The Temperature and Type of Intracellular Ice Formation in Preimplantation Mouse Embryos as a Function of the Developmental Stage

Shinsuke Seki and Peter Mazur

Fundamental and Applied Cryobiology Group, Department of Biochemistry and Cellular and Molecular Biology, The University of Tennessee, Knoxville, Tennessee

ABSTRACT

Our studies the past 5 yr have concentrated on intracellular ice formation (IIF) in mature mouse oocytes at the metaphase stage of meiosis II. Here we report an analogous investigation of the temperature of intracellular ice nucleation in preimplantation embryo stages from one-cell to early morula suspended in 1 M ethylene glycol/PBS and cooled at 20°C/min to −70°C. Physical modeling indicates that oocytes and preimplantation embryos undergo very little osmotic shrinkage at that cooling rate. As a consequence, their interior becomes increasingly supercooled until the supercooling is abruptly terminated by IIF. Four categories of IIF were observed. The first two were 1) those undergoing IIF at temperatures well below the temperature of external ice formation (EIF; −7.2°C) vs. 2) those undergoing IIF within 1°C of the EIF temperature. The other two categories were those multicellular stages in which 3) all the blastomeres underwent IIF simultaneously vs. 4) those in which blastomeres underwent IIF sequentially. Embryos in categories 1 and 3 constituted the majority (80–90%), and for them, the mean IIF temperatures of one-cell, two-cell, four- to six-cell, and early eight-cell ranged from −37°C to −43°C, temperatures that indicate that IIF is a consequence of homogeneous nucleation. However, the IIF nucleation temperature of early morulae in categories 1 and 3 was markedly higher; namely, −23.1 ± 1.5°C. This marked rise in nucleation temperature coincides with the appearance of aquaporin 3 and gap junctions in early morulae (compacted eight-cell), and is presumably causally related.

INTRODUCTION

The formation of ice crystals within cells is almost always lethal. The classical way to avoid it is to cool cells slowly enough so that they lose nearly all their freezable water osmotically before they have cooled to the temperature at which ice nucleation of the remaining supercooled intracellular water can occur. Thus, whether intracellular ice forms or not depends on two factors. One is the rate at which the cells are cooled, for it determines how much supercooled water remains in the cells as a function of temperature. The second is the nucleation temperature of that supercooled water. We have reported [1] that the nucleation temperature of mature mouse oocytes (metaphase II [MII] stage) in 1 M ethylene glycol (EG) is −35°C. The question we are addressing in this report is whether that temperature is the same or different in preimplantation embryos of various developmental stages.

We had reason to believe that it would be different in compacted eight-cell embryos or early morulae because the structure of their membranes is changed in several important respects from that of uncompacted eight-cell and earlier stages. Mazur et al. [2] had reported in 1976 that the permeability of mouse embryos to glycerol was 10-fold higher at the eight-cell stage than at the one-cell stage. However, they did not distinguish between pre- and postcompacted eight-cell embryos. In 1995, Valdimarsson and Kidder [3] reported that functional gap junctions form between the blastomeres of compacted eight-cell embryos, and still more recently, Edashige et al. [4, 5] and Matsuo et al. [6] have demonstrated the presence of the aquaglyceroporin AQP 3 (and possibly AQP 9) in early morulae. One manifestation of this is a 5-fold increase in the permeability coefficient or hydraulic conductivity of water (Lp). These changes are summarized in Table 1. Consequently, a comparison between the freezing properties of early morulae and earlier stages should provide valuable information on the role of endogenous membrane pores in the formation and propagation of intracellular ice.

Prior to compaction, the blastomeres of the embryos lie in physical juxtaposition, much like a stack of cannonballs, but are not functionally connected. However, after compaction, their plasma membranes have formed tight junctions with their neighbors, and, as indicated, gap junctions have formed in these tight junctions. In that sense, then, the postcompaction eight-cell embryo or early morula is a true multicellular system, and, as such, it may be a good model for more complex multicellular systems like tissues and organs.

MATERIALS AND METHODS

Many of the methods were described in detail in [1]; consequently, here we give details only for those aspects that differed. The procedures for obtaining and manipulating the mouse oocytes and embryos were carried out under the University of Tennessee Institutional Animal Care and Use Committee protocol 911-0607, approved 28 June 2007, and the Animal Ethics Committee of the College of Agriculture, Kochi University.

