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# An investigation of the effects of laser-assisted zona pellucida drilling on the preimplantation mouse embryo and the competency of embryo implantation

--Manuscript Draft--

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<b>Corresponding Author:</b>	Kevin Coward University of Oxford Nuffield Department of Women's and Reproductive Health UNITED KINGDOM
<b>Corresponding Author Secondary Information:</b>	
<b>Corresponding Author's Institution:</b>	University of Oxford Nuffield Department of Women's and Reproductive Health
<b>Corresponding Author's Secondary Institution:</b>	
<b>First Author:</b>	Yaqiong Liu
<b>First Author Secondary Information:</b>	
<b>Order of Authors:</b>	Yaqiong Liu
	Celine Jones
	Kevin Coward
<b>Order of Authors Secondary Information:</b>	
<b>Abstract:</b>	<p><b>Objective :</b> To investigate the impact of laser-assisted zona pellucida (ZP) drilling on the mouse embryo, with particular emphasis on molecular mechanisms, and the efficiency of embryo attachment capability using an in vitro model of implantation.</p> <p><b>Design:</b> Experimental study.</p> <p><b>Setting:</b> Academic research laboratory.</p> <p><b>Patients/Animals:</b> C57BL/6J0laHsd mouse embryos and B6C3F1 × B6D2F1 mouse embryos.</p> <p><b>Interventions:</b> 8-cell stage mouse embryos were randomly assigned to a laser-assisted ZP drilling group (n=343), a ZP-partial drilling group (n=312), a ZP-quarter thinning group (n=289), and a control group (n=353). Embryos were cultured in vitro from E2.5 to E4.5 for 48 hours. To investigate the capacity to implant, E4.5 embryos (laser-assisted drilling group (n=46), ZP-partial drilling group (n=28), ZP-quarter thinning group (n=26), and control group (n=36)), were then transferred onto an attachment model based on Ishikawa cells and cultured for another 72 hours.</p> <p><b>Main Outcome Measures :</b> Blastocyst formation, hatching status, and hatching morphology at E4.5. Blastocyst cell components, the extent of apoptosis in embryonic cells (DNA fragmentation, caspase-3 activation, and the expression of apoptosis-related genes), the expression of HSP70, and differentially expressed genes (DEGs) generated by RNA-sequencing. Fully hatched embryo rate and stable attachment rate at E7.5 in the in vitro attachment model.</p> <p><b>Results:</b> There were no significant differences between the laser-assisted ZP manipulation groups and the control group with respect to the formation of blastocysts, cell number, embryonic cell apoptosis, and cellular stress. All three of the laser-assisted ZP manipulations significantly increased the hatching rate at E4.5 when compared with control group, especially the ZP drilling group. However, only ZP drilling group was associated with a significantly higher proportion of '8'-shape hatching blastocysts. Furthermore, RNA-sequencing identified 48 DEGs between blastocysts</p>

	<p>from the laser-assisted drilling group and the control group; the metabolic pathways were significantly enriched in these DEGs. In addition, there were no significant differences between the laser-assisted ZP manipulation groups and the control group with respect to the rate of stable attachment at E7.5, although a significantly higher entrapment rate was observed in the ZP drilling group.</p> <p>Conclusions: Laser-assisted ZP manipulations did not induce cellular apoptosis or stress in mouse blastocysts. Nevertheless, for the first time, we found that laser-assisted ZP drilling could alter the embryonic transcriptome and may affect metabolic activity. Furthermore, although laser-assisted ZP manipulations can enhance the initiation of hatching, it is evident that ZP drilling comes with a potential risk of embryo entrapment.</p>
<b>Suggested Reviewers:</b>	
<b>Opposed Reviewers:</b>	
<b>Response to Reviewers:</b>	<p>We have revised manuscript according to the editor's requirement:</p> <p>We have (1) added the running title as 'Laser-assisted drilling and embryos' ; (2) included the academic degree of each author; (3) revised the structured abstract; (4) justified the reference style accordingly; (5) re-named the tables; (6) changed the name of supplemental figure; and (7) submitted the supplemental table as an Excel spreadsheet.</p>
<b>Additional Information:</b>	
<b>Question</b>	<b>Response</b>

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Corresponding author: Yaqiong Liu, Celine Jones, Kevin Coward

Authors may either sign the same form or submit individually

I am an author on this submission, have adhered to all editorial policies for submission as described in the Information for Authors, attest to having met all authorship criteria, and disclosed all potential conflicts of interest for inclusion on the title page of the submission.

Signatures are required - typed signatures are unacceptable.

Typed or CLEARLY Printed Name:

Signature:

DR YAQIONG LIU

Typed or CLEARLY Printed Name:

Signature:

DR KEVIN COWARD

Typed or CLEARLY Printed Name:

Signature:

MRS CELINE JONES

Typed or CLEARLY Printed Name:

Signature:

Typed or CLEARLY Printed Name:

Signature:

Typed or CLEARLY Printed Name:

Signature:

Typed or CLEARLY Printed Name:

Signature:

Typed or CLEARLY Printed Name:

Signature:

Kevin Coward  
Director, MSc in Clinical Embryology  
Research Group Leader  
Lecturer in Medicine, Trinity College  
Senior Fellow, Higher Education Academy  
Fellow, Royal Society of Biology

1<sup>st</sup> August 2021

Dear Professor Catherino and Professor Treff,

**RE: An investigation of the effects of laser-assisted zona pellucida drilling on the preimplantation mouse embryo and the competency of embryo implantation (Liu *et al.*)**

We wish to re-submit a style revision of this manuscript. In this revised manuscript, we have added the running title as 'Laser-assisted drilling and embryos' and included the academic degree of each author. We have also revised the structured abstract and justified the reference style accordingly. Besides, we re-named the tables and supplemental figures. In addition, we submitted the supplemental table as an Excel spreadsheet.

We would love to thank the referees and the editors for re-reviewing and considering accepting this manuscript.

Yours sincerely,

A handwritten signature in blue ink that reads "Kevin Coward".

**Kevin Coward PhD PGDipLATHE SFHEA FRSB CBiol CSciTeach**

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**An investigation of the effects of laser-assisted zona pellucida drilling on the  
preimplantation mouse embryo and the competency of embryo implantation**

Running title: Laser-assisted drilling and embryos

Yaqiong Liu, MRes, Celine Jones, Kevin Coward, PhD \*

Nuffield Department of Women's and Reproductive Health, University of Oxford, Level 3,  
Women's Centre, John Radcliffe Hospital, Headington, Oxford, OX3 9DU, UK.

\*Corresponding author

Email: [kevin.coward@wrh.ox.ac.uk](mailto:kevin.coward@wrh.ox.ac.uk); Telephone: +44 [0] 1865 618900; Fax: +44 [0] 1865  
618900

Conflicts of interest: None.



## Abstract

**Objective:** To investigate the impact of laser-assisted zona pellucida (ZP) drilling on the mouse embryo, with particular emphasis on molecular mechanisms, and the efficiency of embryo attachment capability using an *in vitro* model of implantation.

**Design:** Experimental study.

**Setting:** Academic research laboratory.

**Patients/Animals:** C57BL/6JOlaHsd mouse embryos and B6C3F1 × B6D2F1 mouse embryos.

**Interventions:** 8-cell stage mouse embryos were randomly assigned to a laser-assisted ZP drilling group (n=343), a ZP-partial drilling group (n=312), a ZP-quarter thinning group (n=289), and a control group (n=353). Embryos were cultured *in vitro* from E2.5 to E4.5 for 48 hours. To investigate the capacity to implant, E4.5 embryos (laser-assisted drilling group (n=46), ZP-partial drilling group (n=28), ZP-quarter thinning group (n=26), and control group (n=36)), were then transferred onto an attachment model based on Ishikawa cells and cultured for another 72 hours.

**Main Outcome Measures:** Blastocyst formation, hatching status, and hatching morphology at E4.5. Blastocyst cell components, the extent of apoptosis in embryonic cells (DNA fragmentation, caspase-3 activation, and the expression of apoptosis-related genes), the expression of HSP70, and differentially expressed genes (DEGs) generated by RNA-sequencing. Fully hatched embryo rate and stable attachment rate at E7.5 in the *in vitro* attachment model.

**Results:** There were no significant differences between the laser-assisted ZP manipulation groups and the control group with respect to the formation of blastocysts, cell number, embryonic cell apoptosis, and cellular stress. All three of the laser-assisted ZP manipulations

significantly increased the hatching rate at E4.5 when compared with control group, especially the ZP drilling group. However, only ZP drilling group was associated with a significantly higher proportion of '8'-shape hatching blastocysts. Furthermore, RNA-sequencing identified 48 DEGs between blastocysts from the laser-assisted drilling group and the control group; the metabolic pathways were significantly enriched in these DEGs. In addition, there were no significant differences between the laser-assisted ZP manipulation groups and the control group with respect to the rate of stable attachment at E7.5, although a significantly higher entrapment rate was observed in the ZP drilling group.

**Conclusions:** Laser-assisted ZP manipulations did not induce cellular apoptosis or stress in mouse blastocysts. Nevertheless, for the first time, we found that laser-assisted ZP drilling could alter the embryonic transcriptome and may affect metabolic activity. Furthermore, although laser-assisted ZP manipulations can enhance the initiation of hatching, it is evident that ZP drilling comes with a potential risk of embryo entrapment.

**Keywords:** Laser-assisted drilling; Embryo development; Cell apoptosis, RNA-sequencing; Embryo attachment

## Introduction

As a powerful tool in assisted reproductive technology (ART), the non-contact infrared diode laser serves as a reliable technique for manipulations of the embryonic zona pellucida (ZP) with high levels of consistency and reduced procedure time (1,2). At present, laser-assisted ZP drilling is widely applied in various clinical applications, including embryo biopsy, intracytoplasmic sperm injection (ICSI), and embryo cryopreservation (3). Drilling an opening in the ZP is one of the key steps to undertake when biopsying an embryo; the most popular technique for this purpose is laser-assisted drilling, a technique that can be applied immediately prior to biopsy (4). In addition, laser-assisted pre-drilling can also be applied for trophectoderm (TE) biopsy, a technique that is commonly performed on day 3 - 4 (3-6). Apart from ZP drilling, laser-assisted ZP-partial drilling as well as ZP- quarter thinning, with the intention to thin but not breach the ZP, are also applied in the clinic to improve embryo implantation rate (3,7,8).

Laser-assisted ZP manipulation involves the application of a high energy form of light that produces heat to dissolve or ablate the ZP (9). This heat is dissipated and transferred through culture media, thus creating a temperature gradient in the culture media immediately surrounding an embryo (10,11). Furthermore, as a critical structure, the ZP protects an embryo from detrimental factors that may be present in artificial media, including toxins, microorganisms, and antibodies (12,13). Laser-assisted drilling, as an invasive technique, inevitably exposes an embryo to the external environment and may cause the loss of blastomeres and a reduction in embryonic autocrine secretion, thus creating a potential risk to embryo development (8,14,15). Therefore, the energy delivered to an embryo during laser-assisted ZP drilling or ZP thinning, and the drilling technique itself, might affect the quality of an embryo and its ability to implant.

Several research studies have attempted to evaluate the laser-assisted manipulations on human embryos or embryos acquired from animal models. On a positive note, many of these studies failed to detect any adverse effects on embryo quality and preimplantation development potential, especially when applying a laser beam to manipulate the ZP at high power laser and appropriate durations of time (16-18). As a vital indicator of developmental potential, embryonic cells are positively correlated with blastocyst quality (19). Worryingly, some studies have reported a reduction in the number of embryonic cells following laser-assisted treatment (14,20-22). Aside from developmental potential, cellular apoptosis is another biological process that can serve as a useful indicator of an inadequate or suboptimal *in vitro* environment or the detrimental effects of manipulation. A recent study demonstrated that laser-assisted ZP drilling at either 2-cell and 6 to 9-cell stage embryos significantly induced embryo DNA damage (21); although the specificity of the TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) assay used to detect DNA fragmentation in this previous study was relatively low (21). Consequently, it is evident that future studies must use other methods to detect cellular apoptosis, such as the expression levels of apoptosis- and stress-related genes and the activation of caspase-3 (14,23,24).

Although laser-assisted drilling has been used in clinics for a long period of time, very few studies have specifically investigated the impact of this technique on embryos at the molecular level; furthermore, the most comprehensive of these previous studies used mouse models (25,26). As yet, none of these previous studies have detected any significant changes in the expression levels of genes related to heat shock stress (25) or epigenetic markers (26) following laser-assisted drilling at the 8-cell stage. However, insufficient evidence is available at this time to confirm the safety of laser-assisted drilling on the embryonic transcriptome since only a limited number of genes have been investigated thus far, such as *Dnmt3a*, *Dnmt3b*, and *Hsp70i*.

On the other hand, the precise efficacy of laser-assisted ZP manipulations on clinical outcomes also remains controversial. Some studies demonstrated that laser-assisted drilling on day 3 embryos could result in poor pregnancy outcomes when applied for assisted hatching (7,12) and for pre-hatching prior to embryo biopsy (4,27). Some researchers have suggested that laser-assisted ZP thinning could provide the better pregnancy outcomes when compared with control or ZP drilling groups (7,28,29). Embryo implantation is known to be affected by both embryo competency and the receptivity of the endometrium (30). In addition, the hatching process is also affected by zona lysis in the uterus (31,32). Thus, it is evident that there are numerous factors that could confound data when attempting to evaluate the efficacy of laser ZP drilling on embryo competency *in vivo*. A better model for studying the efficacy of laser-assisted ZP manipulations would be the *in vitro* attachment model, particularly when investigating the efficiency of hatching. By focusing solely on the competency of the embryo to implant, the *in vitro* attachment model avoids potential maternal confounding factors, such as components in the uterine fluid that may enhance blastocyst development and endometrium receptivity (33).

In the present study, we investigated the effect of laser-assisted ZP drilling, ZP- partial drilling, and ZP-quarter thinning on mouse embryos from a variety of biological and molecular aspects. We found that these techniques did not affect the formation of blastocysts and did not induce cellular apoptosis or stress. However, RNA-sequencing demonstrated, for the first time, that laser-assisted ZP drilling can result in changes to the embryonic transcriptome. This indicated a potential impact on embryonic metabolism. Finally, experiments involving an *in vitro* embryo attachment model demonstrated that laser-assisted ZP drilling, but not partial ZP drilling and ZP-quarter thinning, had an adverse impact on the completion of embryo hatching but not attachment capability.

## Materials and methods

### Embryo collection and assessment

Mice were raised in the Biomedical Services (BMS) Unit at the University of Oxford. All experiments and animal handling were conducted in accordance with the institutional guidelines for animal experimentation after obtaining prior approval from the Ethics Committee of the Department of Physiology, Anatomy and Genetics, University of Oxford (License number: 30/3301). Super-ovulation strategies and general maintenance were performed under protocol BMS//JRSOP285. The day of human chorionic gonadotrophin (hCG) injection and mating was defined as E 0. Females with vaginal plugs were sacrificed, and the reproductive tracts were harvested on E 2.0. Eight-cell stage embryos (C57BL/6J OlaHsd) were obtained from the junction of the oviduct by mincing the oviducts and uterus using fine forceps under microscopy. The morphology of the collected 8-cell stage embryos was then evaluated; embryos with more than 6 equivalent size blastomeres were then pooled and allocated into two groups by defocusing the microscope to prevent any selection bias. In contrast, embryos containing cytoplasmic fragments, unevenly sized blastomere, and damaged ZP were excluded from study in accordance with previous criteria (34).

