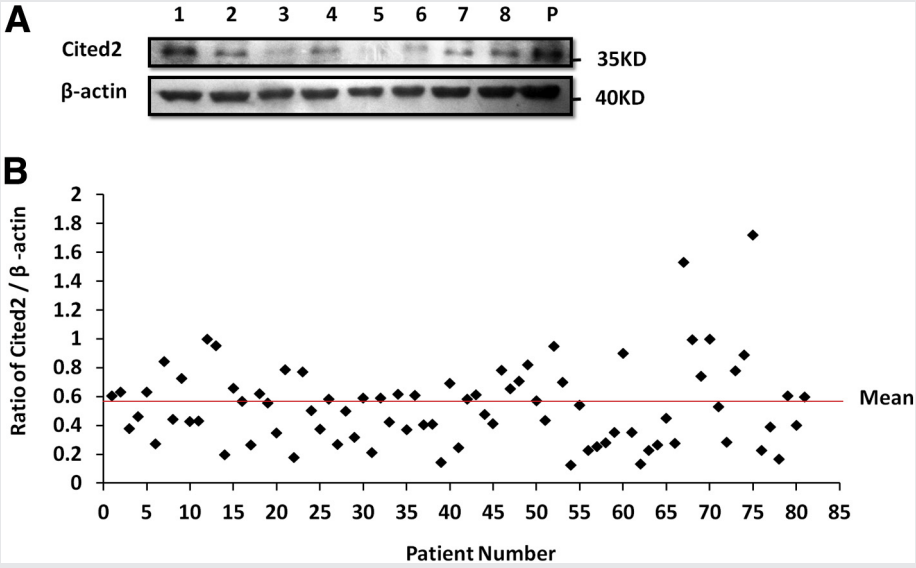
SUPPLEMENTAL TABLE 1

Primer sequences.

Gene	Sequence
PR	Forward 5′-CCCAAGGAAGATTCCCGCTT-3′ Reverse 5′-GCGTGATTGAGAGGCAGGAT-3′
CYP11A1	Forward 5′-CCCACCTTCTTCTTCGACCC-3′ Reverse 5′-AGGAGATGGGCTTTTCAGGC-3′
CYP19A1	Forward 5′-ATGCGAGTCTGGATCTCTGGA-3′ Reverse 5′-TTGGTCACCTCCTCCAACCT-3′
StAR	Forward 5′-GTGACTTTGTGAGCGTGCG-3′ Reverse 5′-ACTTCCAGCCAACGGGTGAA-3′
PPARγ	Forward 5′-GCAATCAAAGTGGAGCCTGC-3′ Reverse 5′-TCTCCGGAAGAAACCCTTGC-3′
PGC-1α	Forward 5′-CAGACCTGACACAACACGGA-3′ Reverse 5′-GCAGCAAAAGCATCACAGGT-3′
PCK1	Forward 5′-AAGGGCCATCAACCCAGAAA-3′ Reverse 5′-AGGTTCCCCATCCTCTGAGC-3′
GLUT1	Forward 5′-CATTGGCTCCGGTATCGTCA-3′ Reverse 5′-GCCAGGACCCACTTCAAAGA-3′

Fang. Cited2 level reflects embryo quality of patient. Fertil Steril 2016.

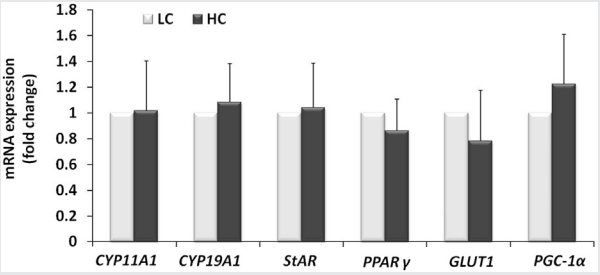
SUPPLEMENTAL FIGURE 1



Detection of Cited2 protein in human CCs using Western blotting. (**A**) A representative blot showing Cited2 protein levels in cells obtained from different patients. (**B**) Quantification of Cited2 protein level in CCs from different patients and positive sample using β-actin as an internal control. On the basis of the results of the assay, patients were divided into two groups (*red line*) according to the levels of the Cited2/β-actin ratio detected in the CCs from each individual patient. 1–9 indicate different patients; P = positive standard sample.

Fang. Cited2 level reflects embryo quality of patient. Fertil Steril 2016.

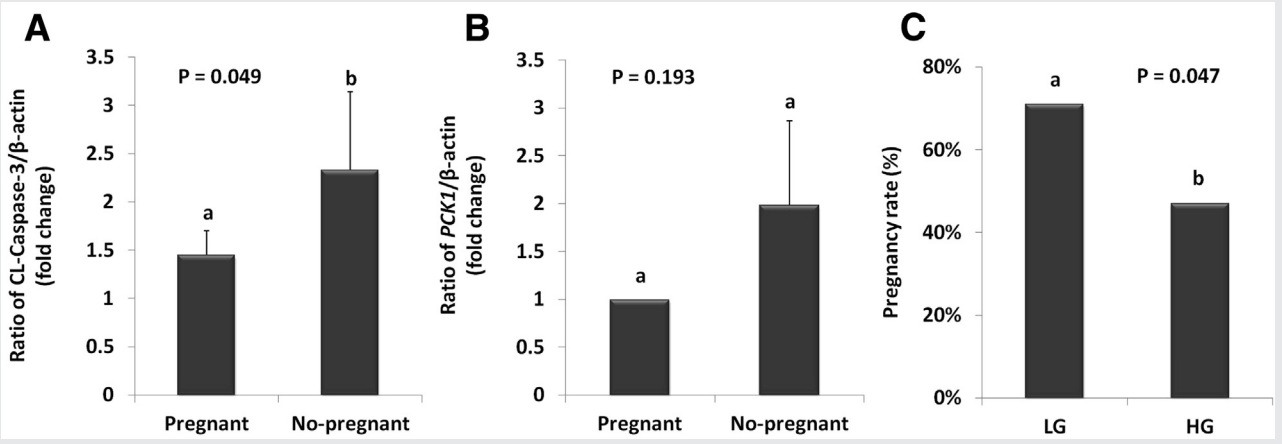
SUPPLEMENTAL FIGURE 2



Relationship between Cited2 levels and its related gene levels. HC = high Cited2 group; LC = low Cited2 group. The mRNA expression levels (relative to internal control *β*-actin) of *StAR*, *CYP11A1*, *CYP19A1*, *PPARγ*, *PGC-1α*, and *GLUT1* in patients with different Cited2 levels were determined using qPCR. Data are presented relative to the values in the LC groups. *Bars with different letters* (a, b) are significantly different ($P<.05$).

Fang. Cited2 level reflects embryo quality of patient. Fertil Steril 2016.

SUPPLEMENTAL FIGURE 3



Relationship between pregnancy outcome and related factors of Cited2. (**A**) The CL-caspsase-3 levels (relative to internal control *β*-actin) in patients with different pregnancy outcomes. (**B**) The mRNA expression levels (relative to *β-actin*) of *PCK1* in patients with different pregnancy outcomes. Data are presented relative to the values in the pregnant groups. (**C**) Pregnant outcomes from patients with different glucose levels in CCs. The pregnancy rates between high and glucose level patients were statistically compared by χ^2 test. LG = low glucose group; HG = high glucose group. *Bars with different letters* (a, b) are significantly different ($P<.05$).

Fang. Cited2 level reflects embryo quality of patient. Fertil Steril 2016.