Collection of Oocytes and Embryos

Mouse oocytes at the MII stage and embryos at the one-cell, two-cell, four-cell, eight-cell, and early morula stages were used in the study. Mature female mice of the ICR strain were induced to superovulate with intraperitoneal injections of 5 IU of equine chorionic gonadotropin (Sigma) and 5 IU of human chorionic gonadotropin (hCG; Sigma) given 48 h later. For the collection of embryos, females were mated with mature males of the same strain. For the collection of oocytes, matured oocytes surrounded by cumulus cells were collected from the ampullar portion of the oviducts at 13 h after hCG injection and were freed from cumulus cells by suspending them in modified phosphate-buffered saline (PBS) containing 0.5 mg/ml hyaluronidase followed by washing with fresh PBS medium. For the collection of one-cell, two-cell, four-cell, and eight-cell embryos, ovauidts of mated animals were flushed with PBS medium at 25, 44, 55, and 68 h, respectively, after the injection of hCG. For the collection of early morulae, the uteri of mated animals were flushed with PBS medium at 25, 44, 55, and 68 h, respectively, after the injection of hCG.
### Vitrifying Oocytes/Embryos and Shipment

Some of the oocytes and most of the embryos were collected in P.M.'s laboratory at the University of Tennessee; the remainder, however, were collected at Kochi University in Japan, vitrified under the supervision of Drs. M. Kasai and K. Edashige, and shipped to Tennessee at under 5% CO₂. Several lines of evidence [1, p. 48] support the view that the droplets of M16 medium in which they were incubated for some 2 h at 37°C were transferred rapidly from LN2 to a water bath at 23°C, and then plunged into LN₂. The measured cooling rate from 20°C to −150°C above which the blackening first appeared.

For vitrification, oocytes at MII stage were suspended in EAFS 10/10 for 2 min, two-cell embryos in EFS 20 for 2 min and EFS 40 for 1 min, four-to-eight-cell embryos in EFS 30, and morulae in EFS 40. In these acronyms E, A, F, and S refer to EG, acetamide, Ficoll, and sucrose. The "10", "20" and "40" refer to the weight percentages of EG and acetamide when present. Details on their preparation are given in [1, 8–10]. The following were then successively aspirated into 0.25-ml straws: a 60-mm column of 0.5 M sucrose in PB1, a 20-mm air bubble, a 5-mm column of EAFS or EFS, a 5-mm air bubble, and a 12-mm column of vitrification solution.

For vitrification, oocytes at MII stage were suspended in EAFS 10/10 for 2 min, two-cell embryos in EFS 20 for 2 min and EFS 40 for 1 min, four-to-eight-cell embryos in EFS 30, and morulae in EFS 40. During these exposure times, the oocytes and embryos were transferred into 0.25-ml straws: a 60-mm column of 0.5 M sucrose in PB1, a 20-mm air bubble, a 5-mm column of EAFS or EFS, a 5-mm air bubble, and a 12-mm column of vitrification solution.

For vitrification, oocytes at MII stage were suspended in EAFS 10/10 for 2 min, two-cell embryos in EFS 20 for 2 min and EFS 40 for 1 min, four-to-eight-cell embryos in EFS 30, and morulae in EFS 40. During these exposure times, the oocytes and embryos were transferred into 0.25-ml straws: a 60-mm column of 0.5 M sucrose in PB1, a 20-mm air bubble, a 5-mm column of EAFS or EFS, a 5-mm air bubble, and a 12-mm column of vitrification solution.

### Table 1. Presence (+) or absence (−) of aquaporin water channels and gap junctions in mouse oocytes and embryos of various stages.

<table>
<thead>
<tr>
<th>Developmental stage</th>
<th>Aquaporin (AQP)</th>
<th>Gap junction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metaphase II (MII) oocyte</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Zygote (1-cell), 2-cell, 4-cell</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>8-Cell</td>
<td>+ (AQP9)</td>
<td>−</td>
</tr>
<tr>
<td>Morula (compact 8-cell)</td>
<td>+ (AQP3, AQP9)</td>
<td>+</td>
</tr>
</tbody>
</table>

* The plasma membrane of the in situ MII oocytes forms gap junctions with the membrane extensions of the surrounding cumulus cells. These connections and junctions are broken during the experimental processing of the oocytes.

