Effect of the expression of aquaporins 1 and 3 in mouse oocytes and compacted eight-cell embryos on the nucleation temperature for intracellular ice formation

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Abstract

The occurrence of intracellular ice formation (IIF) is the most important factor determining whether cells survive a cryopreservation procedure. What is not clear is the mechanism or route by which an external ice crystal can traverse the plasma membrane and cause the heterogeneous nucleation of the supercooled solution within the cell. We have hypothesized that one route is through preexisting pores in aquaporin (AQP) proteins that span the plasma membranes of many cell types. Since the plasma membrane of mature mouse oocytes expresses little AQP, we compared the ice nucleation temperature of native oocytes with that of oocytes induced to express AQP1 and AQP3. The oocytes were suspended in 1.0 M ethylene glycol in PBS for 15 min, cooled in a Linkam cryostage to -7.0°C , induced to freeze externally, and finally cooled at $20^{\circ}\text{C}/\text{min}$ to -70°C . IIF that occurred during the $20^{\circ}\text{C}/\text{min}$ cooling is manifested by abrupt black flashing. The mean IIF temperatures for native oocytes, for oocytes sham injected with water, for oocytes expressing AQP1, and for those expressing AQP3 were -34 , -40 , -35 , and -25°C respectively. The fact that the ice nucleation temperature of oocytes expressing AQP3 was 10 – 15°C higher than the others is consistent with our hypothesis. AQP3 pores can supposedly be closed by low pH or by treatment with double-stranded *Aqp3* RNA. However, when morulae were subjected to such treatments, the IIF temperature still remained high. A possible explanation is suggested.

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Introduction

The most important factor determining the success of cryopreservation is whether a cell undergoes intracellular ice formation (IIF) during freezing. In classical slow freezing, IIF is avoided by cooling cells sufficiently slowly so that osmotic dehydration results in water remaining in near chemical potential equilibrium with the outside solution and ice. When cells are cooled too rapidly to achieve this, they become increasingly supercooled, and at some subzero temperature, they undergo IIF. In some cases, IIF occurs at or very near the lowest temperature at which the supercooled state can be maintained, the so-called temperature for homogeneous nucleation (T_h). In cells, the size of mouse oocytes that temperature is approximately -40°C (Mazur *et al.* 2005b). But in many other cases, IIF occurs at temperatures well above T_h and is said to occur by heterogeneous nucleation.

We have argued that the heterogeneous nucleator of the supercooled water in cells is external ice. One route

by which an external ice crystal might traverse the lipid bilayer membrane is through pores in preexisting transmembrane proteins. One family of such proteins that are constitutively present in the membranes of many cell types and that contain central pores are the aquaporins (AQPs). In AQP1, and probably in the others, the pore is hourglass shaped and the diameter at the neck is so narrow that the pore should not allow the passage of more than one molecule of water at a time, much less allow the passage of the assembly of ice molecules that constitute even a tiny ice crystal. But, we consider it possible that the force generated by ice crystal growth might be sufficient to enlarge the pore.

No constitutive AQPs have been found in mouse oocytes or in mouse embryos ranging from the one-cell (zygote) through the uncompact eight-cell (although the eight-cell may possibly express traces of AQP9; Barcroft *et al.* 2003, Matsuo *et al.* 2008). However, Edashige *et al.* (2003) have developed a molecular procedure for inducing the expression of AQP3 in mouse

oocytes. They have achieved similar results with oocytes of *Xenopus* and fish, which also lack constitutive AQPs (Valdez *et al.* 2006, Yamaji *et al.* 2006, Seki *et al.* 2007). The first line of evidence that the Edashige *et al.* procedures resulted in the expression of functional AQP3 in mouse oocytes was a several-fold increase in the permeability of the oocytes to water and to the low molecular weight nonelectrolyte solutes glycerol and ethylene glycol (EG). AQP3 is a glyceroaquaporin. It increases water permeability (L_p) somewhat with low activation energy (E_a), but mostly it enhances the permeability of low molecular weight nonelectrolytes like glycerol and EG. The second piece of evidence was that immunofluorescence showed the AQP3 protein to be localized at the plasma membrane. The procedure was the same as that described here. In the present paper, using the same two criteria, we report a similar procedure for obtaining the functional expression of AQP1 in mouse oocytes.