For the RNA sequencing, we needed to ensure the sample size (no less than 8 samples/group). In addition, we needed to harvest a sufficient amount of embryonic cDNA for qRT-PCR (quantitative real-time polymerase chain reaction) in every experimental repeat. To facilitate these, we purchased cryopreserved 8-cell stage mouse embryos (B6C3F1  $\times$  B6D2F1) from Embryotech Laboratories (USA) for these experiments.

## Laser-assisted ZP manipulation and embryo culture

Embryos were cultured individually by transferring one embryo into one drop of potassium-supplemented simplex optimized medium with  $\frac{1}{2}$  Amino Acids (KSOM-AA) (EmbryoMax KSOM Mouse Embryo Media, Millipore, Abingdon, UK) (20  $\mu$ L) covered with mineral oil (FertiPro NV, Beernen, Belgium) and incubated at 5% CO<sub>2</sub> and 37°C for at least 10 minutes prior to ZP manipulation. All embryo manipulations were performed using a Flexipet (EZ-Grip) and a transfer pipette (EZ-Tip) (CooperSurgical, Falmouth, UK).

Laser-assisted ZP manipulations involved a Saturn 5 Laser System (Cooper Surgical Fertility Companies, Denmark) set to a wavelength of 1.48  $\mu$ m, a power of 400 mW, and a laser diameter of 7.5  $\mu$ m. The laser was calibrated prior to the experiment, and these parameters were used consistently for all manipulations. In addition, a red pilot beam was used to ensure targeting optimization on the microscope and guaranteed that the laser was initiated in a position that was sufficiently far away from the blastomeres to avoid damage during manipulation. Laser-assisted ZP drilling was conducted by targeting the ZP with two consecutive laser pulses, from the outer side to the inner side (**Fig. 1 B-1**). We confirmed that the ZP had been breached by observing fluid flowing out of the perivitelline space. If the ZP was not drilled completely with 2 pulses, an extra laser pulse would be given. For ZP-partial drilling, one laser pulse was fired to drill through half of the ZP thickness (**Fig. 1 C-1**). The ZP-quarter thinning was performed to ablate the one quarter of the ZP circumference by half of the ZP thickness (**Fig. 1 D-1**). Embryos in the control group were placed onto a pre-warmed stage for a similar period of time but without laser manipulation (**Fig. 1**). Following manipulation of the ZP manipulation, embryos were cultured in an incubator with 5% CO<sub>2</sub> at 37°C, as described in previous studies (14,16,22,28) (starting from 12 pm).

## Embryo development and embryo hatching patterns

After 48 hours of *in vitro* culture, embryos were observed on a pre-warmed microscope stage (at the E4.5 stage; 12 pm) (**Fig. 1**) and the number of developed blastocysts and hatching blastocysts were quantified. The hatching rate was calculated by dividing the number of hatching blastocysts by the number of existing blastocysts  $\times 100$  (%). As we noticed an overt difference in the pattern of hatching at the assessment time-point, we further evaluated the hatching pattern and categorized these patterns according to different morphological features (35,36): an '8'-shape hatching blastocyst featured two blastocoels divided by the ZP with an opening in the ZP of  $\leq 25 \mu\text{m}$  at the time of observation. Conversely, when the opening in the ZP was  $\geq 25 \mu\text{m}$ , the blastocysts were regarded as 'U'-shape hatching blastocysts. Blastocysts with multiple hatching/breaching points ( $\geq 2$ ) were referred to as multiple-point hatching/breaching blastocysts (**Fig. 1**).

## Immunofluorescence staining

Immunofluorescence staining was used to determine the expression levels of a marker for the inner cell mass (ICM) (OCT-4), marker for trophoctoderm (TE) cells (CDX2), and heat shock protein (HSP70). In brief, the collected E4.5 blastocysts were washed with 0.01% (V/V) poly (vinyl alcohol) (PVA)-phosphate-buffered saline (PBS) buffer prior to fixation with 4% paraformaldehyde (PFA) for 20 minutes and was then permeabilized with 0.05% Triton X-100 for 10 minutes. Embryos were then incubated overnight at 4°C with primary antibodies (OCT-3/4 (Santa Cruz, UK), CDX2 (Novus Biological, UK), and HSP70 (Invitrogen, UK)). The following morning, embryos were washed and incubated with a fluorescein isothiocyanate (FITC)-conjugated secondary antibody (Invitrogen, Thermo Fisher, UK). DAPI (Vector Laboratories, UK) was then used to visualize all cell nuclei. Image J software was used to quantify the number of cells in the ICM and the TE, and to measure the mean gray value of



HSP70 (in arbitrary units). For Heat Shock Protein 70 (HSP70) staining, heat shock (HS) treated embryos were used as a positive control. Before staining, HS was induced by placing the blastocyst at 43°C for 30 minutes, followed by a recovery period at 37°C for 30 minutes to produce HSP70 (37). Then, antibody staining was performed as described above.

### **The assessment of cell apoptosis**

Embryonic cell apoptosis was assessed by evaluating DNA fragmentation and the activation of caspase-3. The extent of DNA fragmentation was assessed using a TdT-mediated dUTP nick-end labelling (TUNEL) kit (Promega, UK) in accordance with the manufacturer's instructions. The positive control for TUNEL assay involved the treatment of embryos with DNase I (50 IU/ml) for 10 minutes at room temperature prior to fixation. The negative control was created by treating embryos with DNase but without labelled TdT reagent. The apoptotic index of DNA fragmentation (%) (the number of the TUNEL-positive cells / the number of the DAPI-positive cells) was then determined for each embryo (**Supplemental Fig. 1-A**).

Furthermore, we analyzed Caspase-3/7 activation using a fluorescent inhibitor of caspase (FLICA) kit (Invitrogen, UK). Prior to embryo labelling, the positive control embryos were incubated with 1 µM Staurosporine (Sigma, UK) for 2 hours at 37°C. The kit was used in accordance with the instructions provided by the manufacturer. Green fluorescence represented the activated Caspase-3/7 (**Supplemental Fig. 1-B**). Activated Caspase-3/-7 was quantified with Image J software by measuring mean grey values (arbitrary units).

### **RNA-sequencing (RNA-seq) and the analysis of differentially expressed genes (DEGs)**

RNA-sequencing experiments involved 8 hatching blastocysts in the control group and 8 hatching blastocysts in the laser-drilling group. For cDNA synthesis, each single blastocyst was lysed in 2.3 µl of lysis buffer (0.8% (vol/vol) Triton X-100 and 2 U/µl of RNase inhibitor

(both from Thermo Fisher, UK)) using the SmartSeq2 method in accordance with Picelli et al. (38). Libraries were then prepared using Nextera-XT. ERCC RNA Spike-in mix (4456740, ThermoFisher) was then added at a dilution of 1/100,000. Amplified libraries were analysed for size distribution using the Agilent TapeStation High Sensitivity D1000 kit and then quantified using PicoGreen; relative volumes were then pooled accordingly. Sequencing was performed as 75bp paired-end reads on a HiSeq4000 according to Illumina specifications.

RNA-seq reads were aligned to the mouse reference genome (GRCm38) using HISAT2 (39), and duplicate reads were removed using the Picard 'MarkDuplicates' tool [<http://broadinstitute.github.io/picard>]. Reads that were mapping uniquely to Ensembl-annotated genes (~5 million per sample) were summarised using featureCounts program (40). The raw gene count matrix was imported into the R/BioConductor environment (41) for quality control and data normalisation. The edgeR package (42) was used to test for differentially expressed genes between the control and laser-drilling groups. We also included the embryo batch as an additional explanatory variable. Raw P-values were adjusted for multiple testing using the Benjamini-Hochberg (BH) procedure (43) to control the false discovery rate at 5%.

### **Downstream pathway analysis**

DEGs were imported into Ingenuity Pathway Analysis (IPA, QIAGEN Inc) software for Canonical Pathway Analysis. In addition, Over Representation Analysis (ORA) was also used to identify enrichment of the DEGs with respect to Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways and Reactome pathways.

### **cDNA synthesis from blastocysts and quantitative polymerase chain reaction (PCR)**

cDNA was generated from three blastocysts using the cell-to-cDNA kit (Invitrogen, UK) in accordance with the manufacturer's instructions. Pre-amplification of target cDNA was

performed using the TaqMan PreAmp Master Mix Kit (Applied Biosystems, UK). In brief, pooled TaqMan assays were created by combining an equal volume of the target TaqMan probes: [*Mvk* (Mm00445773\_m1), *Mvd* (Mm00507014\_m1), *Dnmt3l* (Mm00457635\_m1), *Lmna* (Mm00497783\_m1), *Cdx2* (Mm01212280\_m1), *B2m* (Mm00437762\_m1), *Ywhaz* (Mm05674356\_s1), *Bax* (Mm00432051\_m1), *Bcl2l2* (Mm00432054\_m1), *Sod1* (Mm01344233\_g1), *Gpx7* (Mm00481133\_m1), and *Gapdh* (Mm99999915\_g1)]. Quantitative RT-PCR was then carried out using the TaqMan Gene Expression System (Applied Biosystem, UK) on an Applied Biosystems QuantStudio 3 (Thermo Fisher, UK) using standard thermocycler parameters. After validation, the mean CT values of *B2m* and *Ywhaz* were used as internal controls for comparing gene expression levels between blastocysts from the laser-drilling and control groups for RNA-seq validation. The relative expression levels of all target genes were calculated using the  $2^{(-\Delta\Delta Ct)}$  method (44) by normalizing to the internal control, and the differences in gene expression level are presented as the fold change relative to the mean Ct value of the control group.

### Cell culture and the *in vitro* model of embryo attachment

Ishikawa cells (ECACC 99040201), a human endometrial adenocarcinoma cell line of epithelial origin, were maintained in conditional medium (1:1 Dulbecco's modified Eagle's medium: Ham's F12 (Sigma-Aldrich, UK)) containing 10% fetal bovine serum (FBS) supplemented with 100 µg/ml streptomycin, 100 IU/ml penicillin, and 20 µg/ml gentamicin (Sigma-Aldrich, UK). Ishikawa cells were then grown to full confluence in 24-well plates on 13 mm glass coverslips coated with 2% growth phenol red-free factor-reduced Matrigel (Corning, UK). The monolayer of Ishikawa cells was then washed and replenished with a co-culture medium (0.1% FBS in conditional medium) prior to embryo transfer. One to five E4.5 blastocysts (beyond the expanded blastocysts) were collected and transferred non-selectively

1 onto the Ishikawa cell monolayer, following by culture at 37°C in 5% CO<sub>2</sub> for 72 hours to E7.5.  
2 For embryos showing a delay in blastulation, E4.5 embryos were cultured one more day *in*  
3 *vitro*. Then, the E5.5 blastocysts (if they grew to an expanded blastocyst or beyond) were then  
4 collected and transferred onto an *in vitro* co-culture system.  
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10 Hatching ability and attachment stability were evaluated every 24 hours. The fully hatched rate  
11 was calculated as the number of hatched blastocysts divided by the number of transferred  
12 blastocysts  $\times 100$  (%). Embryos that failed to hatch out at 72 hours were defined as trapped  
13 embryos. In accordance with a previous study (45), the stability of embryo attachment to the  
14 Ishikawa monolayer was assessed using a four-point scale of blastocyst behavior upon agitation  
15 of the co-culture system (by gently pressing the microscope table 2-3 times): not attached, weak  
16 attachment (major oscillation around an attachment point), intermediate attachment (minor  
17 oscillation), and fully attached (no oscillation). The stable attachment rate was calculated as  
18 the number of fully attached blastocysts divided by the number of transferred blastocysts  $\times 100$   
19 (%).  
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### 34 35 36 **Statistical analysis**

37 Quantitative data were analyzed using Prism 8 software (GraphPad Software, CA, USA). Data  
38 were compared between four groups using the one-way analysis of variance (ANOVA) test (if  
39 normally distributed) or Kruskal-Wallis non-parametric test (if not normally distributed) with  
40 relevant post-hoc tests for multiple comparison between each treatment and control groups.  
41 Normality was evaluated with the D'Agostino-Pearson omnibus (K2) test. Differences in  
42 proportions between treatment and control groups were assessed using the  $\chi^2$  test in the SPSS  
43 version 26.0 software package (SPSS Inc., Chicago, IL, USA). A probability (*P*) value  $< 0.05$   
44 was considered to be statistically significant.  
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## Results

### The incidence of hatching and hatching patterns at E4.5

Blastocyst formation rates were similar between the control and the treatment groups. However, when compared with the control group, the embryo hatching rate was significantly higher in the laser-ZP drilling group ( $P<0.0001$ ), ZP-partial drilling group ( $P=0.034$ ), and ZP-quarter thinning group ( $P=0.003$ ). In addition, a significant disparity was shown with regards to hatching patterns in the ZP-drilling group in the comparison with the control group ( $P<0.0001$ ): 48.9% of embryos in the control group were beginning to hatch at E4.5, while 75% of blastocysts in the ZP drilling group adopted an ‘8-shape’ morphology. In addition, 25.8% of hatching blastocysts in the ZP-quarter thinning groups were adapted in ‘U-shape’ morphology (**Table 1**). Nevertheless, no significant difference in E4.5 hatching pattern was detected between ZP-partial drilling, ZP-quarter thinning, and the control group ( $P>0.05$ ).

### Embryonic cell number, cellular apoptosis, and stress at E4.5

Embryos were stained with OCT-3/4 and CDX2 to visualize the inner cell mass (ICM) and trophectoderm (TE). There were no significant differences across four groups in terms of embryonic cell number of blastocysts. Interestingly, the total cell number of the hatching blastocysts in the laser-assisted ZP drilling group was significantly lower than that in the control group ( $50.92 \pm 2.69$  vs  $66.71 \pm 7.04$ ,  $P=0.035$ ); a similar pattern was also found with the number of cells in the TE of hatching blastocysts, although this was not statistically significant (**Fig. 2A**).

Next, we analyzed cellular apoptosis from several different aspects. As shown in **Fig. 2B**, DNA fragmentation and caspase-3 activation levels were comparable in the four groups. Furthermore, these laser-assisted ZP treatments did not change the mRNA expression of apoptosis-related

genes (*Bax*, *Bcl2l2*, *Sod1*, and *Gpx7*) ( $P>0.05$ ). These results suggested that laser-assisted ZP manipulations would not result in a significant increase in apoptosis in the embryonic cells.