** The expression of AQP9 has been reported by Barcroft et al. [7].

medium 78 h after the injection of hCG. The oocytes and embryos were washed and pooled in fresh PB1 medium in a culture dish under paraffin oil to await each suite of experiments.

### The Linkam Cryostage, Freezing Protocols, and Ramps

Using LN, vapor for cooling and electrical resistors for heating, the Linkam cryostage with its associated control hardware and Pateit software allows samples to be subjected to sequential ramps in which cooling rate, limiting temperature, holding time, and warming rate can be specified. The ramps used here are shown in Table 2. The procedure was as follows: the oocytes/embryos were cooled rapidly to −5.0°C, and then to −8.0°C (ramps 1 and 2). External ice formation (EIF) occurred at a mean of −7.2 ± 0.06°C. The sample was then warmed (ramp 3) to 3.2°C, which is just at the melting point of the medium. Most, but not all, of the external ice melted. Recooling was then initiated in ramp 4 after a 10-sec hold at the end of ramp 3. The purpose of ramp 3 was to provide time for the external liquid medium, the external ice, and the supercooled water in the cell to come to near equilibrium before recociling began. If ramp 3 was omitted, the observed temperatures of IIF were about 20°C higher [13].

### Glycyrhrhetic Acid Experiments

The compound 18β-glycyrrhetic acid (GA) is a known gap junction blocker [14]. To determine whether it affected sequential EIF in morulae, 30 μM GA (Sigma) was added to the final 1.0 M EG/PBS solution in which the morulae were suspended for 15 min at 25°C prior to freezing in the Linkam cryostage. In some experiments, we used a PBS that lacked the normal Ca²⁺.

### Table 2. Cooling and warming rates programmed into the Linkam cryostage for oocytes and embryos frozen in 1.0 M ethylene glycol/PBS.

<table>
<thead>
<tr>
<th>Ramp no.</th>
<th>Rate (°C/min)</th>
<th>Limit (°C)</th>
<th>Hold (sec)</th>
<th>Capture intervals*</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>−10</td>
<td>−5.0</td>
<td>0</td>
<td>30</td>
<td>Cooling</td>
</tr>
<tr>
<td>2</td>
<td>−2</td>
<td>−8.0</td>
<td>10</td>
<td>10</td>
<td>Cooling: EIF</td>
</tr>
<tr>
<td>3</td>
<td>+2</td>
<td>−3.2</td>
<td>10</td>
<td>10</td>
<td>Warming: partial thawing</td>
</tr>
<tr>
<td>4</td>
<td>−10</td>
<td>−7.0</td>
<td>0</td>
<td>10</td>
<td>Cooling</td>
</tr>
<tr>
<td>5</td>
<td>−20</td>
<td>−70.0</td>
<td>0</td>
<td>10</td>
<td>Cooling: EIF</td>
</tr>
<tr>
<td>6</td>
<td>+10</td>
<td>−5.0</td>
<td>30</td>
<td>20</td>
<td>Warming and thawing</td>
</tr>
<tr>
<td>7</td>
<td>+10</td>
<td>+20</td>
<td>60</td>
<td>20</td>
<td>Warming and thawing</td>
</tr>
</tbody>
</table>

* Time interval in seconds between the storage of successive images on the hard drive.

† EIF, extracellular ice formation; IIF, intracellular ice formation.
and Mg\(^{++}\). The absence of Ca\(^{++}\) in particular is supposed to disaggregate the compacted blastomeres, and that action would be expected to disrupt gap junctions.

Statistics

Plus/minus values in tables and error bars in figures are standard errors (standard deviations of the mean). Two-tailed t-tests and chi-square analysis were used to assess significant differences.

RESULTS

Two Types of IIF

Two types of flashing or IIF were observed in this study. In one case (simultaneous IIF), all the blastomeres in a given embryo darkened simultaneously and uniformly. The rate at which they darkened depended on the temperature at which flashing was initiated [1]. When the temperature was above \(-30^\circ C\), complete darkening took only a fraction of a second. When nucleation occurred below \(-30^\circ C\), darkening took a number of seconds. In the other type (sequential), the flashing of an individual blastomere or a small group of blastomeres was separated in time (and therefore in temperature) from the flashing of neighboring blastomeres or small groups of blastomeres in the same embryo. The two types are illustrated in Figure 1 for a two-cell embryo. Figure 1–1C shows simultaneous IIF in the two blastomeres at \(-31.8^\circ C\). Figure 1–1B and 1–2D shows sequential IIF in each blastomere 2.2°C apart and 6.4 sec apart. The “A” photographs in the first column show their appearance before the start of the experiment. The “B” photographs in the second column show the appearances after EIF occurred at \(-7.0^\circ C\) but before the occurrence of IIF.