Compacted eight-cell embryos (=early morulae) are different from oocytes in that they express AQP3 natively (Barcroft *et al.* 2003, Edashige *et al.* 2006, 2007). As evidence of this, Edashige *et al.* (2006) found their L_p to be some fivefold greater than that of oocytes.

In the process of compaction, the blastomeres of the eight-cell embryo are transformed from an assembly of independent stacked cannon balls to a 'tissue' in which contiguous plasma membranes between two blastomeres form tight junctions and gap junctions (Valdimarsson & Kidder 1995).

We (Seki & Mazur 2010a) have recently determined that the IIF temperature in mouse oocytes and in embryos ranging from zygotes to uncompact eight-cell embryos is essentially the same and very low (-35 to -43 °C). However, in compacted eight-cell (early morulae), it is -23 °C. The fact that the abrupt ~ 17 °C rise in the IIF temperature occurs at exactly the same developmental stage as the appearance of functional AQP3 and gap junctions strongly suggests a cause and effect relationship.

A central aim of this research was to elucidate whether extracellular ice can traverse the plasma membrane through AQP pores and can act as the heterogeneous nucleator of the supercooled water in cells. To achieve this, we have compared the ice nucleation behavior of normal mouse oocytes (which lack detectable AQP channels) with that of oocytes induced to express AQP3 or AQP1 and with that of early morulae, which natively express AQP3, but not AQP1.

Results

The detection of intracellular AQP3 and AQP1 proteins by immunofluorescence

Edashige *et al.* (2003, 2007) demonstrated by immunofluorescence that after exogenous expression procedures, AQP3 proteins were located in the plasma

membrane of mouse oocytes at metaphase II stage. We have now demonstrated the same in AQP1. Figure 1 shows that AQP1 protein has been synthesized in the cell and is preferentially located at or near the plasma membrane. Although we can confirm the expression of AQP1 protein in the cytoplasm near the plasma membrane, we could not clearly confirm it to be in the plasma membrane. However, their permeability to water is 2.99 ($\mu\text{m}/\text{min}$ per atm), which is four to five times higher than that (0.70) in intact oocytes (Table 1), thus strongly indicating that AQP1 protein is located in the plasma membrane.

The L_p of oocytes expressing AQP1 or AQP3

The L_p of each oocyte that had been injected with AQP1 or Aqp3 cRNA was determined as indicated in the Materials and Methods section. The L_p of normal untreated oocytes and morulae was measured on randomly selected samples. The results are summarized in Table 1. The L_p of the AQP1- and AQP3-expressing oocytes was $4\times$ and $9\times$ than that of controls respectively. The difference is probably more a matter of differences in the number of AQPs inserted in the plasma membranes than of inherent differences in the L_p of each AQP assembly.

The effect of AQP1 and AQP3 expression on the temperature of IIF in mouse oocytes

Table 2 shows the IIF temperatures of mouse oocytes that were induced to express AQP1 or AQP3. The IIF temperature of water-injected control oocytes (-40.4 °C) and that of the oocytes injected with AQP1 cRNA and expressing AQP1 (-34.8 °C) were not significantly different from that of normal control oocytes (-34.4 °C). But, the IIF temperature of oocytes induced to express AQP3 (-24.5 °C) was

Figure 1 The detection of AQP1 proteins in oocytes by immunofluorescence. Mouse oocytes at the germinal vesicle stage were injected with cRNA of human AQP1 and cultured for 12–14 h. The expression of AQP1 was detected by immunofluorescent-staining using anti-rat AQP1 rabbit antibody and FITC-conjugated anti-rabbit immunoglobulin goat antibody. (A) An uninjected oocyte (right) and a cRNA-injected oocyte (left); (B) the same two cells under the fluorescence microscope. The bar indicates $50\ \mu\text{m}$. Edashige *et al.* (2003, 2007) have obtained analogous immunofluorescence data for AQP3 proteins in mouse oocytes.

