# Laser zona drilling does not induce *hsp70i* transcription in blastomeres of eight-cell mouse embryos

To assess whether zona drilling with a 1,480-nm laser induces heat shock in eight-cell embryos, we measured *hsp70i* RNA levels in sets of single blastomeres isolated after laser treatment of mouse embryos that had or had not been heated at 43°C. Unlike heating, laser zona drilling did not stimulate *hsp70i* expression, even in the blastomeres closest to the laser beam, corroborating the safety of this procedure for assisted reproduction. (Fertil Steril® 2005;84:1547–50. ©2005 by American Society for Reproductive Medicine.)

The perforation of the zona pellucida (ZP) of embryos fertilized in vitro is known to enhance hatching and facilitate implantation (1) and is a necessary step for the isolation of single blastomeres or polar bodies for genetic analysis (2). The recent availability of lasers suitable for zona drilling has spurred numerous studies aimed at establishing the validity of this procedure. The mode and intensity of heating generated by the laser have been scrutinized with particular attention because hyperthermia is a known cause of teratogenesis and embryonic death (3).

Diode 1,480-nm lasers (4) present ideal characteristics to avoid blastomere damage. They emit a long-wavelength, nonmutagenic radiation that is deliverable in noncontact mode directly through a microscope lens. Quantification of the heat produced by a 1,480-nm laser beam has shown that short pulses at high power minimize thermal excursion in the blastomeres' proximity while allowing precise zona cutting (5). Several studies employing this type of laser on human embryos have addressed the issue of treatment safety by looking at outcomes such as blastocyst development (6–8), implantation rate (9), or pregnancy success (10, 11) and have concluded that laser ablation of the zona is as good or better than any other available method. The effects of laser treatment, however, have not been investigated at the molecular level.

We have recently have demonstrated that embryos at the eight-cell stage are able to respond to thermal shock by activating heat shock proteins (HSPs) production, as shown by the sharp increase in *hsp70i* transcription that follows embryo exposure to elevated temperature (12). In the present study, we measured *hsp70i* RNA levels in individual blastomeres isolated from eight-cell mouse embryos after zona drilling with a 1,480 nm laser and we have compared them to those found in cells of embryos heated at 43°C, using a direct and highly sensitive molecular method in order to evaluate the possibility of heat response in laser-treated embryos.

The designation *hsp70i* includes the two heat-inducible members of the *hsp70* family of genes, *hsp70.1* and *hsp70.3*, that share genomic sequence in the translated region and whose individual expression patterns are still largely unknown but are probably overlapping (12–14).

Mouse embryos (B6C3F1 females bred with B6D2F1 males) were purchased frozen at the two-cell stage from Embryotech Laboratories, Inc. (Wilmington, MA) and cultured in GEM-PS medium (Duncan Holly, Bedford, MA) to the precompaction eight-cell stage, as described elsewhere (15). To induce heat shock, embryos were placed at 43°C for 30 minutes and allowed to recover at 37°C for 2 or 3 hours, during which RNA synthesis took place (12). Because of cell cycle arrest, cell division did not occur in these embryos (henceforth designated as *heat-shocked*) during recovery. Control eight-cell embryos were not heated. Embryos were then collected intact or were dissociated in single cells after laser zona drilling according to our standard protocol (12, 15), which requires three 1-ms pulses with a noncontact 1,480-nm diode laser beam (ZILOS-tk™ zona infrared laser optical system; Hamilton Thorne Biosciences, Inc., Beverly, MA). Blastomeres were harvested immediately after zona drilling (12, 15) so that *hsp70i* RNA transcription had time to take place after the heating step, but not as a result of laser treatment.

An alternative protocol was used for embryos designated as *laser-treated,* which were not incubated at 43°C. Samples underwent laser zona drilling and were then placed back at 37°C for 2 hours before either cell dissociation or harvesting of whole embryos, to permit *hsp70i* RNA accumulation and investigate a possible heat shock effect generated by the laser beam itself. In contrast to heat-shocked embryos, cell division took place in several of these specimens during recovery time, resulting in 9- to 10-cell embryos at collection time.

