Heat shock memory in preimplantation mouse embryos

To investigate the consequences of possible physiologic stress to embryos caused by in vitro fertilization procedures, we used heat shock response in preimplantation mouse embryos as a model. A heat shock "memory" was discovered that renders cleavage-stage embryos more responsive at the transcriptional level to secondary perturbation with very low doses of heat, even several cell cycles after the initial stress has occurred. (Fertil Steril® 2010;93:2760–3. ©2010 by American Society for Reproductive Medicine.)

Over the past 30 years, more than 1% of births in the United States and as many as 4% in Denmark have been initiated using some form of in vitro fertilization (IVF) (1). There is now increasing concern and investigation into whether such procedures have long-term consequences that may or may not be obvious at birth (2, 3). Indeed, recent studies suggest that IVF is associated with some rare genetic syndromes such as Beckwith-Wiedemann syndrome (4, 5) and Angelman syndrome (6). An increased risk of several birth defects in IVF babies has also been reported (7, 8).

In vitro fertilization includes a wide variety of treatments and protocols, and no rigorous investigations of altered gene expression in preimplantation human embryos are available for any of them. However, both short-term and long-term effects of environmental conditions on preimplantation mouse embryos have been analyzed. Ecker et al. (9) transferred genetically marked blastocysts grown in vivo along with blastocysts cultured under different conditions in vitro into the same foster mother and then tested the resulting sibling mice for their capacities to perform in a maze. The results clearly showed that mice born from embryos cultured in vitro were not as maze-bright as mice derived from embryos grown in vivo. Moreover, mice obtained from cultured embryos lacked the caution and fear that normal mice are born with. At the molecular level, genome-wide patterns of DNA methylation and gene expression were different in embryos cultured in vitro for a few days as compared with those grown in vivo to the equivalent stages of development (10, 11).

Heat shock proteins (HSPs) are expressed in virtually all cells in response to environment stressors and serve to protect the cells from heating and toxic substances, which might occur during embryo culture (12–14). The phenomenon of thermotolerance, a first mild heat exposure of the embryo protecting it from a second more severe heat shock, has been observed in postimplantation mouse embryos (15–17). It was suggested that HSP70 has a direct role in the induction of thermotolerance and that the level of thermotolerance can be correlated to the level of HSP70 protein (16). Although inducible expression of the *hsp70i* genes (*hsp70.1* and *hsp70.3*) is observed in mouse embryos starting at the four-cell stage (18), the relationship between *hsp70i* gene expression and thermotolerance in preimplantation embryos is unclear.

Our previous work has shown that ablation of the zona pellucida with a laser does not induce heat shock in preimplantation mouse embryos (19). Here, using *hsp70i* messenger RNA (mRNA) transcription as a model, we show for the first time that a mild heat shock at the eight-cell stage renders mouse embryos more responsive at the level of *hsp70i* mRNA accumulation to a second shorter heat shock delivered even several cell cycles later. Two-cell stage mouse embryos from Embryotech Laboratories (Haverhill, MA) were cultured as described elsewhere (20). Single cells were isolated from eight-cell stage embryos through a hole in the zona pellucida generated using a ZILOS-tk laser optical system (19). The PurAmp single-tube method (21) was used for lysing single cells or whole embryos and for analyzing them by real-time one-step linear-after-the-exponential polymerase chain reaction (RT-LATE-PCR) (22, 23) in the same vessel. This method measured total template copy numbers (*hsp70i* mRNA + *hsp70i* genomic DNA) because the *hsp70i* genes are intronless. The *hsp70i* genomic DNA amounts to four copies per cell. We ran RT-PCR in 50 μL reaction with 50 nM limiting primer (5'-CAGCGTCCTCTTGGCCCTCTCACAC-3'), 2 μM excess primer (5'-GATCGACGACGGCATCTTC-3'), and 500 nM Probe (BHQ1-5'-GATCCTCTTGAACTCCTTC-3'-Cal Orange 560; BHQ1: Black Hole Quencher 1) along with 300 nM Primesafe I (Smith Detection, UK) (24), and 1 μL of SSIII/Platinum Taq mixture (11732; Invitrogen, Carlsbad, CA) in standard PCR buffer (11304; Invitrogen). The cycling profile was 55°C for 15 minutes (RT); 95°C for 5 minutes; 15 cycles consisting of the following three