Table 1 Comparison of the water permeability (Lp) at 25 °C of mouse oocytes expressing AQP1 or AQP3, or morulae embryos versus the Lp of untreated oocytes and water-injected oocytes.

Stage	Injection	Lp ($\mu\text{m}/\text{min}$ per atm)	n
MII oocyte	None	0.70 ± 0.03	20
	Water ^a	0.67 ± 0.20	11
	AQP1 cRNA ^a	2.99 ± 0.94	10
	Aqp3 cRNA ^a	6.29 ± 0.79	22 ^b
Morulae	None	3.25 ± 0.32	6

Edashige *et al.* (2003, 2006) have reported similar values of Lp for these stages and conditions, namely, 0.83, 0.87, and 3.09 for control, water-injected, and Aqp3 cRNA-injected oocytes and 3.93 for uninjected morulae.

^aWater, AQP1 cRNA, or Aqp3 cRNA were injected into oocytes at the germinal vesicle stage and then the oocytes were cultured to the MII stage. ^bLp of two other oocytes were extremely high (50, 94) and were not included in these data.

about 10 °C higher than that in the previous group ($P < 0.05$) and 16 °C higher than that in the water-injected control.

The effect of AQP3 closure in acidic condition on the temperature of IIF in mouse oocytes

Table 3 shows the effects of acidifying the EG/PBS medium on the IIF temperatures of normal oocytes and of oocytes induced to express AQP3. The IIF temperature of the untreated controls is about 14 °C below that of the oocytes expressing AQP3, but there is no effect of pH. This means that acidic conditions did not make the oocytes more fragile and did not affect the IIF temperature. If the closure of AQP3 affects the IIF temperature, one would expect the IIF temperature of AQP3-expressing oocytes at pH 5.7 to be lower than that at pH 7.2. But that was not the case.

The effect of pH on the permeability of mouse morulae to water and cryoprotectants

Figure 2 shows the permeabilities to water in morulae at pH 7.2, 6.0, 5.8, 5.5, and 5.3. The Lp at pH 7.2 was 4.08 ($\mu\text{m}/\text{min}$ per atm), about equal to the Lp of oocytes forced to express AQP3 (Table 3). As the pH of medium was lowered to 5.3, the Lp underwent a fourfold decrease to 0.99 $\mu\text{m}/\text{min}$ per atm. Thus, in morulae, lowering the pH to 6.0 or lower produces a three- to four-fold decrease in Lp (Fig. 2) but no decrease in the IIF temperature (Table 3).

Figure 3 shows analogous data on the effects of pH on the permeability coefficient (Ps) for EG and glycerol in mouse morulae. There is little or no effect of pH on the Ps for glycerol, but there is a large effect on the Ps for EG, namely, the permeability is approximately doubled in going from pH 5.8–7.2 to pH 5.3. This is the opposite of the effect of pH on the Lp of the morulae.

The use of double-stranded Aqp3 RNA to suppress the normal expression of AQP3 in mouse morulae and its effect on the IIF temperature of the morulae

The native early morula expresses AQP3, and Table 1 shows that its Lp is 3.25 $\mu\text{m}/\text{min}$ per atm. This is five times higher than that of the MII oocyte, which does not express AQP3. We have recently shown (Seki & Mazur 2010a) that the ice nucleation temperature of the early morula is also about 12 °C higher than that of the MII oocyte (−23 vs −35 °C). Edashige *et al.* (2007) have shown and we have confirmed that if zygotes are injected with double-stranded (ds) Aqp3 RNA instead of the standard single-stranded form, and allowed to develop, the resulting morulae appear not to express AQP3 as evidenced by a threefold drop in Lp from 3.25 to 1.05 $\mu\text{m}/\text{min}$ per atm, a value close to that of oocytes.

These findings indicate that with ds-Aqp3 RNA, AQP protein is either not incorporated into the plasma membrane or does not form the usual membrane pores. In either case, one would expect that if the pores are absent, they cannot serve as a route for seeding by extracellular ice, and the ice nucleation temperature of the morulae should drop from −23 °C toward −35 °C. But the experiments summarized in Table 4 indicate that this is not the case. The nucleation temperature of the native morula is −23.1 °C and that of morulae derived from ds-cRNA-treated zygotes is −19.4 °C – essentially unchanged. We shall return to this apparent anomaly in the Discussion section.