IIF Temperature of Mouse Embryos at Each Developmental Stage in Which the Blastomeres Flashed Simultaneously

Figure 2 shows the frequency distributions of IIF flashing temperatures for each stage. For two-cell embryos to morulae, the data are for embryos in which the blastomeres underwent simultaneous flashing. The first conclusion is that the distribution of flash temperatures was broader for the MII oocytes and the morulae than for the one-cell to eight-cell embryos. The second conclusion is that the modal value for the flash temperature was around \(-40^\circ C\) for one-cell through eight-cell embryos, but was vaguer and higher for MII oocytes and morulae (much higher for the latter). A third point is that the range of flash temperatures for the morulae overlaps part of the high end temperature distribution for oocytes and two-, four-, and eight-cell embryos, a point that we shall return to in discussion.

Table 3 shows the mean temperature for simultaneous flashing for each developmental stage. In MII oocytes and one-cell, two-cell, four (to six)-cell, and eight-cell embryos, the flash temperatures (\(-34.4, \,-42.9, \,-40.1, \,-38.0,\) and \(-35.4^\circ C\)), are similar, although those for the oocytes (\(-34.4^\circ C\) and eight-cell embryos (\(-35.4^\circ C\)) are slightly but statistically higher than

FIG. 1. Photographs illustrating the two types of intracellular ice formation (IIF) in two-cell mouse embryos. The upper row depicts simultaneous IIF in the two blastomeres at \(-31.8^\circ C\). The bottom row illustrates sequential IIF. One blastomere froze at \(-24.5^\circ C\); the other, at \(-26.7^\circ C\). The first photograph in each row (A) was taken before cooling was initiated. The second photograph (B) was taken at \(-24.3^\circ C\) before IIF occurred, and the third and fourth photographs (C and D) were taken after IIF. The diameter of the faint zona pellucida is \(-75\ \mu m\).

FIG. 2. Frequency distribution of the flash or IIF temperatures for oocytes and the various stage embryos studied here. The bars show the temperature at which all the blastomeres flashed simultaneously.
those for the other embryo stages. In contrast, the mean IIF flash temperature of morulae (−23.1°C) was substantially higher than those of any other stage.

The data in Figure 2 and Table 3 apply to the situation in which all blastomeres flashed simultaneously. The ensuing sections pertain to the situation in which flashing in individual blastomeres or small groups of blastomeres was sequential. The percentages that flashed simultaneously were 91.1%, 82.9%, and 86.0% in two-cell, four-cell, and eight-cell embryos, respectively. With morulae, the percentage was lower—namely, 78.9%—but not significantly so.

Propagation of IIF in Two- to Eight-Cell Embryos and in Early Morulae from a Flashed Blastomere to Neighboring Blastomeres

Table 4 shows that the percentages of embryos in which the blastomeres flashed sequentially were 8.9%, 17.1%, and 14.0% in two-cell, four-cell, and eight-cell embryos. The percentage was 21.1% in morulae. Figure 3 shows photographs of sequential flashing of blastomeres in an eight-cell embryo (Fig. 3–1) and in an early morula (Fig. 3–2). The times shown are the seconds that elapsed between the flashing of one blastomere and that of a next (usually adjoining) one. Because the cells were being cooled at 10 or 20°C/min, the time interval also represents a temperature interval. Two conclusions are evident. One is that the time interval between flashing in the morulae was considerably shorter (Fig. 3–2) than that in the eight-cell embryos (Fig. 3–1). The second point is that this sequential flashing in both stages is occurring at a much higher temperature (−7°C to −18°C) than that observed for simultaneous flashing. The temperature of −7°C is essentially the same as the temperature at which EIF occurred.