Received February 16, 2005; revised and accepted May 16, 2005.
Supported by Brandeis University, Waltham, Massachusetts. A.A. was the recipient of a Howard Hughes Fellowship.
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Nucleic acids preparation, reverse transcription, and real-time polymerase chain reaction (PCR) were performed in a single tube by the PurAmp method (12). Briefly, samples were lysed on a PCR tube lid, releasing both RNA and DNA, and reverse transcription and real-time PCR were then carried out in the same tube. The *hsp70i* PCR assay and the strategy used to calculate *hsp70i* RNA levels were as described elsewhere (12). The *hsp70i* cDNA and genomic DNA templates (total *hsp70i* templates) were identical because the *hsp70i* genes are intronless, and they were coamplified in each sample. Standard curves were obtained from serial dilutions of genomic DNA (12, 15). Mouse DNA contains four *hsp70i* copies per genome, one copy of *hsp70.1* and one copy of *hsp70.3* on each chromosome 17. Four copies per cell were thus subtracted from total *hsp70i* template numbers to calculate *hsp70i* RNA levels.

Heat shock produced a sharp increase in the *hsp70i* RNA levels quantified in embryos at the eight-cell stage, as illustrated in Figure 1A. In that figure, blue bars indicate the *hsp70i* RNA + DNA contents of all single blastomeres that could be recovered from each of three embryos (a, b, and c) after incubation at 43°C. The contribution of *hsp70i* DNA (four copies per cell) is negligible on the scale used, but its presence confirmed that every cell had been delivered to the assay tube even when RNA was not present. (See cell 4a [top panel], which contained only four *hsp70i* templates.)

The data in Figure 1A are plotted according to the cell-picking order for each embryo. Cell 1 was the first to exit through the laser-drilled hole and thus was the closest to the laser beam because embryos do not rotate freely inside the ZP (16). The asterisks on some of the bars in Figure 1 mark pairs of blastomeres, stemming from recent cell division, that required separation with a micropipette. It is evident from the bar graphs in Figure 1A that all but one of the cells recovered from heat-shocked embryos contained hundreds of *hsp70i* RNA copies. The one non-responsive cell derived from a pair, and it is possible that its transcription machinery was slow in restarting after cell division. The proximity of a cell to the laser radiation did not affect its *hsp70i* RNA content, because the *hsp70i* RNA levels of the first-picked blastomeres were not consistently lower than those of the others. Hence, laser treatment does not damage the RNA present in the cells. Conversely, an increase in *hsp70i* transcription caused by the heat generated by the laser would not have the time to occur in this type of experiment because the cells were collected right after laser treatment.

To exclude the possibility that some RNA was degraded during cell biopsy, resulting in artifactually variable response levels, we compared our single-blastomere results with *hsp70i* RNA measurements performed on whole embryos. The average *hsp70i* RNA + DNA contents of the heat-shocked blastomeres recovered from each of the three embryos (green bars in Fig. 1A) are juxtaposed to average *hsp70i* RNA + DNA copy numbers per cell calculated from analyses of whole heat-shocked embryos (red bars in Fig. 1A). The two sets of data are in all cases remarkably similar, demonstrating that the cell isolation procedure does not affect RNA recovery. In the absence of heat shock, *hsp70i* RNA levels were minimal, as indicated by the mean *hsp70i* RNA + DNA content of four untreated whole embryos, corresponding to 19 ± 7 *hsp70i* total templates, or 15 copies of *hsp70i* RNA, per cell (black bars in Fig. 1A).