The expression levels of HSP70 were detected in E4.5 blastocysts. We observed high levels of HSP70 expression in embryos that had been treated with HS (heat shock treatment). Laser-assisted ZP drilling and ZP-partial drilling did not induce a significant increase in the expression levels of HSP70 in the blastocysts (**Fig. 2C**). Interestingly, the level of HSP70 in blastocysts following the laser-assisted ZP-quarter thinning was significantly lower than that of blastocysts in the control group ( $P=0.006$ ).

### **Alterations in the transcriptome following laser-assisted ZP drilling and related pathways**

To investigate the potential transcriptomic changes caused by laser-assisted ZP drilling, we used RNA-seq analysis to determine DEGs between ZP-drilled blastocysts and untreated blastocysts. Overall, we identified 48 genes that were differentially expressed (BH (Benjamini-Hochberg) adjusted  $P < 0.05$ ); of these, 39 genes were up-regulated, and 10 genes were down-regulated (**Fig. 3A**). The fold changes of these DEGs were small; only 13 DEGs exhibited a fold change larger than 2, including 11 up-regulated genes (*Lilra6*, *Maged2*, *Cep250*, *Acbd4*, *Mvk*, *Lmna*, *Acaa1a*, *Aldh18a1*, *Mvd* and *Cdk5rap3*) and two down-regulated genes (*Lmfn* and *Gsk3a*). The DEGs list was presented in the supplemental material. To validate the RNA-seq results, we selected four DEGs (*Mvk*, *Mvd*, *Lmna*, and *Dnmt3l*) and one stably expressed gene (*Cdx2*). As shown in **Fig. 3B**, the results of the qRT-PCR followed the same trends as the RNA-seq findings, although the change in *Dnmt3l* expression level did not reach statistical significance.

Downstream pathway analysis demonstrated that the identified DEGs were significantly enriched in 16 canonical pathways, 2 KEGG pathways, and 1 Reactome pathway (**Fig. 3C**).

Interestingly, many metabolism-related pathways were enriched, particularly those associated with lipids metabolism, including lipid metabolism pathways (*Slc25a1*, *Mvd*, *Acbd4*, *Agpat4*, *Aacs*, *Mvk*, *Acaa1a*, and *Mcat*), mevalonate pathway I (*Mvd* and *Mvk*), cholesterol biosynthesis (*Mvd* and *Mvk*), and docosahexaenoic acid (DHA) signaling (*Gsk3a* and *Pik3c2a*). Furthermore, other metabolic pathways, such as degradation pathways associated with valine, leucine, and isoleucine (*Aldh9a1*, *Aacs*, and *Acaa1a*), the insulin receptor signaling pathway (*Gsk3a*, *Pik3c2a*, and *Trip10*), and xenobiotic metabolism signaling (*Aldh18a1*, *Aldh9a1*, *Keap1*, and *Pik3c2a*) were also significantly enriched according to these DEGs (**Fig. 3C**).

### Embryo hatching completion and attachment from E4.5 to E7.5

Overall, 136 E4.5 mouse blastocysts were transferred onto the *in vitro* attachment model. As shown in **Fig. 4A**, differing hatching statuses and degrees of embryo outgrowth and invasion to the Ishikawa cell layer were observed at the three time-points. When using the *in vitro* attachment model, we were able to observe fully hatched embryos after 24 hours (**Fig. 4B**), while the proportion of stably attached embryos started to increase after 48 hours (**Fig. 4C**). Thus, we compared fully hatched rates from 24 hours and stably attached rates at 48 hours and 72 hours.

As shown in **Table 2**, the fully hatched rate in the laser-assisted ZP drilling group was significantly lower at 48 hours ( $P=0.003$ ) and 72 hours ( $P=0.017$ ) of culture. In contrast, the proportion of trapped embryos was significantly higher in the laser-assisted drilling group than in the control group ( $P=0.007$ ). In addition, 58.7% (27/46) of transferred embryos in ZP drilling group were 8-shaped hatching blastocysts, 70.4% (19/27) of these embryos ended with entrapment. In contrast, there were no differences in fully hatched rates and entrapment rates when compared between the ZP-partial drilling and ZP-quarter thinning groups and the control

group. Interestingly, neither of these laser-assisted ZP manipulations affect the attachment of embryos since no difference was found in the stable attached rate at 48 or 72 hours (**Table 2**).

Furthermore, the blastulation delayed blastocysts, which formed the blastocyst around E5.5, were observed among embryos from the four groups. When transferring these embryos into the *in vitro* model, we observed a lower fully hatched rate (56.3% vs 77.8%) and a significantly lower stably attached rate (50% vs 83.3%,  $P=0.012$ ) in the untreated E5.5 ET embryos when compared with E4.5 untreated embryos. However, in the comparison with the control group, E5.5 embryos in ZP drilling and ZP-quarter thinning groups exhibited substantially higher proportions of fully hatched embryos at 72 hours. In addition, ZP drilling significantly increased the attachment rate ( $P=0.008$ ) (**Table 3**).

## Discussion

Although laser-assisted ZP manipulations, especially ZP drilling, are used widely in clinics, evidence relating to the safety of this technique remains very limited, especially at the molecular level. In the present study, we investigated the effect of laser-assisted ZP manipulations on preimplantation mouse embryos from a variety of different aspects, but with particular focus on embryonic cell number, cell stress and apoptosis, and transcriptomic alternations. We found that laser-assisted ZP-drilling, partial-drilling, and quarter thinning did not interfere with the formation of blastocysts and embryonic cell number; these findings were in line with previous studies (20,22,31). In addition, we also found these laser-assisted ZP manipulations did not induce cellular apoptosis, at least under our experimental conditions. These results contradict those reported by a previous study that showed increased levels of DNA damage following laser-assisted drilling (21). The discrepancies could be associated with a variety of factors related to laser manipulation, such as the duration of laser pulses or the time interval between two consecutive laser pulses. Furthermore, in the present study, we did not



1 detect any significant differences between the laser-assisted ZP manipulation groups and the  
2 control group with respect to the activation of caspase-3/7 and the expression levels of  
3 apoptosis-related genes (*Bax*, *Bcl2l2*, *Sod1*, and *Gpx7*), thus providing compelling evidence  
4 that these ZP manipulations did not induce cell apoptosis.  
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10 The potential thermal damage is one of the major concerns when using laser pulses on embryos  
11 since preimplantation embryos are particularly sensitive to thermal changes (37). A previous  
12 study demonstrated that laser drilling did not induce a rapid upregulation of *Hsp70i* mRNA  
13 expression, even in cells that were closest to the laser beam (25). In that study, the expression  
14 of *Hsp70i* was detected 2 hours after the laser treatment, in order to permit hsp70i RNA  
15 accumulation and investigate the possible heat shock effect generated by laser beam itself.  
16 However, in the present study, we investigated the expression of HSP70 at the blastocyst stage;  
17 therefore, the level of hsp70 expression at this stage would act as an indicator for the cell stress  
18 caused by laser-assisted ZP treatments rather than the laser beam itself. Similarly, we did not  
19 see an increase in the protein expression of HSP70 in the laser-assisted ZP drilling group or  
20 ZP-partial drilling group, thus suggesting that laser-assisted drilling does not induce cellular  
21 stress in preimplantation embryos.  
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41 The evidence in respect to the impact of laser-assisted ZP manipulations on the transcriptome  
42 is sparse. For the first time, this study investigated potential transcriptional alternations  
43 following laser-assisted ZP manipulation with RNA-seq. In our study, however, we focused on  
44 the ZP drilling rather than ZP-thinning, because we assumed that ZP drilling would have the  
45 greatest impact on embryos by exposing them to the external environment. Data analysis  
46 indicated that laser-assisted drilling at the cleavage stage would not induce overt transcriptomic  
47 alternations at the blastocyst stage since only 48 DEGs with small fold changes were identified.  
48 Interestingly, the analysis of downstream pathways further revealed that laser-assisted drilling  
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could potentially affect metabolic activity in blastocysts, at least at the mRNA level. A previous study investigated metabolism changes in the human embryo 24 hours after laser-assisted hatching; however, the component analysis of metabolites failed to detect any significant differences when compared to embryos that did not undergo laser-assisted hatching (16). It is known that embryo metabolism is relatively low prior to the morula stage but becomes highly activated following the formation of blastocyst and until implantation (46,47). Thus, it is understandable that the changes in embryonic metabolic activity from day 2 to day 3 following laser-assisted hatching was not detectable in this previous study (16). In the present study, we cultured embryos for 48 hours from day 3 to day 5 after laser-assisted drilling; the potential changes in embryonic metabolic activity caused by laser-drilling were indicated by the identification of DEGs related to the metabolism of lipids and amino acids. Therefore, the embryonic metabolism alterations caused by laser-assisted drilling may occur at the blastocyst stage. Furthermore, all the related DEGs were upregulated in the laser-drilled blastocysts, indicating that laser ZP drilling might activate embryo metabolism by creating a route for transferring nutrients from the culture medium to embryos. Interestingly, we also observed the enrichment in xenobiotic metabolism signaling (*Aldh18a1*, *Aldh9a1*, *Keap1*, and *Pik3c2a*). A xenobiotic is defined as the presence of an artificial or higher than normal concentration of a substance within an organism. Xenobiotic metabolism aims to remove such xenobiotics by deactivation and excretion (48). Our RNA-seq results indicated that laser-assisted drilling could potentially induce the presence of xenobiotics in embryos, which may be caused by exposing embryos directly to the culture medium before hatching at the blastocyst stage. However, further studies are now needed to confirm these findings by validating these molecules and the pathways involved. In addition, it would be interesting to compare the different impacts between ZP-drilling and ZP-thinning on the embryo transcriptome. Furthermore, it is crucial to understand whether the changes in embryonic metabolism at the

blastocyst stage will have lasting influences on the health of the offspring. Therefore, longer periods of follow-up involving the offspring arising from ZP drilling in mice will be crucial in future studies and should focus particularly on metabolic activity.

In line with previous findings involving mouse embryos (14,17,20,21,49,50) and human embryos (18,51), we found that all three ZP manipulation significantly advanced the initiation of embryo hatching at E4.5, with a highest proportion of embryos hatching found in the ZP-drilling group. In addition, the reduced number cells in hatching embryos from the ZP drilling group also supported the fact that the artificially induced ZP opening allows embryos to begin hatching without reaching a certain embryonic cell number; this observation concurred with previous studies (20,31).

Furthermore, we found that laser-assisted drilling altered the pattern of hatching, and a greater number of embryos adopted an '8'-shaped. This distinct '8'-shape hatching behavior was first reported in 1989 (52) in micromanipulated embryos; the phenomenon by which embryos hatch slowly through a small opening in the ZP is referred to as "herniation" (28). This TE herniation of blastocyst induced by ZP drilling facilitates the separation of the TE during blastocyst biopsy and helps embryologists to arrange their work routine; thus, laser-assisted drilling on day 3 embryos is widely used as a pre-hatching strategy for blastocyst biopsy in clinics (4,53,54).

Nevertheless, although laser-assisted ZP drilling advanced the initiation of hatching, there is a potential risk that this could cause blastocyst entrapment. In this study, a lower hatching completion rate at E7.5, and a higher proportion of trapped embryos, were observed in the laser-assisted drilling group. In particular, around 70% of the '8'-shape hatching blastocysts ended up with entrapment. Hatching entrapment has been observed in several other studies involving mouse embryos (20,28,55) and human embryos (56). Furthermore, animal studies have demonstrated that blastocyst entrapment is related to the size of the opening in the ZP.

1 However, no consensus has been reached with regards to the minimum size of the ZP opening  
2 with which to maintain hatching efficiency (20,49,55). It is not surprising that a large opening  
3 in the ZP could facilitate the completion of embryo hatching, as demonstrated by many  
4 previous studies (22,50,57). However, using a laser to create a small ZP opening is widely  
5 accepted for biopsy purposes (6,53). In clinics, a small ZP opening (5 to 10  $\mu\text{m}$ ) is commonly  
6 applied to day 3 human embryos to assist pre-hatching prior to TE biopsy (4,27,53). On the  
7 other hand, embryo entrapment is rarely reported in clinics since embryos are only cultured  
8 briefly *in vitro* prior to transfer; this means that embryologists cannot observe embryo  
9 entrapment during this short period of culture (4). Using the *in vitro* embryo attachment model,  
10 we demonstrated the risk of blastocyst entrapment following laser-assisted drilling, thus  
11 indicating the importance of refining the size of the ZP opening during clinical application in  
12 order to maintain hatching efficiency.  
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30 Although laser ZP drilling affected the completion of embryo hatching, it did not impact upon  
31 an embryo's capability for *in vitro* attachment to epithelial cells. However, considering that  
32 only fully hatched embryos could ultimately implant into the uterus, the final  
33 implantation/pregnancy rate might be affected following laser-assisted drilling, at least when  
34 the size of the ZP opening is small. This phenomenon partially explains previous observations  
35 that laser ZP drilling in day human 3 embryos is related to the poor pregnancy outcomes when  
36 used for pre-hatching prior to biopsy as well as assisted hatching (4,7,12). Therefore,  
37 considering the potential impact on pregnancy outcomes, it is urgent to investigate the  
38 relationship between the size of the ZP opening and pregnancy outcomes. Furthermore, it is  
39 crucial to refine the ZP drilling protocol for human embryos to avoid damaging the competency  
40 of embryos to implant.  
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On the other hand, the high frequency of '8-shape' hatching following laser-assisted drilling clearly demonstrates that this technique represents a potential risk factor for cleavage of the ICM. A previous study, involving mouse embryos, showed that '8-shape' hatching affects ICM herniation and increases the risk of ICM cleavage (35). Three clinical case reports showed that the transfer of '8-shape' hatching embryos resulted in monozygotic twin (MZT) pregnancy and monochorionic triamniotic triplet's pregnancy (58-60). Furthermore, a recent retrospective study in Japan, involving 937,848 single embryo transfer cycles, revealed that assisted hatching represents a potential risk factor for zygotic splitting. However, no specific details were given regarding the effects of ZP manipulation (61). In contrast, Gu *et al.* (62) found that incarceration of the ICM in 8-shaped blastocysts did not increase the number of MZT pregnancies in patients undergoing PGD/PGS. Since the incidence of MZT is very rare in clinics, large scale follow-up studies are now needed to reveal the specific risk of perinatal complications following laser-assisted drilling.

A previous study indicated that more than 80% of *in vivo* developed embryos underwent the 'U'-shape hatching (35); meanwhile, ZP-quarter thinning could lead to a superior 'U'-shape hatching pattern (14). Therefore, it was suggested that the change of integrity of ZP induced by laser thinning could influence the hatching pattern and facilitate the embryo hatching (14). In this study, a slightly higher proportion of 'U'-shape hatching was also observed in the ZP-quarter thinning group. Interestingly, we also found that the ZP-quarter thinning suppresses the expression of HSP70, which suggesting that this technique could reduce the embryonic cell stress. We assumed that the ZP-quarter thinning, by changing the integrity of the ZP, could reduce pressure within the blastocyst, thus suppressing the expression of HSP70. The superior hatching pattern and decreased cell stress indicated that laser-assisted ZP-quarter thinning might be a better method for assisted hatching. However, further studies are needed to investigate the specific role of HSP70 during the hatching process.