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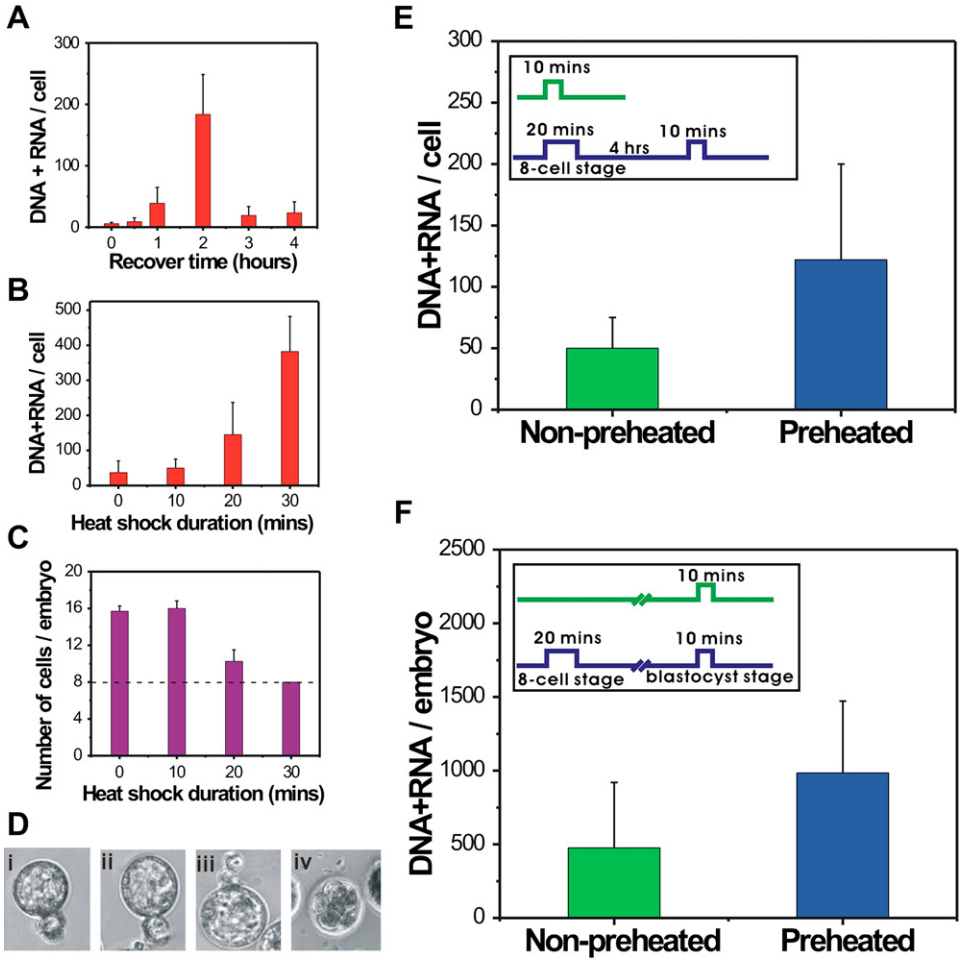
FIGURE 1

(**A**) *hsp70i* messenger RNA (mRNA) transcription in single cells isolated from eight-cell embryos after 20 minutes of heat shock and different recovery times. The bars show average *hsp70i* mRNA + DNA levels per cell plus standard deviation. (**B**) Average *hsp70i* mRNA transcription in single cells from eight-cell embryos after heat shock of different durations. (**C**) Average number of cells per embryo 20 hours after heat shock at the eight-cell stage for different lengths of time. (**D**) Morphologic features of eight-cell embryos 48 hours after heat shock for [i] no heat shock, [ii] 10 minutes, [iii] 20 minutes, and [iv] 30 minutes. (**E**) Double heat-shock effect on *hsp70i* expression in single cells from embryos at the eight-cell stage. The green bar shows average *hsp70i* mRNA + DNA levels in embryos that had been heat-shocked only once, for 10 minutes at the eight-cell stage (cultured for 24 hours). The blue bar shows average *hsp70i* mRNA + DNA levels in embryos that had been preheated for 20 minutes at the eight-cell stage (cultured for 24 hours) and then heated again for 10 minutes after a 4-hour recovery period. The average number of *hsp70i* mRNA + DNA copies per cell was obtained using 30 to 50 single cells from each group of embryos. (**F**) Long-term double heat-shock effect in blastocyst-stage mouse embryos (cultured for 72 hours). The green bar shows average *hsp70i* mRNA + DNA levels in embryos heat-shocked for 10 minutes at the blastocyst stage. The blue bar shows average *hsp70i* mRNA + DNA levels in embryos preheated for 20 minutes at eight-cell stage, then heat-shocked again for 10 minutes at the blastocyst stage.



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steps: 95°C (10 seconds), 63°C (20 seconds), and 72°C (30 seconds); 35 cycles with the following four steps: 95°C (15 seconds), 55°C (25 seconds), 72°C (35 seconds), and 45°C (30 seconds) (fluorescence reading). Standard curves for template copy number quantification were generated with the same pair of primers, using a template of purified mouse genomic DNA (Sigma, D4416) in serial dilutions.

Levels of *hsp70i* mRNA per cell in eight-cell stage embryos (cultured for 24 hours from two-cell stage) heated for 20 minutes at 43°C reached maximum levels 2 hours after treatment and then returned to normal after 4 hours (Fig. 1A). A heating period of only 10 minutes did not result in elevated *hsp70i* mRNA after 2 hours of recovery, while the average number of *hsp70i* templates per cell after 20 or 30 minutes of heating was found to be, respectively, 145 (± 92) and 383 (± 100). Thus, the levels of *hsp70i* mRNA gene expression correlated with the length of heat shock (see Fig. 1B).