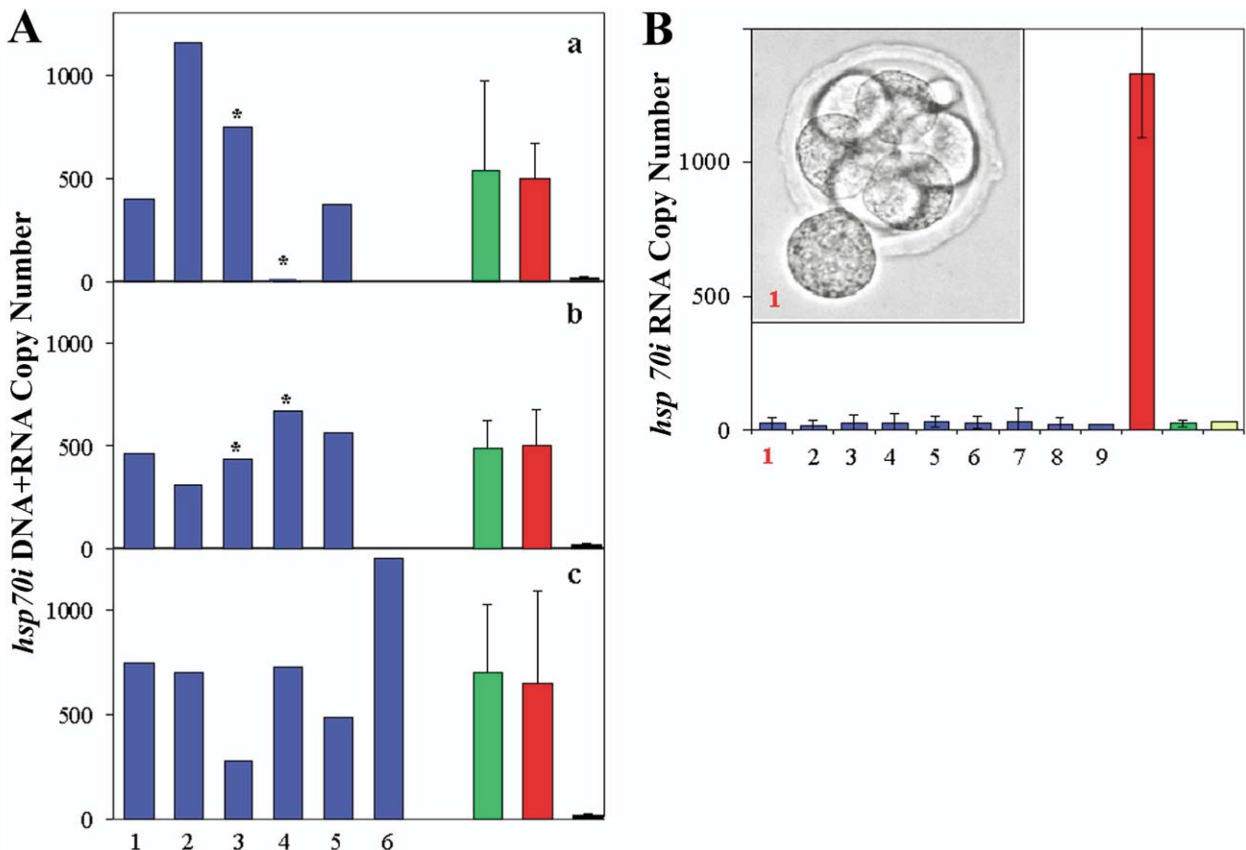
For our second set of experiments, we collected series of individual blastomeres from embryos that had not been purposely exposed to high temperature but had undergone the laser zona drilling procedure necessary for cell harvesting. To investigate the possible occurrence of thermal stress, we allowed enough time for transcript synthesis and accumulation to take place before the embryos were dissociated in individual blastomeres or collected whole.

As shown in Figure 1B, the number of *hsp70i* RNA copies measured in the first blastomere recovered from each of four eight-cell embryos after laser zona drilling averaged 25 ± 22 (blue bar 1), consistent with the average number of *hsp70i* RNA copies per cell (24 ± 16) calculated from whole embryos. Similarly, the average number of *hsp70i* transcripts in the other blastomeres of the same embryos varied from 16 ± 22 to 37 ± 48 copies (blue bars 2–9). As shown above for non-heated embryos, these low levels of *hsp70i* transcription are normally present in embryonic blastomeres. More pulses were used for this experiment (four to ten 1-ms pulses) than were required by our standard protocol, strengthening the conclusion that treatment with this type of laser does not generate thermal shock in embryonic cells. Analysis of a whole eight-cell embryo whose zona was removed with eight 1.5-ms laser pulses (pulses more numerous and longer than usual) gave comparable results (36 copies of *hsp70i* RNA per cell, yellow bar). In contrast, embryos' exposure to 43°C again induced a marked surge in the number of *hsp70i* RNA copies per cell (1,331 ± 238; red bar). Our results, thus, overall prove that laser treatment under the conditions described in this report does not produce a heat shock–like effect on *hsp70i* transcription.