Figure 4 is a plot of the frequency distribution of the temperatures at which the first blastomere or small group flashed (black bars) and the corresponding temperature at which the last group flashed (white bars) in each of the multicellular developmental stages studied. (Note that −6 to −8°C on the far right of the abscissa are repeated two or three times. The reason is that these temperatures are traversed in ramps 2, 3, 4, and 5, as can be seen from inspection of Table 2.) Figure 5 shows the average number of seconds that elapsed between the flashing of the first blastomere in two-, four- (six)-, and eight-cell embryos and early morulae and that of the second, usually adjoining, blastomere. (Strictly speaking, in the four-cell through morula stages, we usually could not observe flashing events in each individual blastomere, but rather separate flashing in small groups of blastomeres. Thus, in four- to six-cell embryos, eight-cell embryos, and morulae, we observed 2–3, 2–4, and 4–11 separate freezing events,

<table>
<thead>
<tr>
<th>Stage</th>
<th>Simultaneous flashing</th>
<th>Sequential flashing</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-Cell</td>
<td>51 ± 0.9 ab</td>
<td>5 ± 0.9</td>
</tr>
<tr>
<td>4-Cell</td>
<td>34 ± 1.0 abc</td>
<td>7 ± 1.0</td>
</tr>
<tr>
<td>8-Cell</td>
<td>43 ± 1.0 abc</td>
<td>7 ± 1.0</td>
</tr>
<tr>
<td>Morulae</td>
<td>30 ± 1.5 d</td>
<td>8 ± 2.1</td>
</tr>
<tr>
<td></td>
<td>n</td>
<td>%</td>
</tr>
</tbody>
</table>

* In 2- to 8-cell embryos, all blastomeres froze simultaneously.
† The means are a combination of data from freshly collected cells and from previously vitrified cells (see Materials and Methods).
‡ Values with different superscripts were significantly different (P < 0.05).

FIG. 3. Photographs illustrating sequential flashing in an eight-cell embryo (1) and a morula embryo (2). Photographs 1A and 2A depict the embryos in 1.0 M EG just prior to EIF; photos 1B and 2B, just after EIF. They then underwent sequential flashing of the blastomeres (1-I, 1-II, 1-III, 1-IV for an eight-cell embryo and 2-I, 2-II, 2-III, 2-IV for a morula embryo) at the temperatures depicted. We also show the time for IIF to spread from one flashed blastomere(s) to an adjoining blastomere(s). The magnification is the same as in Figure 1.
respectively. However, for economy of writing, we will sometimes refer to the flashing of a small group of blastomeres as equivalent to the flashing of a single blastomere.) Figures 4 and 5 both show striking differences between the morulae and the earlier stages. With respect to temperature (Fig. 4), the open bars show the temperatures at which the last blastomere or small group of blastomeres flashed in a given morula. The black bars show the temperatures at which the last group in that morula flashed. The open bars show the temperatures at which the last component to flash in a given embryo (white bars) did so at a still lower temperature. These latter temperatures are somewhat higher than those observed for simultaneous flashing (Fig. 2).

With respect to the time interval between the first flashing event and the second (Fig. 5), the interval in the early morulae was 0.6 ± 0.1 sec, a much shorter time than the intervals of 10 to 18 sec in the three earlier stages. The time differences in the latter group were not significant. The temperature and time abscissas in Figures 4 and 5 are interrelated by the fact that the second factor is the volume of the supercooled component to flash in a given embryo (white bars) did so at a still lower temperature. These later temperatures are somewhat higher than those observed for simultaneous flashing (Fig. 2).

With respect to the time interval between the first flashing event and the second (Fig. 5), the interval in the early morulae was 0.6 ± 0.1 sec, a much shorter time than the intervals of 10 to 18 sec in the three earlier stages. The time differences in the latter group were not significant. The temperature and time abscissas in Figures 4 and 5 are interrelated by the fact that the second factor is the volume of the supercooled component to flash in a given embryo (white bars) did so at a still lower temperature. These later temperatures are somewhat higher than those observed for simultaneous flashing (Fig. 2).

With respect to the time interval between the first flashing event and the second (Fig. 5), the interval in the early morulae was 0.6 ± 0.1 sec, a much shorter time than the intervals of 10 to 18 sec in the three earlier stages. The time differences in the latter group were not significant. The temperature and time abscissas in Figures 4 and 5 are interrelated by the fact that the second factor is the volume of the supercooled component to flash in a given embryo (white bars) did so at a still lower temperature. These latter temperatures are somewhat higher than those observed for simultaneous flashing (Fig. 2).