1 Clinical data suggested that both fresh and cryopreserved embryos, which did not undergo  
2 blastulation until day 6, were associated with lower pregnancy potential (63,64). However, it  
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4 is not clear whether this was due to asynchrony between the embryo and endometrium or due  
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6 to the impaired embryo quality. Here, we showed that delayed blastulation might reduce the  
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8 completion of the embryo hatching and the subsequent attachment capability. Intriguingly, this  
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10 might be rescued by laser-assisted ZP drilling, which facilitated the embryo attachment of E5.5  
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12 ET embryos. Previous clinical data support the notion that assisted-hatching improves clinical  
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14 outcomes when applied to day 6 blastocysts (65), and even day 7 blastocysts (66). However,  
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16 in contrast to clinical practice, the ZP manipulations performed in this study were all carried  
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18 out at E2.5 (equivalent to day 3 in the human embryo). In addition, the sample size for  
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20 blastulation delayed embryos was very limited. Therefore, large scale studies are now needed  
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22 to investigate the effect of laser-assisted ZP manipulations performed on developmental-  
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24 delayed blastocysts.  
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32 There were some limitations to this research that should be considered. For example, we used  
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34 an *in vitro* attachment assay; this may not fully recapitulate the real conditions *in vivo* since the  
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36 hatching process and embryo attachment may vary between *in vitro* and *in vivo* conditions (31).  
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38 Nevertheless, the *in vitro* model of embryo attachment used in the present study was not  
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40 confounded by any of the maternal factors involved in blastocyst hatching or implantation.  
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42 Furthermore, it also allowed us to track individual embryos after transfer; while it is impossible  
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44 to track embryos *in vivo* once embryos have been transferred to the uterus. However, it is  
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46 important to investigate the efficacy of these ZP manipulations in pregnancy outcomes and the  
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48 impact on the offspring health in the future. For example, the treated embryos can be transferred  
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50 into surrogate mice, and the health of the resulting fetuses and newborns could be observed.  
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52 Finally, the embryo hatching process could also be species-dependent, and we should, of course,  
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be very cautious with regards to extrapolating data from the mouse directly to the human scenario.

## Conclusion

In conclusion, our analysis suggested that laser-assisted ZP drilling, ZP-partial drilling, and ZP-quarter thinning, do not impact the formation of blastocysts or induce apoptosis or stress in embryonic cells. However, our data did indicate that the ZP drilling could alter the embryonic transcriptome and potentially affect embryonic metabolism. Furthermore, although ZP manipulations could advance the initiation of hatching, the ZP drilling might also pose a potential risk for hatching alteration and blastocyst entrapment, thus affecting the competency of an embryo to undergo implantation. However, ZP drilling may be beneficial for blastulation delayed embryos. For the first time, this study investigated the effect of laser-assisted drilling on the embryonic transcriptome and revealed the potential safety impacts involved. Our data also highlighted the importance to optimize and refine this clinical technique in future.

## F&S Science Clinical Quick Take

- Laser-assisted ZP manipulations are safe technique that did not interfere with blastocyst formation or cell number and did not induce apoptosis or cellular stress; however, ZP drilling changed the embryonic transcriptome with potential effects on embryonic metabolism.
- Although laser-assisted ZP manipulation advanced the initiation of hatching, the small size of the opening in the ZP created by drilling could cause embryo entrapment, thus potentially affecting the ability of an embryo to implant.
- We identified the risk of laser-assisted drilling on the embryonic transcriptome and the completion of hatching, thus highlighting the importance of optimising this clinical technique and the need to investigate the impact upon embryonic metabolism.

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**Table 1. A comparison of embryo development and hatching pattern at E4.5**

	ZP-drilling (n=343)	ZP-partial drilling (n=312)	ZP-quarter thinning (n=289)	Control (n=353)
Resulting blastocysts	272 (79.3%)	246 (78.9%)	229 (79.2%)	289 (81.9%)
Hatching blastocysts	224 <sup>****</sup> (82.4%)	123 <sup>*</sup> (50.0%)	124 <sup>**</sup> (54.1%)	118 (40.8%)
	ZP-drilling (n=162)	ZP-partial drilling (n=79)	ZP-quarter thinning (n=66)	Control (n=92)
Starting	20 (12.3%) <sup>†</sup>	34 (43%)	29 (43.9%)	45 (48.9%)
8-shape	125 (77.2%) <sup>†</sup>	26 (32.9%)	15 (22.7%)	23 (25.0%)
U-shape	14 (8.6%)	10 (12.7%)	17 (25.8%)	14 (15.2%)
Multiple points hatching	3 (1.9%) <sup>†</sup>	9 (11.4%)	5 (7.6%)	10 (10.9%)

\*ZP-partial drilling vs control group,  $P < 0.05$ ; \*\*ZP-quarter thinning vs control group,  $P < 0.01$ ;

\*\*\*\* ZP drilling vs control group,  $P < 0.0001$

Proportions of four hatching patterns were compared between two groups by applying the  $\chi^2$  test and the z-test. The Bonferroni method was used to adjust  $P$ -values for multiple comparisons. <sup>†</sup> ZP-drilling vs control group,  $P < 0.05$

**Table 2. A comparison of fully hatched rate and stable attachment rate from E4.5 to E7.5**

	Time points	ZP-drilling (n=46)	ZP-partial drilling (n=28)	ZP-quarter thinning (n=26)	Control (n=36)
Fully hatched	24h	5 (10.9%)	3 (10.7%)	6 (23.1%)	3 (8.3%)
	48h	18 (39.1%)**	19 (67.9%)	18 (69.2%)	26 (72.2%)
	72h	24 (52.2%)*	23 (82.1%)	19 (73.1%)	28 (77.8%)
Trapped	72h	19 (41.3%)**	0	3 (11.5 %)	5 (13.9%)
Stable attachment rate	48h	23 (50%)	11 (39.3%)	12 (46.2%)	17 (47.2%)
	72h	36 (78.3%)	19 (67.9%)	22 (84.6%)	30 (83.3%)

\* ZP-drilling group vs control group,  $P<0.05$ ; \*\* ZP-drilling group vs control group,  $P<0.01$

**Table 3. A comparison of fully hatched rate and stable attachment rate from E5.5 to E8.5**

	Time points	ZP-drilling (n=11)	ZP-partial drilling (n=16)	ZP-quarter thinning (n=10)	Control (n=16)
Hatched rate	24h	7 (63.6%)	5 (31.3%)	5 (50%)	7 (43.8%)
	48h	9 (81.8%)	8 (50%)	8 (80%)	9 (56.3%)
	72h	9 (81.8%)	8 (50%)	8 (80%)	9 (56.3%)
Trapped	72h	1 (9.1%)	1(6.3%)	0	1 (6.3%)
Stable attachment rate	48h	6 (54.5%)	6 (37.5%)	6 (60%)	6 (37.5%)
	72h	11(100%)**	7 (43.8%)	8 (80%)	8 (50%)

\*\* ZP-drilling group vs control group,  $P<0.01$

## Figure captions

### Figure 1. Mouse embryos in different ZP manipulation groups and the control group

(A-1) to (A-3): Untreated mouse embryos developed from E2.5 to E4.5; (B-1) to (B-3): Embryo treated with laser-assisted drilling at E2.5 to E4.5: a single hole (yellow arrow) completely drilled through the ZP; (C-1) to (C-3): Embryo treated with ZP-partial drilling at E2.5 to E4.5: a single hole (yellow triangle) created without breaching the inner membrane; (D-1) to (D-3): Embryo treated with laser ZP-quarter thinning at E2.5 to E4.5: around a quarter of the zona pellucida circumference drilled partially (yellow dashed line). (B-3) '8'-shape hatching embryo, (D-3) 'U'-shape hatching embryo, and (C-3) multi-point hatching embryos. (Scale bar=40  $\mu$ m)

### Figure 2. The effects of laser-assisted ZP manipulations on E4.5 mouse embryos

(A) Embryonic TE (trophectoderm) and ICM (inner cell mass) cells were distinguished by immunostaining with CDX2 (red) and OCT-3/4 (green), respectively. DAPI shows nuclear staining (630 $\times$  magnification, Scale bar=50  $\mu$ m). The cell numbers of all blastocysts and hatching blastocysts were compared between treatment groups and control (n= 23-27 (for blastocyst); n= 7-24 (for hatching blastocyst)). (B): The extent of cell apoptosis was evaluated by assessing the DNA fragmentation (n=16-18), the activation of Caspase-3/-7 (n=9-15), and the mRNA expression levels of *Bax*, *Bcl2l2*, *Sod1*, and *Gpx7* (n=3-5). (C) The expression of HSP70 (green) was visualized by confocal microscopy (630 $\times$  magnification, Scale bar=50  $\mu$ m). For the positive control, embryos were incubated at 42°C for 10 minutes and then at 37°C for 30 minutes. Data are presented as mean  $\pm$  SD, except for DNA fragmentation (medium with interquartile range) (\* $P$ <0.05; \*\* $P$ <0.01).

**Figure 3. Transcriptomic changes and related pathways in embryos following laser-assisted ZP drilling**

(A) A volcano plot showing the identification of DEGs between laser-drilled blastocysts and untreated blastocysts: the x-axis shows the fold change in gene expression between samples and the y-axis shows the statistical significance of the differences. Significant up and down regulated genes are highlighted in red and blue, respectively. The top 20 up-regulated genes, and the top 10 down-regulated genes, by fold change, are marked by gene name. The gray points represent non-significant DEGs. (B) Validation of selected DEGs using qRT-PCR; data are presented as mean  $\pm$  SD (\* $P < 0.05$ ). (C) The significantly enriched pathways include IPA Canonical pathways (red), Reactome pathways (blue), and KEGG pathways (green) for DEGs between laser-drilled hatching and control hatching blastocysts. Enrichment analysis was performed using IPA and ClusterProfiler. For each enriched pathway, the significance of pathway enrichment is plotted by the bar ( $-\log(P\text{-value})$ ), while the gene ratios are presented as red dots (x-axis above).

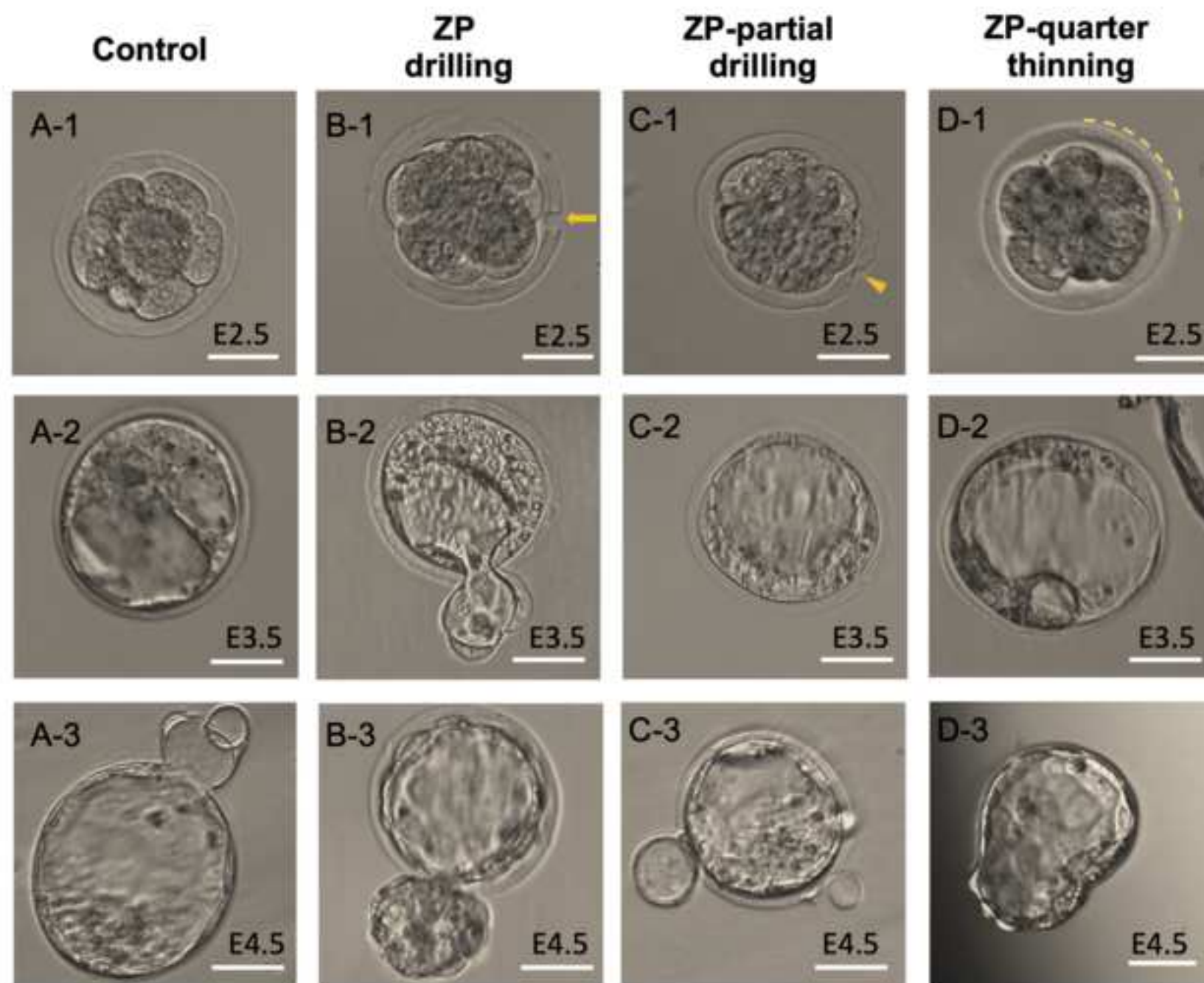
**Figure 4. The *in vitro* attachment model from E4.5 to E7.5**