To corroborate this conclusion, we compared the rates of development to the blastocyst stage of laser-treated and untreated eight-cell embryos. Forty-six hours after treatment, all 24 embryos used for this experiment had become expanded blastocysts and had begun hatching, although hatching was more advanced in the 12 laser-treated embryos. The unchanged rate of development strongly indicates that laser treatment did not induce cell cycle arrest, a well-known effect of hyperthermia (17).

## FIGURE 1

(**A**) *hsp70i* RNA + DNA copy numbers in heat-shocked eight-cell embryos. *Blue bars, hsp70i* RNA + DNA copy numbers in single blastomeres isolated from three embryos (*a*, *b*, and *c*) after heat shock. Blastomere data are graphed according to the cell picking order, left to right in each panel. *Asterisks* mark cells that exited the ZP as pairs and were separated before collection. *Green bars,* average *hsp70i* RNA + DNA copy number (±SD) per recovered heat-shocked blastomere, as calculated from the single blastomere data. *Red bars,* average *hsp70i* RNA + DNA content per cell as calculated from analysis of whole heat-shocked embryos (*a*, *b*: six embryos; *c*: two embryos). Recovery time was as follows: all embryos in *a* and *b*, 2 hours; all embryos in *c*, 3 hours. *Black bars,* average *hsp70i* RNA + DNA content per cell, as calculated from analysis of four whole embryos in the absence of heat shock. (**B**) *hsp70i* RNA levels in laser-treated eight-cell embryos. *Blue bars,* average *hsp70i* RNA copy numbers (±SD), measured in single blastomeres harvested from four embryos through a laser-drilled hole in the ZP. Embryos received between four and ten 1-ms laser pulses in a restricted area of the zona. Blastomere 1 was the first-exiting cell from each embryo and the closest to the laser beam. Average copy numbers were calculated as follows: blastomeres 1 to 6, 4 samples; blastomere 7, 3 samples; blastomere 8, 2 samples. Blastomere 9 was derived from an embryo that underwent cell division during the recovery period after laser treatment. Per-blastomere average *hsp70i* RNA copy numbers calculated from whole-embryo determinations were as follows: *red bar,* heat-shocked embryos (average of two samples); *green bar,* no-treatment controls (average of two samples); *yellow bar,* whole embryo freed from the zona by eight 1.5-ms laser pulses. *Inset,* extrusion of the first blastomere.




*Hartshorn. Zona drilling does not heat shock embryo. Fertil Steril 2005.*

Comparatively longer treatments with lasers of different wavelengths and powers are known to activate the *hsp70* promoter in a number of different systems (18, 19). In contrast, our findings that laser zona drilling does not trigger *hsp70i* expression in eight-cell embryos nor affect the rate of preimplantation development, unlike heating at 43°C, strongly support the conclusion that embryonic cells were not thermally stressed by the procedure employed for this study.

These results have important implications, because an increase in *hsp70i* transcription signals the occurrence of

cell damage and cell cycle arrest even in the absence, or well in advance, of identifiable morphological changes. Such events, even if they are transient, could affect specific blastomeres' survival and developmental fate, as recent data overwhelmingly indicate that the destiny of mammalian blastomeres at the cleavage stages already may be determined and may be linked to the timing of the cell cycle (20, 21).

In conclusion, this study thus provides the first evidence at the molecular level for the safety of ZP perforation with a 1,480-nm diode laser under carefully chosen conditions.

*Acknowledgment:* The laser used in this study was kindly provided by Hamilton Thorne Biosciences, Inc., Beverly, Massachusetts.

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