With respect to the time interval between the first flashing event and the second (Fig. 5), the interval in the early morulae was 0.6 ± 0.1 sec, a much shorter time than the intervals of 10 to 18 sec in the three earlier stages. The time differences in the latter group were not significant. The temperature and time abscissas in Figures 4 and 5 are interrelated by the fact that the second factor is the volume of the supercooled component to flash in a given embryo (white bars) did so at a still lower temperature. These latter temperatures are somewhat higher than those observed for simultaneous flashing (Fig. 2).

The purpose of this study was to determine whether the temperature at which intracellular ice is formed in mouse preimplantation embryos is influenced by the developmental stage. Knowledge of those temperatures is essential for computing the likelihood of IIF as a function of cooling rate. At the cooling rate used here (predominantly 20°C/min), modeling shows that mouse oocytes and embryos lose only −12% of their water osmotically during cooling to −70°C [16, Fig. 1.9]. That means that the chemical potential of water inside the cells will become increasingly higher than that of the ice and solution outside the cell. Put differently, it means that the cell water becomes increasingly supercooled. At some temperature, the supercooling can no longer be maintained. The internal contents will be nucleated, and the cells will freeze internally. There are two forms of ice nucleation. One of them is heterogeneous. It occurs as a consequence of the presence or appearance of a nucleating agent, the most effective nucleating agent being an externally added ice crystal.

The other form of nucleation is homogeneous. It occurs when, by random fluctuations, enough liquid water molecules assume the configuration of an ice crystal of sufficient size to grow spontaneously. Such a crystal is often referred to as a critical size ice embryo. Both theory and experiment show that the homogeneous nucleation temperature ($T_\text{h}$) of water is near −40°C. The exact temperature at which it occurs is affected primarily by two factors. One is the thermodynamic freezing/melting point of the system. If that is suppressed colligatively by added solutes, $T_\text{h}$ is suppressed approximately twice as much. The second factor is the volume of the supercooled...
cases (79% to 91%, depending on the developmental stage), the flashing of individual blastomeres occurred simultaneously; but in a minority of cases (9% to 21%), it occurred sequentially with a variable time interval between blastomere flashing. Morulae had the highest percentages of sequential freezing (21%). They differed even more sharply with respect to the time interval between the flashing of the first blastomere and that of the succeeding one (Fig. 5). In the morulae, that time interval was 0.6 sec. In 2–8-cell embryos, it was 10–18 sec. This difference suggests that the plasma membranes of morulae differ in significant ways from earlier stages. That in fact is the case.

Membrane Changes Associated with Development

Two important structures in the membranes of many cells are aquaporins on cell surfaces and gap junctions between tightly apposed cells in multicellular systems. As shown in Table 1, neither is present at any stage between the MII oocytes and four- to six-cell embryos. The early eight-cell embryo also shows no evidence of the presence of gap junctions or AQP 3; however, its membranes may possibly contain AQP 9 [6]. At this stage, the surfaces of the eight blastomeres are stacked on each other but not structurally joined. Later in the eight-cell stage, the membranes that form the boundary between two blastomeres now form tight junctions, and gap junctions appear. Simultaneously, AQP 3 now becomes detectable in the membranes facing the outside medium.

Gap junctions almost universally form across the dual bilayer constituting the tight junctions between cells. Most gap junctions are formed by a family of connexin proteins, and in the center of these junctions is a transmembrane pore that varies from 4 to 12 Å in radius [18] depending on the connexin that forms them. According to Sáez et al. [19], the pores are hourglass in shape. The outer surfaces have radii of 12–20 Å that narrow to 7.5 Å in the center.

Aquaporins were discovered some 17 yr ago. They too are transmembrane protein complexes that form pores in many animal and plant cells (although by no means all) in the portions of plasma membranes that are exposed to the external medium. These pores are also hourglass shaped. In AQP 1, the diameter tapers from 15 Å at the extracellular side to 2.8 Å at the constriction [20], a width that can just accommodate a single water molecule. If IF in a blastomere facing the medium is caused by contact with external ice, it is difficult to see how anything in the nature of an ice crystal could pass through the 2.8-Å neck unless some force widened the constriction. It is conceivable that external ice in its attempt to grow into the supercooled water inside the cells could provide such a force [21].