Discussion

We reported (Mazur *et al.* 2005b) that IIF in mouse oocytes suspended in 1 M EG in PBS occurs at −35 °C. In a more recent paper (Seki & Mazur 2010a), we have shown that the IIF temperatures of one-cell, two-cell, four- to six-cell, and early eight-cell embryos range from −37 to −43 °C. These temperatures indicate that nucleation of the intracellular supercooled water in these stages is occurring homogeneously, i.e. by the stochastic formation of a critically sized ice embryo that can grow spontaneously. However, the IIF nucleation temperature of compacted eight-cell embryos (early

Table 2 Intracellular flashing temperature of mouse oocytes injected with AQP1 cRNA or Aqp3 cRNA.

Stage	Injection	Flash temperature $\pm$ S.E.M. (°C) ^a	n
MII stage	None	$-34.4 \pm 1.7^*$	57
	Water ^b	$-40.4 \pm 1.3^*$	12
	AQP1 ^b	$-34.8 \pm 2.1^{*+}$	10
	Aqp3 ^b	$-24.5 \pm 2.4^+$	11

Values with different subscripts were significantly different ($P < 0.05$).

^aOocytes suspended in 1 M EG/PBS and subjected to the cooling procedures shown in Table 5. ^bWater, AQP1 cRNA, or Aqp3 cRNA were injected into oocytes at the germinal vesicle stage and the oocytes were cultured to the MII stage.

Table 3 Intracellular flashing temperature of *Aqp3* cRNA-injected mouse oocytes at pH 5.7 vs 7.1.

Stage	Injection	pH	Flash temperature ± S.E.M. (°C) ^a	n
MII stage	None	7.1	−34.4 ± 1.7	57
	None	5.7	−39.3 ± 1.2	10
	<i>Aqp3</i> ^b	7.1	−24.5 ± 2.4	11
	<i>Aqp3</i> ^b	5.7	−24.9 ± 2.9	11

There are no differences between the flash temperatures at pH 5.7 and those at pH 7.1.

^aOocytes suspended in 1 M EG/PBS and subjected to the cooling procedures shown in Table 5. ^b*Aqp3* cRNA was injected into oocytes at the germinal vesicle stage and the oocytes were cultured to the MII stage.

morulae) is markedly higher, namely, −23.1 °C. The developmental stage at which this marked rise in nucleation temperature occurs coincides with the appearance of AQP3 and gap junctions and is presumably causally related to one or both of these events.

Since the nucleation of oocytes or of one- to eight-cell embryos is occurring by homogeneous nucleation, we need not postulate the presence of heterogeneous nucleators or the presence of transmembrane pores through which external ice can grow. But there are challenges in accounting for the much higher nucleation temperatures observed in early morulae and in explaining how ice propagates from one blastomere to another in the morulae. There is considerable evidence that the blastomere to blastomere propagation occurs through the pores in gap junctions that form in the tight junctions between the plasma membranes of contiguous cells. Seki & Mazur (2010a) have presented such evidence in mouse morulae. Acker *et al.* (2001) and Irimia & Karlsson (2002) have presented the evidence in tissue culture cells. According to Saez *et al.* (2003), the pores in gap junctions are hourglass in shape. The outer surfaces have radii of 12–20 Å that narrow to 7.5 Å in the center. Thus, the diameter of the gap junction pore is sufficient to allow such propagation (Seki & Mazur 2010a). In contrast, gap junctions would not be expected to provide a route for the penetration of external ice into the supercooled cytoplasm because they are not present in the single plasma membrane that borders the external compartment.