The fact that longer heating results in higher *hsp70i* mRNA accumulation does not, however, guarantee that embryos treated for 20 or 30 minutes are equally able to recover from stress. Embryos heated for 30 minutes at the eight-cell stage and then cultured for an additional 48 hours at 37°C failed to undergo cell division and

eventually died. In contrast, embryos heated for 10 minutes continued to grow just like control embryos, while embryos treated with heat for 20 minutes paused cell division for several hours (see Fig. 1C) and then resumed growth and development to blastocyst stage 48 hours after heat shock. Thus, although the time needed to initially reach blastocyst stage was not the same for untreated embryos and embryos heated for 20 minutes, both sets of embryos were morphologically similar after 48 hours, with a well-formed blastocoele having an inner cell mass on one side (see Fig. 1D). Both sets of embryos also went on to hatch.

To study preimplantation embryos' response to double heat shock, we exposed embryos at the eight-cell stage to 20 minutes of heat and then, after a gap of 4 hours, tested them for thermotolerance by heating them again for either 10 or 30 minutes. Primary heat treatment for 20 minutes did not prevent developmental arrest and death caused by a 30-minute heating, demonstrating that these embryos were not rendered thermotolerant even though they had actively transcribed *hsp70i* mRNA in response to the first heat treatment.

We next investigated whether a primary exposure to heat could, in fact, sensitize the embryo to secondary heat treatment. One group of eight-cell embryos was heat-treated for 20 minutes, allowed to recover for 4 hours, and then heated again for 10 minutes. The level of *hsp70i* mRNA in the cells of these embryos was compared with that of embryos that had been heated only once for 10 minutes. Because the embryos paused in their development after the 20-minute heat shock (see Fig. 1C), both groups of embryos were at the eight-cell stage during all phases of this experiment. The results in Figure 1E show that single cells from embryos heated only once contained an average of 50 ($\pm$ 25) copies of *hsp70i* mRNA + DNA. In contrast, double heat-shocked embryos had an average of 122 ($\pm$ 78) copies of mRNA + DNA per cell, demonstrating that the initial 20-minute treatment sensitized these cells to secondary heating. We call this phenomenon heat shock "memory" in preimplantation mouse embryos.

These results led us to investigate whether heat shock memory would persist through one or more rounds of DNA replication and cell division. To test this possibility, we once again heated a group of about 20 eight-cell embryos for 20 minutes and then grew them to the blastocyst stage (cultured for 72 hours from two-cell stage). At this point, the embryos were heated a second time for just 10 minutes. A second group of about 20 control embryos was not heated at the eight-cell stage but were exposed to heat for 10 minutes when they reached the blastocyst stage (cultured for 72 hours from two-cell stage). Because single cells cannot readily be dissected out of blastocyst-stage embryos, we analyzed whole embryos and measured the *hsp70i* mRNA + DNA copy numbers 2 hours after the 10-minute heat treatment.

As shown in Figure 1F, the double heat-shocked embryos contained on the average 984 ($\pm$ 478) copies of *hsp70i* templates per embryo while the embryos heat-shocked once yielded only 476 ($\pm$ 444) copies of *hsp70i* templates per embryo, a ratio of about 2:1. The ratio of *hsp70i* mRNA molecules per cell in the double heat-shocked embryos versus embryos heated once, however, is likely to be higher because the first exposure to heat had arrested cell division for several hours. Therefore, the blastocysts preheated at the eight-cell stage contained fewer cells, on average, than the control blastocysts. Heat-shock–induced cell cycle arrest is a well-known phenomenon (25). For instance, the number of cells in heat-shocked bovine preimplantation embryos is two-thirds of that in control embryos (26). The higher response in the blastocysts first heat-treated at the eight-cell stage demonstrates that heat shock memory persists through several cell divisions.

The results presented here raise many intriguing questions and hypotheses. What is the full set of genes in the preimplantation embryo sensitized by primary heat treatment? Can these genes be affected or activated (if stress-inducible) by chemicals that activate heat shock genes in other cell systems (27, 28)? What is the molecular mechanism of long-term heat shock memory? It is known that *hsp70i* genes are methylated in mammalian cells (29). Thus, similar to the findings of Ecker et al. (9), it is plausible that primary heat treatment triggers a change in DNA methylation within the promoters of these genes and that this epigenetic change persists in daughter cells, rendering them more sensitive to secondary heat treatment. If such mechanisms are shown to be true, the same epigenetic changes may be stable through embryonic, fetal, and neonatal development. Such a scenario could mean increased sensitization to a secondary stress, at least in some cell lineages. Validation of these hypotheses will require a great deal more research in the mouse, but the resulting information will have critical importance for the growth and development of human embryos conceived both in vitro and *in utero*.[#]

#" 'The Predestinators send in their figures to the Fertilizers. Who give them the embryos they ask for. And the bottles come in here to be predestinated in detail…. Heat conditioning,' said Mr. Foster. 'Hot tunnels alternated with cool tunnels. Coolness was wedded to discomfort in the form of hard X-ray. By the time they were decanted the embryos had a horror of cold. They were predestined to emigrate to the tropics, to be miners and acetate silk spinners and steel workers…. We condition them to thrive on heat,' concluded Mr. Foster." Aldous Huxley, *Brave New World* (1932).

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