But when ice forms in one blastomere of a morula, the much wider pore in the gap junction might serve as the route by which the ice in this first blastomere nucleates the supercooled water in adjoining blastomeres. This appears to be the case on both theoretical and experimental grounds. The value of 0°C

### Table 5. Freezing point depression (ΔT) of ice in pores of 15 Å and 24 Å diameter vs. the contact angle θ.

<table>
<thead>
<tr>
<th>a (Å)</th>
<th>θ (°)</th>
<th>ΔT (Mazur) (°C)</th>
<th>ΔT (Acker*) (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.5</td>
<td>50°</td>
<td>27.9</td>
<td>33.5</td>
</tr>
<tr>
<td>12</td>
<td>50°</td>
<td>17.6</td>
<td>20.6</td>
</tr>
</tbody>
</table>

* The values are from Acker et al. [18] using either the Mazur version of the Kelvin equation [21] or the Acker modification.
for the melting point of pure ice is the value for a planar ice crystal, i.e., one with an infinite radius of curvature. However, if the ice crystal is convex, its melting point, on thermodynamic grounds, will be suppressed. This is equivalent to saying that the chemical potential of water at the surface of a convex crystal is higher than that at the surface of a planar crystal at the same temperature. The amount by which the melting point of a curved ice crystal in pure water is suppressed below that of a planar crystal is given by the Kelvin equation, namely [21],

\[
\Delta T = 2v T_m \sigma SL \cos \theta / L_f = 2v T_m \sigma SL / r L_f,
\]

where \( v, T_m, \sigma, S, L_f \), and \( L_f \) are the molar volume of water, the melting point of the planar crystal, the interfacial tension between ice and water, the contact angle between ice and the inner wall of the pore (values can be 0–90°), and the latent heat of fusion of ice, respectively. The symbols \( a \) and \( r \) are the radius of the pore and the radius of curvature of the ice crystal (Fig. 6). Values for all except \( \theta \) are known or have been estimated.

Acker et al. [18] state that \( v \) should be the molar volume of ice, not water, and that a correction term needs to be added to the equation to correct for situations where the liquid is a solution, not pure water, and they have calculated \( \Delta T \) for various pore radii and contact angles using both the original and corrected equations. Table 5 shows the calculations for pore radii of 7.5 and 12 Å, and for \( \theta = 50° \) and 75°. There is only about a 3°C difference in \( \Delta T \) with the two equations, mostly due to substituting the molar volume of ice for the molar volume of liquid water. We observed a mean flash temperature of \(-23.1°C\) for the simultaneous freezing of the morulae (Table 3), which is consistent with the \( \Delta T \) values calculated for a gap junction pore radius of 7.5 Å in Table 5.

Additional support for the role of gap junctions in cell-to-cell ice propagation is our finding that the propagation rate is slowed 6-fold by the presence of the gap junction inhibitor GA.

**Interpretation of Findings**

**One-cell through eight-cell embryos.** The mean flash temperature for the one- to eight-cell embryos that flashed simultaneously was \(-38.2°C\) (Table 3). These constituted 87% of the two- to eight-cell embryos studied. That temperature is very close to the calculated homogeneous nucleation temperature of \(-40°C\) for a volume of 1 M EG equal to the volume of water in mouse oocytes and embryos. We conclude from this that the nucleation is homogeneous; i.e., it is not heterogeneously catalyzed by external ice or intracellular nucleators. That in turn means that the plasma membranes of the blastomeres must remain intact throughout all the cooling prior to IIF. Moreover, their plasma membranes must not contain any pores capable of allowing the passage of extracellular ice and none form during cooling. This interpretation is consistent with the fact that stages two- to eight-cell embryos contain no detectable gap junctions, and stages one- to eight-cell embryos show no evidence of aquaporins in their plasma membranes (other than the possibility of AQ9 in eight-cell embryos; Table 1).

However, 13% of embryos in stages two- through eight-cell flashed sequentially; i.e., an observable time (and temperature) interval elapsed between the freezing of one blastomere and that of a neighboring one. The mean time interval was 13.5 sec (Table 5). The mean temperature difference was \(-4.8°C\). The mean temperature of the first freezing event was \(-24.5°C, -24.0°\), and \(-24.3°C\) (excluding one that froze at the EIF temperature of \(-7°C\) for two-cell, four-cell, and eight-cell embryos respectively. That is \(14°C\) higher than the mean flash temperature of \(-38.2°C\) for simultaneous flashing. The mean flash temperature for the second flash in the sequence was \(-29.1°C\). That is 5°C lower than the first flash, but still 9°C higher than the mean for simultaneous flashing.