Conversely, one would not expect AQPs to play a role in the propagation of intracellular ice between individual blastomeres since presumably they are not present in these tight junctions. But they might play a role in the passage of ice from the partly frozen external medium into the supercooled cell interior if they are present in the portion of the plasma membrane of the cell that faces that medium. In Introduction section, we mentioned that, if unaltered, the 2.8 Å (Sui *et al.* 2001) constriction in the pore of AQP1 would be too small to allow the passage of any ice crystal; however, it is possible that it could be enlarged by the forces generated by the growth

of the external ice. While the diameter of the constriction in the pore in AQP3 has not been ascertained, it is believed to be 3.8 Å (King *et al.* 2004); consequently, the same considerations apply.

Evidence that AQP3 channels serve as routes by which external ice can pass through the plasma membrane and cause intracellular ice nucleation

AQP3 has not been found in the membranes of oocytes or of one- to eight-cell embryos. In all these stages, intracellular ice nucleation occurs at or near the homogenous nucleation temperature (Seki & Mazur 2010a). This indicates that IIF is occurring spontaneously without the need to invoke extracellular ice as the nucleating agent.

But AQP3 is present in the plasma membranes of compacted eight-cell embryos (=early morulae; Barcroft *et al.* 2003, Edashige *et al.* 2006, 2007). These morulae exhibit the essential characteristics of membranes with functioning AQPs, namely 1) *Lp* is increased some tenfold over that in earlier stages and 2) the *Ea* of *Lp* is decreased from 12 to 4 kcal/mole. Furthermore, the ice nucleation temperatures rise from −43 to −35 °C for precompaction embryos and MII oocytes to −23.1 °C for morula (Seki & Mazur 2010a; Table 3). This rise indicates that ice nucleation in the supercooled morulae is heterogeneous and that external ice is the likely nucleator.

Although AQP3 is not present in the native oocyte, Edashige *et al.* (2003) have succeeded in expressing it, as evidenced by the immunofluorescent location of the AQP3 protein in the membrane, and by the high *Lp* of such oocytes. Furthermore, we show in Table 4 that the intracellular ice nucleation temperature of these oocytes is 10–15 °C higher than that of untreated oocytes (−24.5 °C vs −34 to −40 °C) and is essentially identical to the nucleation temperature of morulae (−23 °C), which as noted, possess AQP3 endogenously. This rise in nucleation temperature supports the view

Figure 2 Water permeability (*Lp*, μm/min per atm) of early mouse morulae at pH 7.2, 6.0, 5.8, 5.5, and 5.3. The error bars are S.D. of the mean. Values with different letters are significantly different ($P < 0.05$). The numbers of embryos were 9–11 for each bar.

Figure 3 Permeability (P_s , $\times 10^{-3}$ cm/min) of mouse morulae to glycerol and ethylene glycol at pH 7.2, 6.0, 5.8, 5.5, and 5.3. The number of embryos was 2–7 for each bar.

that AQP3 pores may permit external ice to pass into the supercooled cytoplasm.

In contrast, the incorporation of AQP1 pores in the oocyte membranes had no effect on the IIF temperature. The temperatures were -34.8°C for them, -34.4°C for untreated oocytes, and -40.4°C for oocytes sham injected with water (Table 4). All three temperatures are close to the homogeneous nucleation temperature. Since AQP1 has been shown to be incorporated in the plasma membrane of oocytes induced to express it (e.g. it increases L_p some four times (Table 3)), and since this incorporation does not raise the nucleation temperature above T_h , it means that whatever membrane distortion, if any, is associated with the incorporation of AQP1 pores, it does not change the nucleation temperature. We posit that this is also true for AQP3, that is, that the 10 – 15°C rise in nucleation temperature is not due to the production of distortions or defects in the plasma membrane from its incorporation but is due to the ability of the larger AQP3 pore to allow the permeation of external ice.

Consequences of exposure to AQP inhibitors on the IIF temperature

Inhibition by acidic pH

Zeuthen & Klaerke (1999) have reported that acidic conditions ($\text{pH} \leq 6$) have no effect on water flow through AQP1 pores expressed in *Xenopus* oocytes. In contrast, acidic conditions produce a near total abolition of water flow through AQP3 pores induced in *Xenopus* oocytes. If low pH has the same effect on AQP3 pores artificially expressed in mouse oocytes, one might expect the nucleation temperature of AQP3-expressing oocytes to drop from the -24.5°C shown in Table 3 for pH 7.1 to near -40°C if the pH were lowered to 5.7. Clearly, that did not happen. One reason for the discrepancy is suggested from the effect of acid conditions on the L_p of early morulae shown in Fig. 2. Acid conditions clearly decrease the L_p , but they do not reduce it to zero.