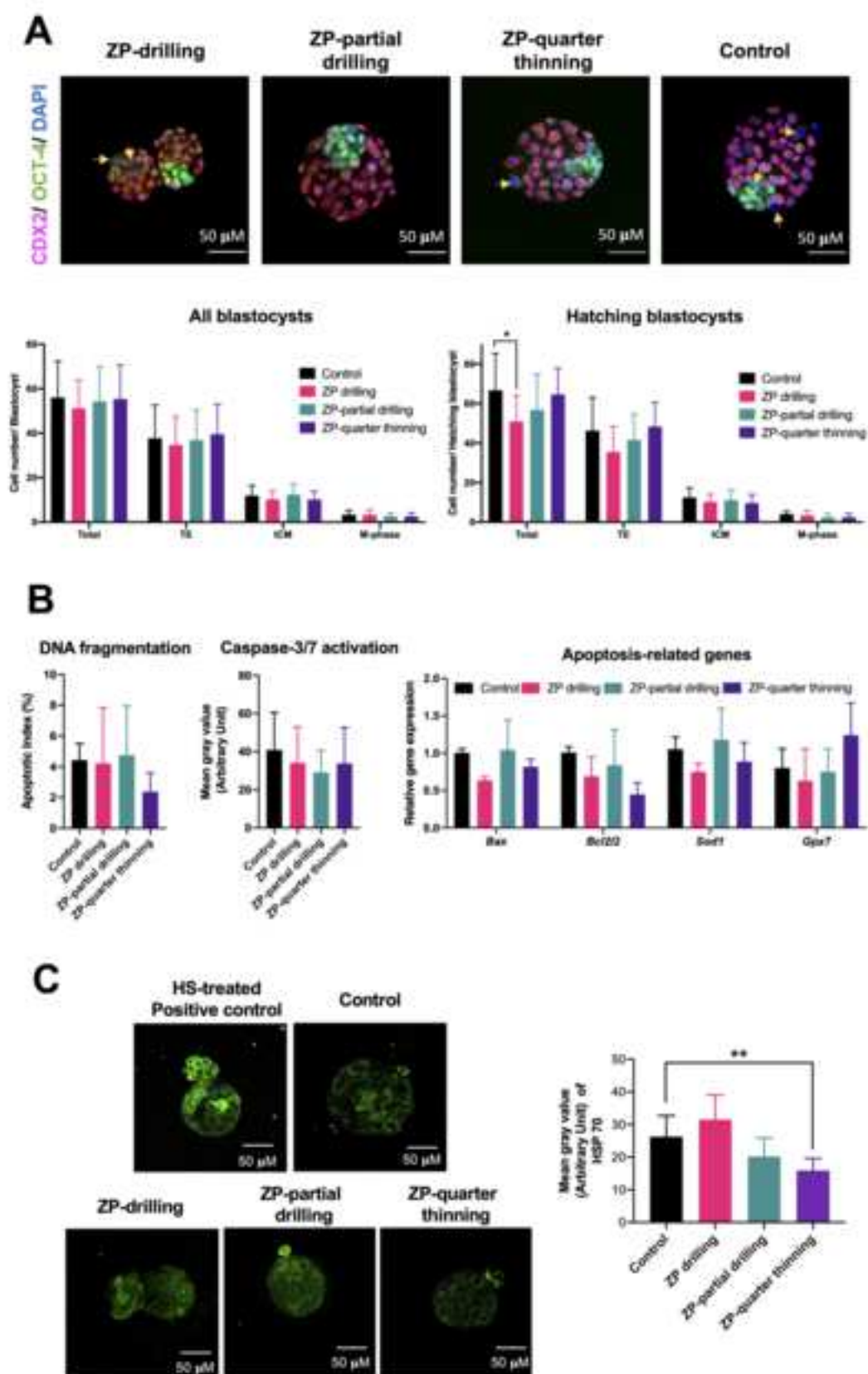
(A): Representative phase-contrast images of co-cultured mouse embryos and Ishikawa cells at 24h, 48h, and 72h following embryo transfer (scale bar= 50  $\mu\text{m}$ ). Blue arrows indicate mouse embryos undergoing hatching. For the embryos attached to Ishikawa cells, red markers indicate the ICM (inner cell mass), thin yellow arrows mark the outgrowth of TE (trophectoderm) cells, and white arrows show the disruption of Ishikawa cells. The outgrowing embryos which breached the Ishikawa cell layer and invaded into Matrigel are indicated with red circles. (B) The proportions of hatching embryos and fully hatched embryos at at 24h, 48h, and 72h of co-culture. (C) The proportions of embryos in different attachment stability at 24h, 48h, and 72h of co-culture.

**Supplemental Figure 1. The assessment of cellular apoptosis in E4.5 mouse embryos**

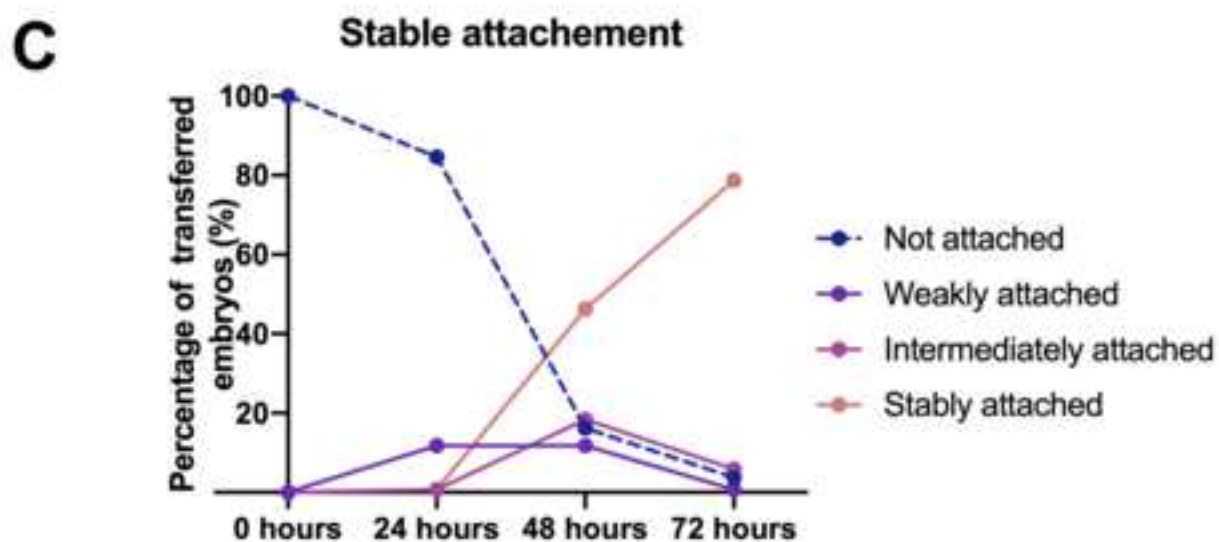
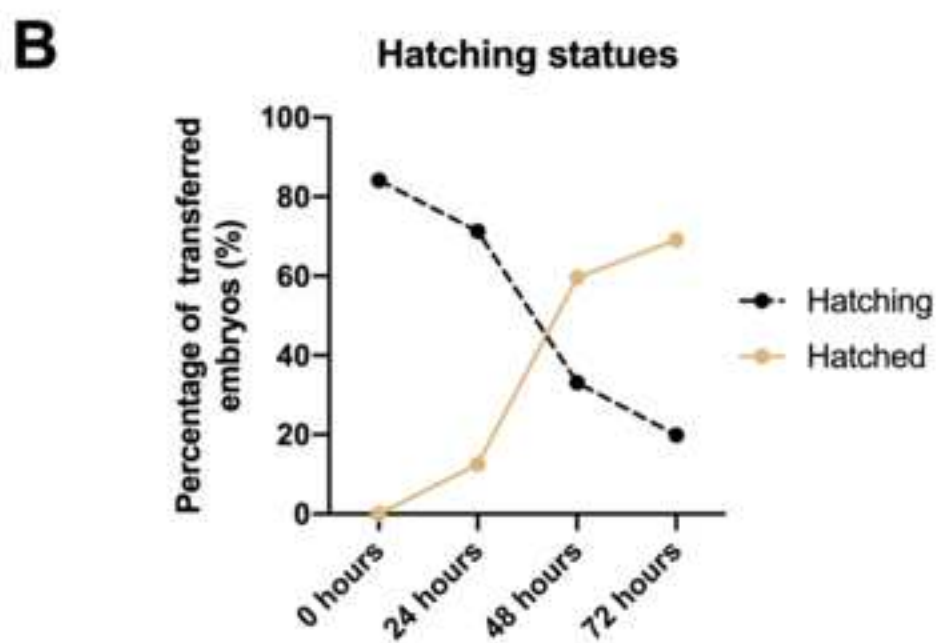
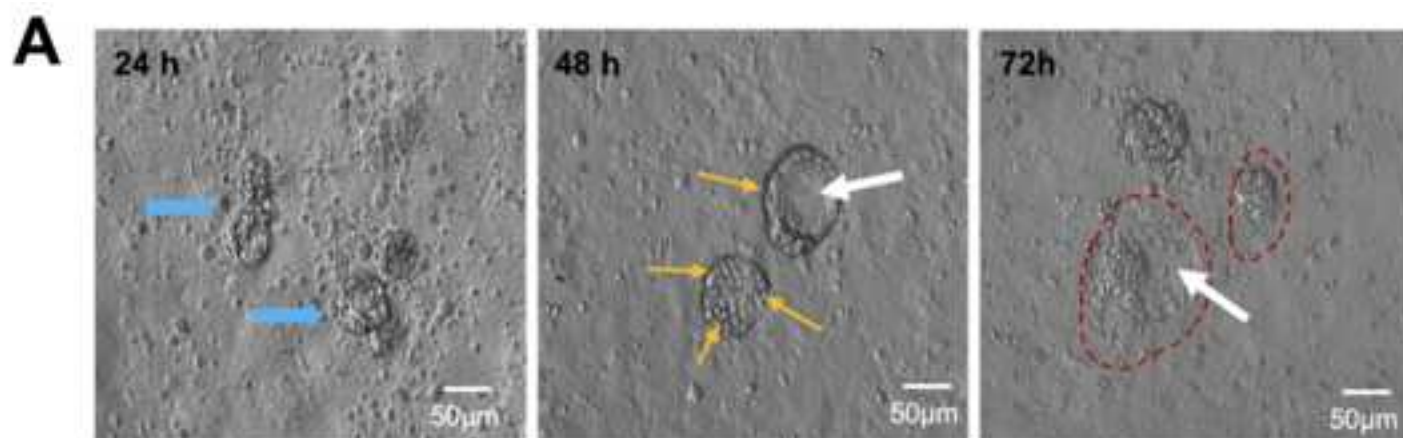
(A) Embryo DNA fragmentation was detected using a TUNEL assay kit (DNA fragmentation (green) and nuclear staining (DAPI, blue)). Positive control: embryos treated with DNase; Negative control: no TdT reagent (400× magnification, Scale bar=50 μm). (B) Cleaved Caspase 3/7 was detected using a Caspase Detection Kit. FAM-DEVD-FMK (green) and nuclear staining (Hoechst 33342, blue) were visualized by fluorescence microscopy. Positive control: embryos treated with 1μM of Staurosporine for 2 hours during culture (400× magnification, Scale bar=50 μm).











# **An investigation of the effects of laser-assisted zona pellucida drilling on the preimplantation mouse embryo and the competency of embryo implantation**

Running title: Laser-assisted drilling and embryos

Yaqiong Liu, MRes, Celine Jones, Kevin Coward, Ph.D \*

Nuffield Department of Women's and Reproductive Health, University of Oxford, Level 3,  
Women's Centre, John Radcliffe Hospital, Headington, Oxford, OX3 9DU, UK.

\*Corresponding author

Email: kevin.coward@wrh.ox.ac.uk; Telephone: +44 [0] 1865 618900; Fax: +44 [0] 1865  
618900

Conflicts of interest: None.

## Abstract

**Objective:** To investigate the impact of laser-assisted ZP drilling on the mouse embryo, with particular emphasis on molecular mechanisms, and the efficiency of embryo attachment capability using an *in vitro* model of implantation.

**Design:** Experimental study.

**Setting:** Academic research laboratory.

**Patients/Animals:** C57BL/6JOlaHsd mouse embryos and B6C3F1 × B6D2F1 mouse embryos.

**Interventions:** 8-cell stage mouse embryos were randomly assigned to a laser-assisted ZP drilling (n=343) group, a ZP-partial drilling group (n=312), a ZP-quarter thinning group (n=289), and a control group (n=354353). Embryos were cultured *in vitro* from E2.5 to E4.5 for 48 hours. To investigate the capacity to implant, E4.5 embryos (laser-assisted drilling group (n=46), ZP-partial drilling group (n=28), ZP-quarter thinning group (n=26), and control group (n=36)), were then transferred onto an attachment model based on Ishikawa cells and cultured for another 72 hours.

**Main Outcome Measures:** Blastocyst formation, hatching status, and hatching morphology at E4.5. Blastocyst cell components, the extent of apoptosis in embryonic cells (DNA fragmentation, caspase-3 activation, and the expression of apoptosis-related genes), the expression of HSP70, and differentially expressed genes (DEGs) generated by RNA-sequencing. Fully hatched embryo rate and stable attachment rate at E7.5 in the *in vitro* attachment model.

**Results:** There were no significant differences between the laser-assisted ZP manipulation groups and the control group with respect to the formation of blastocysts, cell number, embryonic cell apoptosis, and cellular stress. All three of the laser-assisted ZP manipulations



significantly increased the hatching rate at E4.5 when compared with control group, especially the ZP drilling group. However, only ZP drilling group was associated with a significantly higher proportion of '8'-shape hatching blastocysts. Furthermore, RNA-sequencing identified 48 DEGs between blastocysts from the laser-assisted hatching group and the control group; the metabolic pathways were significantly enriched in these DEGs. In addition, there were no significant differences between the laser-assisted ZP manipulation groups and the control group with respect to the rate of stable attachment at E7.5, although a significantly higher entrapment rate was observed in the ZP drilling group.

**Conclusions:** Laser-assisted ZP manipulations did not induce cellular apoptosis or stress in mouse blastocysts. Nevertheless, for the first time, we found that laser-assisted ZP drilling could alter the embryonic transcriptome and may affect metabolic activity. Furthermore, although laser-assisted ZP manipulations can enhance the initiation of hatching, it is evident that ZP drilling comes with a potential risk of embryo entrapment.

**Keywords:** Laser-assisted drilling; Embryo development; Cell apoptosis, RNA-sequencing; Embryo attachment

## Introduction

As a powerful tool in assisted reproductive technology (ART), the non-contact infrared diode laser serves as a reliable technique for manipulations of the embryonic zona pellucida (ZP) with high levels of consistency and reduced procedure times (1,2). At present, laser-assisted ZP drilling is widely applied in various clinical applications, including embryo biopsy, intracytoplasmic sperm injection (ICSI), and embryo cryopreservation (3). Drilling an opening in the ZP is one of the key steps to undertake when biopsying an embryo; the most popular technique for this purpose is laser-assisted drilling, a technique that can be applied immediately prior to biopsy (4). In addition, laser-assisted pre-drilling can also be applied for trophectoderm (TE) biopsy, a technique that is commonly performed on days 3 - 4 (3-6). Apart from ZP drilling, laser-assisted ZP-partial drilling as well as ZP- quarter thinning, with the intention to thin but not breach the ZP, are also applied in the clinic to improve embryo implantation rate (3,7,8).

Laser-assisted ZP manipulation involves the application of a high energy form of light that produces heat to dissolve or ablate the ZP (9). This heat is dissipated and transferred through culture media, thus creating a temperature gradient in the culture media immediately surrounding an embryo (10,11). Furthermore, as a critical structure, the ZP protects an embryo from detrimental factors that may be present in artificial media, including toxins, microorganisms, and antibodies (12,13). Laser-assisted drilling, as an invasive technique, inevitably exposes an embryo to the external environment and may cause the loss of blastomeres and a reduction in embryonic autocrine secretion, thus creating potential risk to embryo development (8,14,15). Therefore, the energy delivered to an embryo during laser-assisted ZP drilling or ZP thinning, and the drilling technique itself, might affect the quality of an embryo and its ability to implant.