We can only speculate as to the cause of the \(14°C\) difference between the flash temperature of the 87% of the two- to eight-cell embryos in which the blastomeres flashed simultaneously and the \(14°C\)-higher temperature of the 13% in which they flashed sequentially. Because \( T_b \) should be constant, the latter cells must be freezing heterogeneously. It is possible, but seems highly unlikely, that these 13% contained a weak nucleator whereas the other 87% did not. These stages contain no aquaporins that might permit the passage of ice from the medium and no gap junctions that would permit blastomere-to-blastomere propagation of ice. More likely, we think, is that a defect develops in the plasma membrane during the cooling of the 13%, a defect that allows external ice to pass through the membrane. Perhaps this defect is a result of forces generated by EIF. Pedro et al. [22] have reported that the ability of mouse oocytes/embryos to survive the osmotic swelling resulting from exposure to hypotonic saline solutions depends significantly on their developmental stage. One-cell embryos (zygotes) were the most resistant; eight-cell embryos and early morulae were the least resistant. Perhaps the putative defect mentioned in the previous sentence is a manifestation of that fact.

**IIF in Morulae** Flash temperatures in the 38 morulae examined fell into three groups. In the first group, three morulae flashed simultaneously at \(-40°C\) and one at \(-34°C\). The second group comprised 24 morulae that underwent simultaneous flashing between \(-14°\) and \(-26°C\). The third group consisted of two morulae that flashed simultaneously at \(-8°\) and \(-10°C\) and eight morulae that flashed sequentially at \(-6°\) and \(-8°C\). The four morulae in group 1 show that morulae are capable of cooling to \( T_b \) before nucleating. Therefore, these four possessed intact membranes during cooling and possessed no internal heterogeneous nucleators. Because the 24 morulae in the second group flash at much higher temperatures, they must be nucleating heterogeneously. We suggest the following interpretation of their behavior. One blastomere undergoes IIF at, say, \(-20°C\), perhaps because its membrane is damaged or perhaps for stochastic reasons. (Note that the range of \(-14°C\) to \(-26°C\) for morula flashing overlaps the higher end of the temperature range of flashing in the earlier stages.) At \(-20°C\), the Kelvin effect is strong enough to permit ice in that blastomere to easily pass through the gap junctions into neighboring blastomeres to initiate IIF in them.

The third and final group includes the morulae that flashed between \(-6°C\) and \(-8°C\), 80% of which flashed sequentially. It would seem more than a coincidence that these flashing events occurred right at the temperature at which extracellular freezing occurred (mean of \(-7.3°C\)). Some flashed almost immediately after EIF in ramp 2; some flashed during the warming segment of ramp 3; and some flashed during the subsequent cooling of ramp 4. If this is more than a coincidence, it suggests that external ice crystals exert a force on the morula that produces defects in the plasma membrane of one or more of the blastomeres that lead to IIF in it or them. In group 2 morulae, the temperature is low enough to permit that internal ice to propagate swiftly through gap junctions to nucleate neighboring blastomeres. But in these group 3 morulae, the temperature is \(-6°\) to \(-8°C\), and that may be marginal with respect to the existence of any ice crystal with a radius of curvature small enough to pass through the pore in a gap junction. The conclusion that the ice is propagating from one cell in a morula to others through the pores in gap junctions is supported by the
finding that GA, which reportedly closes the pores, increases the propagation time 6-fold.

NOTE ADDED IN PROOF

During the production of this paper, a paper by Seki and Mazur showing that the temperatures of intracellular ice formation of oocytes and embryos subjected to a prior vitrification are the same as those of freshly collected oocytes and embryos was accepted for publication [23].

ACKNOWLEDGMENTS

We appreciate the assistance of the following undergraduate and graduate students of Drs. Edashige and Kasai in collecting and vitrifying the oocytes/embryos sent to Knoxville: Dr. B. Jin, M. Tanaka, S. Ohta, K. Matsuo, T. Kuwano, M. Fuchiwaki, T. Kouya, T. Hara, and S. Takahashi.

REFERENCES

23. Seki S, Mazur P. Comparison between the temperatures of intracellular ice formation in fresh mouse oocytes and embryos and those previously subjected to a vitrification procedure. Cryobiology 2010; (in press).