It remains about 25% of the control value at pH 7.2. This could either mean that 25% of the AQP3 pores are still open at say pH 5.3–5.8 and 75% are completely closed, or it could mean that the pores in 100% of the AQPs are 75% closed, or it could be some percentage in between. In theory, it only takes ice propagation through a single pore of proper dimensions to initiate IIF, and the membrane contains thousands of AQP3 pores. As mentioned, the structure of the AQP3 channel has not yet been determined, but the narrow constriction is believed to be about $1 \text{ \AA}$ wider than that in AQP1 (Sui *et al.* 2001, King *et al.* 2004).

Inhibition by ds-Aqp3 RNA

The expression of functional AQP3 in morulae can also be inhibited by treating zygotes with ds-RNA. We find that it does so based on its reducing the permeability of the morulae to water threefold – from $3.25 \mu\text{m}^3/\mu\text{m}^2$ per atm (Table 1) to 1.05 ± 0.06 ($n=7$). But as is the case with acidification, there is no corresponding decrease in the intracellular ice nucleation temperature. It is -23.1°C in native morulae and -19.4°C in morulae previously treated with ds-Aqp3 cRNA (Table 4).

Thus, both acidification and treatment with ds-Aqp3 RNA inhibit the functional expression of AQP and both produce a substantial reduction in the L_p of morulae. But neither produces any lowering of their ice nucleation temperature. The answer to this apparent paradox is, we believe, the following. With cells possessing AQP pores in their plasma membranes, the overall permeability to water depends on the ratio of the aggregate cross-sectional area of all the pores to the area of the lipids in the bilayer and to the relative permeability per unit area of each component. The L_p values in Table 1 suggest that water permeation through the bilayer is $0.7 \mu\text{m}^3/\mu\text{m}^2$ per min per atm and that through AQP3 channels in the morula is $6.29 - 0.7 = 5.6 \mu\text{m}^3/\mu\text{m}^2$ per min per atm. When treated with ds-Aqp3 RNA, the permeability of water through AQP3 channels drops to $1.05 - 0.7 = 0.35 \mu\text{m}^3/\mu\text{m}^2$ per min per atm. In other words, with this simplistic analysis, treatment with ds-Aqp3 RNA reduces the permeability to water through AQP3 pores to $0.35/5.6$, or 6% of normal. In this scenario, if the surface membranes of the blastomeres of a morula normally contain 100 000 functional AQP3 pores, treatment with

Table 4 Intracellular flashing temperature of mouse morulae developed from zygotes injected with ds-Aqp3 RNA.

Stage	Injection	Flash temperature $\pm$ S.E.M. ($^\circ\text{C}$)	<i>n</i>
MII stage	None	$-34.4 \pm 1.7^*$	57
Morulae	None	$-23.1 \pm 1.5^\dagger$	30
	ds-Aqp3 RNA	$-19.4 \pm 1.5^\dagger$	7

Values with different superscripts were significantly different ($P < 0.05$). ds-Aqp3 RNA was injected into zygotes and the embryos were cultured to the morulae stage.

ds-*Aqp3* RNA would reduce this to 6000 fully functional pores (Alternatively, one could hypothesize that all 100 000 pores have their properties changed to reduce their L_p to 6% of normal.).

We argue that the ability of AQP3 pores to permit the passage of extracellular ice and the ice nucleation of supercooled cytoplasm has a very different functional relationship than does L_p to the number of functional pores present. The ability of a pore to permit the passage of external ice ought to depend much more on the diameters of the pores than on their number. At the limit, only one pore of appropriate diameter is necessary for ice nucleation. Continuing with our hypothetical example, we argue that the 6000 residual functional pores in the morula treated with ds-*Aqp3* RNA will permit nucleation at the same temperature as 100 000 pores.