Several research studies have attempted to evaluate the laser-assisted manipulations on human embryos or embryos acquired from animal models. On a positive note, many of these studies failed to detect any adverse effects on embryo quality and preimplantation development potential, especially when applying a laser beam to manipulate the ZP at high power laser and appropriate durations of time (16-18). As a vital indicator of developmental potential, embryonic cells are positively correlated with blastocyst quality (19). Worryingly, some studies have reported a reduction in the number of embryonic cells following laser-assisted treatment (14,20-22). Aside from developmental potential, cellular apoptosis is another biological process that can serve as a useful indicator of an inadequate or suboptimal *in vitro* environment or the detrimental effects of manipulation. A recent study demonstrated that laser-assisted ZP drilling at either 2-cell and 6 to 9-cell stage embryos significantly induced embryo DNA damage (21); although the specificity of the TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) assay used to detect DNA fragmentation in this previous study was relatively low (21). Consequently, it is evident that future studies must use other methods to detect cellular apoptosis, such as the expression levels of apoptosis- and stress-related genes and the activation of caspase-3 (14,23,24).

Although laser-assisted drilling has been used in clinics for a long period of time, very few studies have specifically investigated the impact of this technique on embryos at the molecular level; furthermore, the most comprehensive of these previous studies used mouse models (25,26). As yet, none of these previous studies have detected any significant changes in the expression levels of genes related to heat shock stress (25) or epigenetic markers (26) following laser-assisted drilling at the 8-cell stage. However, insufficient evidence is available at this time to confirm the safety of laser-assisted drilling on the embryonic transcriptome since only a limited number of genes have been investigated thus far, such as *Dnmt3a*, *Dnmt3b*, and *Hsp70i*.

On the other hand, the precise efficacy of laser-assisted ZP manipulations on clinical outcomes also remains controversial. Some studies demonstrated that laser-assisted drilling on day 3 embryos could result in poor pregnancy outcomes when applied for assisted hatching (7,12) and for pre-hatching prior to embryo biopsy (4,27). Some researchers have suggested that laser-assisted ZP thinning could provide the better pregnancy outcomes when compared with control or ZP drilling groups (7,28,29). Embryo implantation is known to be affected by both embryo competency and the receptivity of the endometrium (30). In addition, the hatching process is also affected by zona lysis in the uterus (31,32). Thus, it is evident that there are numerous factors that could confound data when attempting to evaluate the efficacy of laser ZP drilling on embryo competency *in vivo*. A better model for studying the efficacy of laser-assisted ZP manipulations would be the *in vitro* attachment model, particularly when investigating the efficiency of hatching. By focusing solely on the competency of the embryo to implant, the *in vitro* attachment model avoids potential maternal confounding factors, such as components in the uterine fluid that may enhance blastocyst development and endometrium receptivity (33).

In the present study, we investigated the effect of laser-assisted ZP drilling, ZP- partial drilling, and ZP-quarter thinning on mouse embryos from a variety of biological and molecular aspects. We found that these techniques did not affect the formation of blastocysts and did not induce cellular apoptosis or stress. However, RNA-sequencing demonstrated, for the first time, that laser-assisted ZP drilling can result in changes to the embryonic transcriptome. This indicated a potential impact on embryonic metabolism. Finally, experiments involving an *in vitro* embryo attachment model demonstrated that laser-assisted ZP drilling, but not partial ZP drilling and ZP-quarter thinning, had an adverse impact on the completion of embryo hatching but not attachment capability.

## **Materials and methods**

### **Embryo collection and assessment**

Mice were raised in the Biomedical Services (BMS) Unit at the University of Oxford. All experiments and animal handling were conducted in accordance with the institutional guidelines for animal experimentation after obtaining prior approval from the Ethics Committee of the Department of Physiology, Anatomy and Genetics, University of Oxford (License number: 30/3301). Super-ovulation strategies and general maintenance were performed under protocol BMS//JRSOP285. The day of human chorionic gonadotrophin (hCG) injection and mating was defined as E0. Females with vaginal plugs were sacrificed, and the reproductive tracts were harvested on E2.0. Eight-cell stage embryos (C57BL/6J0laHsd) were obtained from the junction of the oviduct by mincing the oviducts and uterus using fine forceps under microscopy. The morphology of the collected 8-cell stage embryos was then evaluated; embryos with more than 6 equivalent size blastomeres were then pooled and allocated into two groups by defocusing the microscope to prevent any selection bias. In contrast, embryos containing cytoplasmic fragments, unevenly sized blastomere, and damaged ZP were excluded from study in accordance with previous criteria (34).

For the RNA sequencing, we needed to ensure the sample size (no less than 8 samples/group). In addition, we needed to harvest a sufficient amount of embryonic cDNA for qRT-PCR (quantitative real-time polymerase chain reaction) in every experimental repeat. To facilitate these, we purchased cryopreserved 8-cell stage mouse embryos (B6C3F1  $\times$  B6D2F1) from Embryotech Laboratories (USA) for these experiments.

## **Laser-assisted ZP manipulation and embryo culture**

Embryos were cultured individually by transferring one embryo into one drop of potassium-supplemented simplex optimized medium with  $\frac{1}{2}$  Amino Acids (KSOM-AA) (EmbryoMax KSOM Mouse Embryo Media, Millipore, Abingdon, UK) (20  $\mu$ L) covered with mineral oil (FertiPro NV, Beernen, Belgium) and incubated at 5% CO<sub>2</sub> and 37°C for at least 10 minutes prior to ZP manipulation. All embryo manipulations were performed using a Flexipet (EZ-Grip) and a transfer pipette (EZ-Tip) (CooperSurgical, Falmouth, UK).

Laser-assisted ZP manipulations involved a Saturn 5 Laser System (Cooper Surgical Fertility Companies, Denmark) set to a wavelength of 1.48  $\mu$ m, a power of 400 mW, and a laser diameter of 7.5  $\mu$ m. The laser was calibrated prior to the experiment, and these parameters were used consistently for all manipulations. In addition, a red pilot beam was used to ensure targeting optimization on the microscope and guaranteed that the laser was initiated in a position that was sufficiently far away from the blastomeres to avoid damage during manipulation. Laser-assisted ZP drilling was conducted by targeting the ZP with two consecutive laser pulses, from the outer side to the inner side (**Fig. 1 B-1**). We confirmed that the ZP had been breached by observing fluid flowing out of the perivitelline space. If the ZP was not drilled completely with 2 pulses, an extra laser pulse would be given. For ZP-partial drilling, one laser pulse was fired to drill through half of the ZP thickness (**Fig. 1 C-1**). The ZP-quarter thinning was performed to ablate the one quarter of the ZP circumference by half of the ZP thickness (**Fig. 1 D-1**). Embryos in the control group were placed onto a pre-warmed stage for a similar period of time but without laser manipulation (**Fig. 1**). Following manipulation of the ZP manipulation, embryos were cultured in an incubator with 5% CO<sub>2</sub> at 37°C, as described in previous studies (14,16,22,28) (starting from 12 pm).

## Embryo development and embryo hatching patterns

After 48 hours of *in vitro* culture, embryos were observed on a pre-warmed microscope stage (at the E4.5 stage; 12 pm) (**Fig. 1**) and the number of developed blastocysts and hatching blastocysts were quantified. The hatching rate was calculated by dividing the number of hatching blastocysts by the number of existing blastocysts  $\times 100$  (%). As we noticed an overt difference in the pattern of hatching at the assessment time-point, we further evaluated the hatching pattern and categorized these patterns according to different morphological features (35,36): an ‘8’-shape hatching blastocyst featured two blastocoels divided by the ZP with an opening in the ZP of  $\leq 25 \mu\text{m}$  at the time of observation. Conversely, when the opening in the ZP was  $\geq 25 \mu\text{m}$ , the blastocysts were regarded as ‘U’-shape hatching blastocysts. Blastocysts with multiple hatching/breaching points ( $\geq 2$ ) were referred to as multiple-point hatching/breaching blastocysts (**Fig.1**).

## Immunofluorescence staining

Immunofluorescence staining was used to determine the expression levels of a marker for the inner cell mass (ICM) (OCT-4), marker for trophoctoderm (TE) cells (CDX2), and heat shock protein (HSP70). In brief, the collected E4.5 blastocysts were washed with 0.01% (V/V) poly (vinyl alcohol) (PVA)-phosphate-buffered saline (PBS) buffer prior to fixation with 4% paraformaldehyde (PFA) for 20 minutes and was then permeabilized with 0.05% Triton X-100 for 10 minutes. Embryos were then incubated overnight at 4°C with primary antibodies (OCT-3/4 (Santa Cruz, UK), CDX2 (Novus Biological, UK), and HSP70 (Invitrogen, UK)). The following morning, embryos were washed and incubated with a fluorescein isothiocyanate (FITC)-conjugated secondary antibody (Invitrogen, Thermo Fisher, UK). DAPI (Vector Laboratories, UK) was then used to visualize all cell nuclei. Image J software was used to quantify the number of cells in the ICM and the TE, and to measure the mean gray value of

HSP70 (in arbitrary units). For Heat Shock Protein 70 (HSP70) staining, heat shock (HS) treated embryos were used as a positive control. Before staining, HS was induced by placing the blastocyst at 43°C for 30 minutes, followed by a recovery period at 37°C for 30 minutes to produce HSP70 (37). Then, antibody staining was performed as described above.

### **The assessment of cell apoptosis**

Embryonic cell apoptosis was assessed by evaluating DNA fragmentation and the activation of caspase-3. The extent of DNA fragmentation was assessed using a TdT-mediated dUTP nick-end labelling (TUNEL) kit (Promega, UK) in accordance with the manufacturer's instructions. The positive control for TUNEL assay involved the treatment of embryos with DNase I (50 IU/ml) for 10 minutes at room temperature prior to fixation. The negative control was created by treating embryos with DNase but without labelled TdT reagent. The apoptotic index of DNA fragmentation (%) (the number of the TUNEL-positive cells / the number of the DAPI-positive cells) was then determined for each embryo (**Supplemental Fig. S1-A**).

Furthermore, we analyzed Caspase-3/7 activation using a fluorescent inhibitor of caspase (FLICA) kit (Invitrogen, UK). Prior to embryo labelling, the positive control embryos were incubated with 1  $\mu$ M Staurosporine (Sigma, UK) for 2 hours at 37°C. The kit was used in accordance with the instructions provided by the manufacturer. Green fluorescence represented the activated Caspase-3/7 (**Supplemental Fig. S1-B**). Activated Caspase-3/7 was quantified with Image J software by measuring mean grey values (arbitrary units).

### **RNA-sequencing (RNA-seq) and the analysis of differentially expressed genes (DEGs)**

RNA-sequencing experiments involved 8 hatching blastocysts in the control group and 8 hatching blastocysts in the laser-drilled group. For cDNA synthesis, each single blastocyst was lysed in 2.3  $\mu$ l of lysis buffer (0.8% (vol/vol) Triton X-100 and 2 U/ $\mu$ l of RNase inhibitor (both

from Thermo Fisher, UK)) using the SmartSeq2 method in accordance with Picelli et al. (38). Libraries were then prepared using Nextera-XT. ERCC RNA Spike-in mix (4456740, ThermoFisher) was then added at a dilution of 1/100,000. Amplified libraries were analysed for size distribution using the Agilent TapeStation High Sensitivity D1000 kit and then quantified using PicoGreen; relative volumes were then pooled accordingly. Sequencing was performed as 75bp paired-end reads on a HiSeq4000 according to Illumina specifications.

RNA-seq reads were aligned to the mouse reference genome (GRCm38) using HISAT2 (39), and duplicate reads were removed using the Picard 'MarkDuplicates' tool [<http://broadinstitute.github.io/picard>]. Reads that were mapping uniquely to Ensembl-annotated genes (~5 million per sample) were summarised using featureCounts program (40). The raw gene count matrix was imported into the R/BioConductor environment (41) for quality control and data normalisation. The edgeR package (42) was used to test for differentially expressed genes between the control and laser-drilling groups. We also included the embryo batch as an additional explanatory variable. Raw P-values were adjusted for multiple testing using the Benjamini-Hochberg (BH) procedure (43) to control the false discovery rate at 5%.

### **Downstream pathway analysis**

DEGs were imported into Ingenuity Pathway Analysis (IPA, QIAGEN Inc) software for Canonical Pathway Analysis. In addition, Over Representation Analysis (ORA) was also used to identify enrichment of the DEGs with respect to Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways and Reactome pathways.

### **cDNA synthesis from blastocysts and quantitative polymerase chain reaction (PCR)**

cDNA was generated from three blastocysts using the cell-to-cDNA kit (Invitrogen, UK) in accordance with the manufacturer's instructions. Pre-amplification of target cDNA was

performed using the TaqMan PreAmp Master Mix Kit (Applied Biosystems, UK). In brief, pooled TaqMan assays were created by combining an equal volume of the target TaqMan probes: [*Mvk* (Mm00445773\_m1), *Mvd* (Mm00507014\_m1), *Dnmt3l* (Mm00457635\_m1), *Lmna* (Mm00497783\_m1), *Cdx2* (Mm01212280\_m1), *B2m* (Mm00437762\_m1), *Ywhaz* (Mm05674356\_s1), *Bax* (Mm00432051\_m1), *Bcl2l2* (Mm00432054\_m1), *Sod1* (Mm01344233\_g1), *Gpx7* (Mm00481133\_m1), and *Gapdh* (Mm99999915\_g1)]. Quantitative RT-PCR was then carried out using the TaqMan Gene Expression System (Applied Biosystem, UK) on an Applied Biosystems QuantStudio 3 (Thermo Fisher, UK) using standard thermocycler parameters. After validation, the mean CT values of *B2m* and *Ywhaz* were used as internal controls for comparing gene expression levels between blastocysts from the laser-drilling and control groups for RNA-seq validation. The relative expression levels of all target genes were calculated using the  $2^{(-\Delta\Delta Ct)}$  method (44) by normalizing to the internal control, and the differences in gene expression level are presented as the fold change relative to the mean Ct value of the control group.

### **Cell culture and the *in vitro* model of embryo attachment**

Ishikawa cells (ECACC 99040201), a human endometrial adenocarcinoma cell line of epithelial origin, were maintained in conditional medium (1:1 Dulbecco's modified Eagle's medium: Ham's F12 (Sigma-Aldrich, UK)) containing 10% fetal bovine serum (FBS) supplemented with 100 µg/ml streptomycin, 100 IU/ml penicillin, and 20 µg/ml gentamicin (Sigma-Aldrich, UK). Ishikawa cells were then grown to full confluence in 24-well plates on 13 mm glass coverslips coated with 2% growth phenol red-free factor-reduced Matrigel (Corning, UK). The monolayer of Ishikawa cells was then washed and replenished with a co-culture medium (0.1% FBS in conditional medium) prior to embryo transfer. One to five E4.5 blastocysts (beyond the expanded blastocysts) were collected and transferred non-selectively



onto the Ishikawa cell monolayer, following by culture at 37°C in 5% CO<sub>2</sub> for 72 hours to E7.5. For embryos showing a delay in blastulation, E4.5 embryos were cultured one more day *in vitro*. Then, the E5.5 blastocysts (if they grew to an expanded blastocyst or beyond) were then collected and transferred onto an *in vitro* co-culture system.

Hatching ability and attachment stability were evaluated every 24 hours. The fully hatched rate was calculated as the number of hatched blastocysts divided by the number of transferred blastocysts  $\times 100$  (%). Embryos that failed to hatch out at 72 hours were defined as trapped embryos. In accordance with a previous study (45), the stability of embryo attachment to the Ishikawa monolayer was assessed using a four-point scale of blastocyst behavior upon agitation of the co-culture system (by gently pressing the microscope table 2-3 times): not attached, weak attachment (major oscillation around an attachment point), intermediate attachment (minor oscillation), and fully attached (no oscillation). The stable attachment rate was calculated as the number of fully attached blastocysts divided by the number of transferred blastocysts  $\times 100$  (%).

### **Statistical analysis**

Quantitative data were analyzed using Prism 8 software (GraphPad Software, CA, USA). Data were compared between four groups using the one-way analysis of variance (ANOVA) test (if normally distributed) or Kruskal-Wallis non-parametric test (if not normally distributed) with relevant post-hoc tests for multiple comparison between each treatment and control groups. Normality was evaluated with the D'Agostino-Pearson omnibus (K2) test. Differences in proportions between treatment and control groups were assessed using the  $\chi^2$  test in the SPSS version 26.0 software package (SPSS Inc., Chicago, IL, USA). A probability (*P*) value  $< 0.05$  was considered to be statistically significant.

## Results

### The incidence of hatching and hatching patterns at E4.5

Blastocyst formation rates were similar between the control group and the treatment groups. However, when compared with control groups, the embryo hatching rate was significantly higher in the laser-ZP drilling group ( $P<0.0001$ ), ZP-partial drilling group ( $P=0.034$ ), and ZP-quarter thinning group ( $P=0.003$ ). In addition, a significant disparity was shown with regards to hatching patterns in the ZP-drilling group in the comparison with the control group ( $P<0.0001$ ): 48.9% of embryos in the control group were beginning to hatch at E4.5, while 75% of blastocysts in the ZP drilling group adopted an ‘8-shape’ morphology. In addition, 25.8% of hatching blastocysts in the ZP-quarter thinning groups were adapted in ‘U-shape’ morphology (**Table 1**). Nevertheless, no significant difference in E4.5 hatching pattern was detected between the ZP-partial drilling, ZP-quarter thinning, and the control group ( $P>0.05$ ).

### Embryonic cell number, cellular apoptosis, and stress at E4.5

Embryos were stained with OCT-3/4 and CDX2 to visualize the inner cell mass (ICM) and trophectoderm (TE). There were no significant differences between blastocysts across four groups in terms of embryonic cell number. Interestingly, the total cell number of the hatching blastocysts in the laser-assisted ZP drilling group was significantly lower than that in the control group ( $50.92 \pm 2.69$  vs  $66.71 \pm 7.04$ ,  $P=0.035$ ); a similar pattern was also found with the number of cells in the TE of hatching blastocysts, although this was not statistically significant (**Fig.2A**).

Next, we analyzed cellular apoptosis from several different aspects. As shown in **Fig. 2B**, DNA fragmentation and caspase-3 activation levels were comparable in the four groups. Furthermore, these laser-assisted ZP treatments did not change the mRNA expression of apoptosis-related

genes (*Bax*, *Bcl2l2*, *Sod1*, and *Gpx7*) ( $P>0.05$ ). These results suggested that laser-assisted ZP manipulations would not result in a significant increase in apoptosis in the embryonic cells.

The expression levels of HSP70 were detected in E4.5 blastocysts. We observed high levels of HSP70 expression in embryos that had been treated with HS (heat shock treatment). Laser-assisted ZP drilling and ZP-partial drilling did not induce a significant increase in the expression levels of HSP70 in the blastocysts (**Fig. 2C**). Interestingly, the level of HSP70 in blastocysts following the laser-assisted ZP-quarter thinning was significantly lower than that of blastocysts in the control group ( $P=0.006$ ).

### **Alterations in the transcriptome following laser-assisted ZP drilling and related pathways**

To investigate the potential transcriptomic changes caused by laser-assisted ZP drilling, we used RNA-seq analysis to determine DEGs between ZP-drilled blastocysts and untreated blastocysts. Overall, we identified 48 genes that were differentially expressed (BH (Benjamini-Hochberg) adjusted  $P < 0.05$ ); of these, 39 genes were up-regulated, and 10 genes were down-regulated (**Fig. 3A**). The fold changes of these DEGs were small; only 13 DEGs exhibited a fold change larger than 2, including 11 up-regulated genes (*Lilra6*, *Maged2*, *Cep250*, *Acbd4*, *Mvk*, *Lmna*, *Acaa1a*, *Aldh18a1*, *Mvd* and *Cdk5rap3*) and two down-regulated genes (*Lmfn* and *Gsk3a*). The DEGs list was presented in the supplementary material. To validate the RNA-seq results, we selected four DEGs (*Mvk*, *Mvd*, *Lmna*, and *Dnmt3l*) and one stably expressed gene (*Cdx2*). As shown in **Fig. 3B**, the results of the qRT-PCR followed the same trends as the RNA-seq findings, although the change in *Dnmt3l* expression level did not reach statistical significance.

Downstream pathway analysis demonstrated that the identified DEGs were significantly enriched in 16 canonical pathways, 2 KEGG pathways, and 1 Reactome pathway (**Fig. 3C**).

Interestingly, many metabolism-related pathways were enriched, particularly those associated with lipids metabolism, including lipid metabolism pathways (*Slc25a1*, *Mvd*, *Acbd4*, *Agpat4*, *Aacs*, *Mvk*, *Acaa1a*, and *Mcat*), mevalonate pathway I (*Mvd* and *Mvk*), cholesterol biosynthesis (*Mvd* and *Mvk*), and docosahexaenoic acid (DHA) signaling (*Gsk3a* and *Pik3c2a*). Furthermore, other metabolic pathways, such as degradation pathways associated with valine, leucine, and isoleucine (*Aldh9a1*, *Aacs*, and *Acaa1a*), the insulin receptor signaling pathway (*Gsk3a*, *Pik3c2a*, and *Trip10*), and xenobiotic metabolism signaling (*Aldh18a1*, *Aldh9a1*, *Keap1*, and *Pik3c2a*) were also significantly enriched according to these DEGs (**Fig. 3C**).

### **Embryo hatching completion and attachment from E4.5 to E7.5**

Overall, 136 E4.5 mouse blastocysts were transferred onto the *in vitro* attachment model. As shown in **Fig. 4A**, differing hatching statuses and degrees of embryo outgrowth and invasion to the Ishikawa cell layer were observed at the three time-points. When using the *in vitro* attachment model, we were able to observe fully hatched embryos after 24 hours (**Fig. 4B**), while the proportion of stably attached embryos started to increase after 48 hours (**Fig. 4C**). Thus, we compared fully hatched rates from 24 hours and stably attached rates at 48 hours and 72 hours.

As shown in **Table H2**, the fully hatched rate in the laser-assisted ZP drilling group was significantly lower at 48 hours ( $P=0.003$ ) and 72 hours ( $P=0.017$ ) of culture. In contrast, the proportion of trapped embryos was significantly higher in the laser-assisted drilling group than in the control group ( $P=0.007$ ). In addition, 58.7% (27/46) of transferred embryos in ZP drilling group were 8-shaped hatching blastocysts, 70.4% (19/27) of these embryos ended with entrapment. In contrast, there were no differences in fully hatched rates and entrapment rates when compared between the ZP-partial drilling and ZP-quarter thinning groups and the control

group. Interestingly, neither of these laser-assisted ZP manipulations affect the attachment of embryos since no difference was found in the stable attached rate at 48 or 72 hours (**Table H2**).

Furthermore, the blastulation delayed blastocysts, which formed the blastocyst around E5.5, were observed among embryos from the four groups. When transferring these embryos into the *in vitro* model, we observed a lower fully hatched rate (56.3% vs 77.8%) and a significantly lower stably attached rate (50% vs 83.3%,  $P=0.012$ ) in the untreated E5.5 ET embryos when compared with E4.5 untreated embryos. However, in the comparison with the control group, E5.5 embryos in ZP drilling and ZP-quarter thinning groups exhibited substantially higher proportions of fully hatched embryos at 72 hours. In addition, ZP drilling significantly increased the attachment rate ( $P=0.008$ ) (**Table H3**).

## Discussion

Although laser-assisted ZP manipulations, especially ZP drilling, are used widely in clinics, evidence relating to the safety of this technique remains very limited, especially at the molecular level. In the present study, we investigated the effect of laser-assisted ZP manipulations on preimplantation mouse embryos from a variety of different aspects, but with particular focus on embryonic cell number, cell stress and apoptosis, and transcriptomic alternations. We found that laser-assisted ZP-drilling, partial-drilling, and quarter thinning did not interfere with the formation of blastocysts and embryonic cell number; these findings were in line with previous studies (20,22,31). In addition, we also found these laser-assisted ZP manipulations did not induce cellular apoptosis, at least under our experimental conditions. These results contradict those reported by a previous study that showed increased levels of DNA damage following laser-assisted drilling (21). The discrepancies could be associated with a variety of factors related to laser manipulation, such as the duration of laser pulses or the time interval between two consecutive laser pulses. Furthermore, in the present study, we did not

detect any significant differences between the laser-assisted ZP manipulation groups and the control group with respect to the activation of caspase-3/7 and the expression levels of apoptosis-related genes (*Bax*, *Bcl2l2*, *Sod1*, and *Gpx7*), thus providing compelling evidence that these ZP manipulations did not induce cell apoptosis.

The potential thermal damage is one of the major concerns when using laser pulses on embryos since preimplantation embryos are particularly sensitive to thermal changes (37). A previous study demonstrated that laser drilling did not induce a rapid upregulation of *Hsp70i* mRNA expression, even in cells that were closest to the laser beam (25). In that study, the expression of *Hsp70i* was detected 2 hours after the laser treatment, in order to permit hsp70i RNA accumulation and investigate the possible heat shock effect generated by laser beam itself. However, in the present study, we investigated the expression of HSP70 at the blastocyst stage; therefore, the level of hsp70 expression at this stage would act as an indicator for the cell stress caused by laser-drilling treatment rather than the laser beam itself. Similarly, we did not see an increase in the protein expression of HSP70 in the laser-assisted ZP drilling group or ZP-partial drilling group, thus suggesting that laser-assisted drilling does not induce cellular stress in preimplantation embryos.

The evidence in respect to the impact of laser-assisted ZP manipulations on the transcriptome is sparse. For the first time, this study investigated potential transcriptional alternations following laser-assisted ZP manipulation with RNA-seq. In our study, however, we focused on the ZP drilling rather than ZP-thinning, because we assumed that ZP drilling would have the greatest impact on embryos by exposing them to the external environment. Data analysis indicated that laser-assisted drilling at the cleavage stage would not induce overt transcriptomic alternations at the blastocyst stage since only 48 DEGs with small fold changes were identified. Interestingly, the analysis of downstream pathways further revealed that laser-assisted drilling

could potentially affect metabolic activity in blastocysts, at least at the mRNA level. A previous study investigated metabolism changes in the human embryo 24 hours after laser-assisted hatching; however, the component analysis of metabolites failed to detect any significant differences when compared to embryos that did not undergo laser-assisted hatching (16). It is known that embryo metabolism is relatively low prior to the morula stage but becomes highly activated following the formation of blastocyst and until implantation (46,47). Thus, it is understandable that the changes in embryonic metabolic activity from day 2 to day 3 following laser-assisted hatching was not detectable in this previous study (16). In the present study, we cultured embryos for 48 hours from day 3 to day 5 after laser-assisted drilling; the potential changes in embryonic metabolic activity caused by laser-drilling were indicated by the identification of DEGs related to the metabolism of lipids and amino acids. Therefore, the embryonic metabolism alterations caused by laser-assisted drilling may occur at the blastocyst stage. Furthermore, all the related DEGs were upregulated in the laser-drilled blastocysts, indicating that laser ZP drilling might activate embryo metabolism by creating a route for transferring nutrients from the culture medium to embryos. Interestingly, we also observed enrichment in xenobiotic metabolism signaling (*Aldh18a1*, *Aldh9a1*, *Keap1*, and *Pik3c2a*). A xenobiotic is defined as the presence of an artificial or higher than normal concentration of a substance within an organism. Xenobiotic metabolism aims to remove such xenobiotics by deactivation and excretion (48). Our RNA-seq results indicated that laser-assisted drilling could potentially induce the presence of xenobiotics in embryos, which may be caused by exposing embryos directly to the culture medium before hatching at the blastocyst stage. However, further studies are now needed to confirm these findings by validating these molecules and the pathways involved. In addition, it would be interesting to compare the different impacts between ZP-drilling and ZP-thinning on the embryo transcriptome. Furthermore, it is crucial to understand whether the changes in embryonic metabolism at the

blastocyst stage will have lasting influences on the health of the offspring. Therefore, longer periods of follow-up involving the offspring arising from ZP drilling in mice will be crucial in future studies and should focus particularly on metabolic activity.

In line with previous findings involving mouse embryos (14,17,20,21,49,50) and human embryos (18,51), we found that all three ZP manipulation significantly advanced the initiation of embryo hatching at E4.5, with a highest proportion of embryos hatching found in the ZP-drilling group. In addition, the reduced number cells in hatching embryos from the ZP drilling group also supported the fact that the artificially induced ZP opening allows embryos to begin hatching without reaching a certain embryonic cell number; this observation concurred with previous studies (20,31).

Furthermore, we found that laser-assisted drilling altered the pattern of hatching, and a greater number of embryos adopted an '8'-shaped. This distinct '8'-shape hatching behavior was first reported in 1989 (52) in micromanipulated embryos; the phenomenon by which embryos hatch slowly through a small opening in the ZP is referred to as "herniation" (28). This TE herniation of blastocyst induced by ZP drilling facilitates the separation of the TE during blastocyst biopsy and helps embryologists to arrange their work routine; thus, laser-assisted drilling on day 3 embryos is widely used as a pre-hatching strategy for blastocyst biopsy in clinics (4,53,54).