As mentioned, the structure of the AQP3 channel has not yet been determined by X-ray diffraction. However, the narrow constriction is believed to be about 1 Å wider than that in AQP1 (Sui *et al.* 2001, King *et al.* 2004). That is consistent with the fact that AQP3 is a glyceroaquaporin and as such it also enhances the permeation of EG and glycerol. Figure 3 shows an interesting and so far unexplained result, namely, in sharp contrast to its effect on L_p , acidity increases the permeability of mouse morulae to EG. Strangely, it has no effect on glycerol permeation.

Inhibition by mercuric compounds

More than 50 years ago, long before the discovery of the water channel AQP1 and its high abundance in human erythrocytes, Paganelli & Solomon (1957) reported that the hydraulic conductivity of these cells was very high. Thirteen years later, Macey & Farmer (1970) found that this very high L_p was reduced by a factor of 10 by exposure to mercuric compounds. Later, it was found that mercuric compounds interacted with the AQPs.

Consequently, we tried to use HgCl_2 to inhibit water permeation through AQP3 pores expressed in mouse oocytes. Normal oocytes (which lack AQP pores) were supercooled to -35°C in 1 M EG, but when they were treated with HgCl_2 , they underwent IIF immediately after extracellular ice formation (EIF) at about -7°C . Clearly, the HgCl_2 exposure caused major damage to the plasma membranes. We did not attempt experiments with other mercuric compounds.

Conclusions

When mouse MII oocytes in 1 M EG or glycerol are rapidly cooled at $20^\circ\text{C}/\text{min}$, they do not undergo IIF until -35 or -40°C even though ice formed in the outside medium at about -7°C . This means that the cell interior supercools some 30°C before it freezes. This in turn has to mean that the plasma membrane prevents external ice from crossing it. Thus, IIF occurs by

homogeneous nucleation. The above also applies to all embryonic stages from the zygote to the early eight-cell. But it does not apply to compacted eight-cell embryos (early morulae). The reason, we suggest, is that the plasma membranes in this latter stage natively express AQP3 and the central pore in that protein permits the passage of external ice. This hypothesis is supported by the fact that the IIF temperature in the early morula rises from the approximately -40°C in earlier stages to -23°C . The mouse oocyte does not natively express AQPs, but it can be induced to do so. When it is induced to express AQP3, its IIF temperature rises to -24.5°C , about the same as with the morulae.

One would expect that if the expression of AQP3 causes the oocytes/morulae to undergo IIF at markedly higher temperatures, the inhibition of AQP expression would have the opposite effect, i.e. it would lower the IIF temperature. That turned out not to be the case. Acidification and the use of ds-*Aqp3* RNA have both been shown to substantially reduce L_p , but neither produces the expected result of lowering the ice nucleation temperature. We speculated earlier in this discussion as to why this might be the case.

Also, as mentioned earlier, another type of pore forms in compacted eight-cell embryos and morulae, namely, the central pore in the gap junction that forms across the dual bilayer constituting the tight junctions between blastomeres. As discussed in our recent paper (Seki & Mazur 2010a), there is accumulating evidence that the cell-to-cell propagation of intracellular ice occurs through the pores in the gap junctions in mouse morulae. But the initial intracellular freezing event in multicellular systems occurs through the portions of the plasma membranes that face the freezing external medium and that portion does not contain fully formed gap junctions. Nor can IIF occur through the channels in gap junctions of single cells, for single cells lack gap junctions.

Materials and Methods

Creating AQP1- and AQP3-expressing oocytes requires special micromanipulation and injection equipment and considerable experience in these techniques and the relevant molecular biology, both of which were available in Dr Edashige's laboratory in Japan but are lacking in Knoxville. Consequently, to determine their ice nucleation behavior, it was necessary to ship the oocytes/embryos from Japan to Knoxville in the vitrified state. Since the AQP-expressing oocytes were to be vitrified in Japan, we considered it essential that at least initially the control oocytes be from the identical source and be subjected to vitrification in the same laboratory. We have since reported in some detail our evidence that in terms of osmotic behavior, plasma membrane integrity, and ice nucleation behavior the control oocytes used in our experiments were not affected by their previous vitrification (Seki & Mazur 2010b).