Nevertheless, although laser-assisted ZP drilling advanced the initiation of hatching, there is a potential risk that this could cause blastocyst entrapment. In this study, a lower hatching completion rate at E7.5, and a higher proportion of trapped embryos, were observed in the laser-assisted drilling group. In particular, around 70% of the '8'-shape hatching blastocysts ended up with entrapment. Hatching entrapment has been commonly observed in several other studies involving mouse embryos (20,28,55) and human embryos (56). Furthermore, animal studies have demonstrated that blastocyst entrapment is related to the size of the opening in the



ZP. However, no consensus has been reached with regards to the minimum size of the ZP opening with which to maintain hatching efficiency (20,49,55). It is not surprising that a large opening in the ZP could facilitate the completion of embryo hatching, as demonstrated by many previous studies (22,50,57). However, using a laser to create a small ZP opening is widely accepted for biopsy purposes (6,53). In clinics, a small ZP opening (5 to 10  $\mu\text{m}$ ) is commonly applied to day 3 human embryos to assist pre-hatching prior to TE biopsy (4,27,53). On the other hand, embryo entrapment is rarely reported in clinics since embryos are only cultured briefly *in vitro* prior to transfer; this means that embryologists cannot observe embryo entrapment during this short period of culture (4). Using the *in vitro* embryo attachment model, we demonstrated the risk of blastocyst entrapment following laser-assisted drilling, thus indicating the importance of refining the size of the ZP opening during clinical application in order to maintain hatching efficiency.

Although laser ZP drilling affected the completion of embryo hatching, it did not impact upon an embryo's capability for *in vitro* attachment to epithelial cells. However, considering that only fully hatched embryos could ultimately implant into the uterus, the final implantation/pregnancy rate might be affected following laser-assisted drilling, at least when the size of the ZP opening is small. This phenomenon partially explains previous observations that laser ZP drilling in day human 3 embryos is related to the poor pregnancy outcomes when used for pre-hatching prior to biopsy as well as assisted hatching (4,7,12). Therefore, considering the potential impact on pregnancy outcomes, it is urgent to investigate the relationship between the size of the ZP opening and pregnancy outcomes. Furthermore, it is crucial to refine the ZP drilling protocol for human embryos to avoid damaging the competency of embryos to implant.

On the other hand, the high frequency of ‘8-shape’ hatching following laser-assisted drilling clearly demonstrates that this technique represents a potential risk factor for cleavage of the ICM. A previous study, involving mouse embryos, showed that ‘8-shape’ hatching affects ICM herniation and increases the risk of ICM cleavage (35). Three clinical case reports showed that the transfer of ‘8-shape’ hatching embryos resulted in monozygotic twin (MZT) pregnancy and monochorionic triamniotic triplet’s pregnancy (58-60). Furthermore, a recent retrospective study in Japan, involving 937,848 single embryo transfer cycles, revealed that assisted hatching represents a potential risk factor for zygotic splitting. However, no specific details were given regarding the effects of ZP manipulation (61). In contrast, Gu *et al.* (62) found that incarceration of the ICM in 8-shaped blastocysts did not increase the number of MZT pregnancies in patients undergoing PGD/PGS. Since the incidence of MZT is very rare in clinics, large scale follow-up studies are now needed to reveal the specific risk of perinatal complications following laser-assisted drilling.

A previous study indicated that more than 80% of *in vivo* developed embryos underwent the ‘U’-shape hatching (35); meanwhile, ZP-quarter thinning could lead to a superior ‘U’-shape hatching pattern (14). Therefore, it was suggested that the change of integrity of ZP induced by laser thinning could influence the hatching pattern and facilitate the embryo hatching (14). In this study, a slightly higher proportion of ‘U’-shape hatching was also observed in the ZP-quarter thinning group. Interestingly, we also found that the ZP-quarter thinning suppresses the expression of HSP70, which suggesting that this technique could reduce the embryonic cell stress. We assumed that the ZP-quarter thinning, by changing the integrity of the ZP, could reduce pressure within the blastocyst, thus suppressing the expression of HSP70. The superior hatching pattern and decreased cell stress indicated that laser-assisted ZP-quarter thinning might be a better method for assisted hatching. However, further studies are needed to investigate the specific role of HSP70 during the hatching process.

Clinical data suggested that both fresh and cryopreserved embryos, which did not undergo blastulation until day 6, were associated with lower pregnancy potential (63,64). However, it is not clear whether this was due to asynchrony between the embryo and endometrium or due to the impaired embryo quality. Here, we showed that delayed blastulation might reduce the completion of the embryo hatching and the subsequent attachment capability. Intriguingly, this might be rescued by laser-assisted ZP drilling, which facilitated the embryo attachment of E5.5 ET embryos. Previous clinical data support the notion that assisted-hatching improves clinical outcomes when applied to day 6 blastocysts (65), and even day 7 blastocysts (66). However, in contrast to clinical practice, the ZP manipulations performed in this study were all carried out at E2.5 (equivalent to day 3 in the human embryo). In addition, the sample size for blastulation delayed embryos was very limited. Therefore, large scale studies are now needed to investigate the effect of laser-assisted ZP manipulations performed on developmental-delayed blastocysts.

There were some limitations to this research that should be considered. For example, we used an *in vitro* attachment assay; this may not fully recapitulate the real conditions *in vivo* since the hatching process and embryo attachment may vary between *in vitro* and *in vivo* conditions (31). Nevertheless, the *in vitro* model of embryo attachment used in the present study was not confounded by any of the maternal factors involved in blastocyst hatching or implantation. Furthermore, it also allowed us to track individual embryos after transfer; while it is impossible to track embryos *in vivo* once embryos have been transferred to the uterus. However, it is important to investigate the efficacy of these ZP manipulations in pregnancy outcomes and the impact on the offspring health in the future. For example, the treated embryos can be transferred into surrogate mice, and the health of the resulting fetuses and newborns could be observed. Finally, the embryo hatching process could also be species-dependent, and we should, of course,

be very cautious with regards to extrapolating data from the mouse directly to the human scenario.

## **Conclusion**

In conclusion, our analysis suggests that laser-assisted ZP drilling, ZP-partial drilling, and ZP-quarter thinning, do not impact the formation of blastocysts or induce apoptosis or stress in embryonic cells. However, our data did indicate that the ZP drilling could alter the embryonic transcriptome and potentially affect embryonic metabolism. Furthermore, although ZP manipulations could advance the initiation of hatching, the ZP drilling might also pose a potential risk for hatching alteration and blastocyst entrapment, thus affecting the competency of an embryo to undergo implantation. However, ZP drilling may be beneficial for blastulation delayed embryos. For the first time, this study investigated the effect of laser-assisted drilling on the embryonic transcriptome and revealed the potential safety impacts involved. Our data also highlighted the importance to optimize and refine this clinical technique in future.

## **F&S Science Clinical Quick Take**

- Laser-assisted ZP manipulations are safe technique that did not interfere with blastocyst formation or cell number and did not induce apoptosis or cellular stress; however, ZP drilling changed the embryonic transcriptome with potential effects on embryonic metabolism.
- Although laser-assisted ZP manipulation advanced the initiation of hatching, the small size of the opening in the ZP created by drilling could cause embryo entrapment, thus potentially affecting the ability of an embryo to implant.
- We identified the risk of laser-assisted drilling on the embryonic transcriptome and the completion of hatching, thus highlighting the importance of optimising this clinical technique and the need to investigate the impact upon embryonic metabolism.

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**Table 11. A comparison of embryo development and hatching pattern at E4.5**

	ZP-drilling (n=343)	ZP-partial drilling (n=312)	ZP-quarter thinning (n=289)	Control (n=353)
Resulting blastocysts	272 (79.3%)	246 (78.9%)	229 (79.2%)	289 (81.9%)
Hatching blastocysts	224 <sup>****</sup> (82.4%)	123 <sup>*</sup> (50.0%)	124 <sup>**</sup> (54.1%)	118 (40.8%)
	ZP-drilling (n=162)	ZP-partial drilling (n=79)	ZP-quarter thinning (n=66)	Control (n=92)
Starting	20 (12.3%) <sup>†</sup>	34 (43%)	29 (43.9%)	45 (48.9%)
8-shape	125 (77.2%) <sup>†</sup>	26 (32.9%)	15 (22.7%)	23 (25.0%)
U-shape	14 (8.6%)	10 (12.7%)	17 (25.8%)	14 (15.2%)
Multiple points hatching	3 (1.9%) <sup>†</sup>	9 (11.4%)	5 (7.6%)	10 (10.9%)

\*ZP-partial drilling vs control group,  $P < 0.05$ ; \*\*ZP-quarter thinning vs control group,  $P < 0.01$ ;

\*\*\*\* ZP drilling vs control group,  $P < 0.0001$

Proportions of four hatching patterns were compared between two groups by applying the  $\chi^2$  test and the z-test. The Bonferroni method was used to adjust  $P$ -values for multiple comparisons. <sup>†</sup> ZP-drilling vs control group,  $P < 0.05$

**Table 22. A comparison of fully hatched rate and stable attachment rate from E4.5 to E7.5**

	Time points	ZP-drilling (n=46)	ZP-partial drilling (n=28)	ZP-quarter thinning (n=26)	Control (n=36)
Fully hatched	24h	5 (10.9%)	3 (10.7%)	6 (23.1%)	3 (8.3%)
	48h	18 (39.1%)**	19 (67.9%)	18 (69.2%)	26 (72.2%)
	72h	24 (52.2%)*	23 (82.1%)	19 (73.1%)	28 (77.8%)
Trapped	72h	19 (41.3%)**	0	3 (11.5 %)	5 (13.9%)
Stable attachment rate	48h	23 (50%)	11 (39.3%)	12 (46.2%)	17 (47.2%)
	72h	36 (78.3%)	19 (67.9%)	22 (84.6%)	30 (83.3%)

\* ZP-drilling group vs control group,  $P<0.05$ ; \*\* ZP-drilling group vs control group,  $P<0.01$

**Table 33. A comparison of fully hatched rate and stable attachment rate from E5.5 to E8.5**

	Time points	ZP-drilling (n=11)	ZP-partial drilling (n=16)	ZP-quarter thinning (n=10)	Control (n=16)
Hatched rate	24h	7 (63.6%)	5 (31.3%)	5 (50%)	7 (43.8%)
	48h	9 (81.8%)	8 (50%)	8 (80%)	9 (56.3%)
	72h	9 (81.8%)	8 (50%)	8 (80%)	9 (56.3%)
Trapped	72h	1 (9.1%)	1(6.3%)	0	1 (6.3%)
Stable attachment rate	48h	6 (54.5%)	6 (37.5%)	6 (60%)	6 (37.5%)
	72h	11(100%)**	7 (43.8%)	8 (80%)	8 (50%)

\*\* ZP-drilling group vs control group,  $P<0.01$

## Figure captions

### Figure 1. Mouse embryos in different ZP manipulation groups ~~the laser-assisted drilling~~ and the control group

(A-1) to (A-3): Untreated mouse embryos developed from E2.5 to E4.5; (B-1) to (B-3): Embryo treated with laser-assisted drilling at E2.5 to E4.5: a single hole (yellow arrow) completely drilled through the ZP; (C-1) to (C-3): Embryo treated with ZP-partial drilling at E2.5 to E4.5: a single hole (yellow triangle) created without breaching the inner membrane; (D-1) to (D-3): Embryo treated with laser ZP-quarter thinning at E2.5 to E4.5: around a quarter of the zona pellucida circumference drilled partially (yellow dashed line). (B-3) ‘8’-shape hatching embryo, (D-3) ‘U’-shape hatching embryo, and (C-3) multi-point hatching embryos. (Scale bar=40  $\mu\text{m}$ )

### Figure 2. The effects of laser-assisted ZP manipulations on E4.5 mouse embryos

(A) Embryonic TE (trophectoderm) and ICM (inner cell mass) cells were distinguished by immunostaining with CDX2 (red) and OCT-3/4 (green), respectively. DAPI shows nuclear staining (630 $\times$  magnification, Scale bar=50  $\mu\text{m}$ ). The cell numbers of all blastocysts and hatching blastocysts were compared between treatment groups and control (n= 23-27 (for blastocyst); n= 7-24 (for hatching blastocyst)). (B): The extent of cell apoptosis was evaluated by assessing the DNA fragmentation (n=16-18), the activation of Caspase-3/-7 (n=9-15), and the mRNA expression levels of *Bax*, *Bcl2l2*, *Sod1*, and *Gpx7* (n=3-5). (C) The expression of HSP70 (green) was visualized by confocal microscopy (630 $\times$  magnification, Scale bar=50  $\mu\text{m}$ ). For the positive control, embryos were incubated at 42°C for 10 minutes and then at 37°C for 30 minutes. Data are presented as mean  $\pm$  SD, except for DNA fragmentation (medium with interquartile range) (\* $P$ <0.05; \*\* $P$ <0.01).

**Figure 3. Transcriptomic changes and related pathways in embryos following laser-assisted ZP drilling**

(A) A volcano plot showing the identification of DEGs between laser-drilled blastocysts and untreated blastocysts: the x-axis shows the fold change in gene expression between samples and the y-axis shows the statistical significance of the differences. Significant up and down regulated genes are highlighted in red and blue, respectively. The top 20 up-regulated genes, and the top 10 down-regulated genes, by fold change, are marked by gene name. The gray points represent non-significant DEGs. (B) Validation of selected DEGs using qRT-PCR; data are presented as mean  $\pm$  SD (\* $P < 0.05$ ). (C) The significantly enriched pathways include IPA Canonical pathways (red), Reactome pathways (blue), and KEGG pathways (green) for DEGs between laser-drilled hatching and control hatching blastocysts. Enrichment analysis was performed using IPA and ClusterProfiler. For each enriched pathway, the significance of pathway enrichment is plotted by the bar ( $-\log(P\text{-value})$ ), while the gene ratios are presented as red dots (x-axis above).

**Figure 4. The *in vitro* attachment model from E4.5 to E7.5**

(A): Representative phase-contrast images of co-cultured mouse embryos and Ishikawa cells at 24h, 48h, and 72h following embryo transfer (scale bar= 50  $\mu\text{m}$ ). Blue arrows indicate mouse embryos undergoing hatching. For the embryos attached to Ishikawa cells, red markers indicate the ICM (inner cell mass), thin yellow arrows mark the outgrowth of TE (trophectoderm) cells, and white arrows show the disruption of Ishikawa cells. The outgrowing embryos which breached the Ishikawa cell layer and invaded into Matrigel are indicated with red circles. (B) The proportions of hatching embryos and fully hatched embryos at 24h, 48h, and 72h of co-culture. (C) The proportions of embryos in different attachment stability at 24h, 48h, and 72h of co-culture.

**Supplemental Figure 1. The assessment of cellular apoptosis in E4.5 mouse embryos**

(A) Embryo DNA fragmentation was detected using a TUNEL assay kit (DNA fragmentation (green) and nuclear staining (DAPI, blue)). Positive control: embryos treated with DNase; Negative control: no TdT reagent\_ (400× magnification, Scale bar=50 μm). (B) Cleaved Caspase 3/7 was detected using a Caspase Detection Kit. FAM-DEVD-FMK (green) and nuclear staining (Hoechst 33342, blue) were visualized by fluorescence microscopy. Positive control: embryos treated with 1μM of Staurosporine for 2 hours during culture\_ (400× magnification, Scale bar=50 μm).



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