Collection of oocytes/embryos

Approximately half of the control oocytes and morulae used in this study were collected in Japan and vitrified before shipment to Tennessee. The other half were collected in our laboratory at Tennessee and used without prior vitrification.

Mature female mice of the ICR colony were induced to superovulate with i.p. injections of 5 IU equine chorionic gonadotropin (eCG; Sigma) and 5 IU human chorionic gonadotropin (hCG; Sigma) given 48 h later. For the collection of embryos, females were mated with mature males of the same strain.

To obtain oocytes at metaphase II, matured oocytes surrounded by cumulus cells were collected from the ampullar portion of the oviducts 13 h after hCG injection and were freed from cumulus cells by suspending them in modified PBS (PB1; Whittingham 1971) containing 0.5 mg/ml hyaluronidase followed by washing with fresh PB1 medium (non-injected oocytes).

The preparation of the mouse metaphase II oocytes that express AQP1 or AQP3 artificially was carried out in Kochi University, Japan. Oocytes at the germinal vesicle stage (immature oocytes) were obtained by puncturing the follicles on the ovaries of female mice without injection of hCG, 46–50 h after the injection of eCG. Then, these oocytes were injected with water (control), AQP1 cRNA, or Aqp3 cRNA and cultured for 12 h until they matured to the metaphase II stage as described previously.

To obtain normal untreated embryos, mature female ICR mice injected with eCG and hCG were mated with male ICR mice. Oviducts of mated animals were flushed with PB1 medium 25 h after the injection of hCG and one-cell embryos collected and cultured for 56–68 h until they developed to the morulae stage.

All experiments were approved by IACUC protocol 911-0607 of University of Tennessee and the Animal Care and Use Committee of College for Kochi University.

Preparation of AQP1 and AQP3 cRNA

Human AQP1 cDNA was cloned at Kochi University from commercial plasmid (American Type Culture Collection, Manassas, VA, USA; ATCC number, 99583) and rat Aqp3 cDNA was cloned from rat kidney cDNA by PCR as described previously (Edashige *et al.* 2003, 2007, Valdez *et al.* 2006, Yamaji *et al.* 2006, Seki *et al.* 2007). The sense strands for AQP1 cDNA cloning was 5'-CGGAATTCAG-CATGGCCAGCGAGTTC-3' and the antisense strand was 5'-CGTCTAGATCTATTGGGCTTCATCTCC-3' (underlined sequences indicate inserted EcoRI and XbaI sites respectively). The sense strands for Aqp3 cloning was 5'-CGGAATTC-CATGGGTGACAGAAAGGAGTT-3' and the antisense strand was 5'-GCTCTAGAGATCTGCTCCTTGCTTCA-3' (underlined sequences indicate inserted EcoRI and XbaI sites respectively). These primers were derived from the human AQP1 sequence (Moon *et al.* 1993; GenBank Accession No. U41517) and the rat Aqp3 sequence (Echevarria *et al.* 1994; GenBank Accession No. L35108). The following profile was used for PCR: 30 cycles of 94 °C for 1.0 min, 58 °C for

0.5 min, and 72 °C for 1.0 min. The PCR product included the open reading frame of human AQP1 or rat Aqp3. The fragments of the PCR product was subcloned into the EcoRI/XbaI site for AQP1 or Aqp3 of a pTNT vector (Promega). Capped cRNA of AQP1 or Aqp3 were synthesized using SP6 polymerase (Takara, Otsu, Japan) after digestion of the constructed plasmid DNA by DraIII (New England Biolabs, Ipswich, MA, USA) or BamHI (Takara) respectively. The linearized plasmid DNA was digested using a DNase I Kit (Ambion, Austin, TX, USA), and the resulting capped RNA was further purified with an RNeasy Mini Kit (Qiagen). The synthesized cRNA was resuspended in RNase-free water (1 pg/pl).

Preparation of ds-Aqp3 RNA

As described in Edashige *et al.* (2007), to synthesize Aqp3 dsRNA, the cDNA of mouse Aqp3 was cloned from mouse kidney cDNA by PCR with the sense primer 5'-TAATACGACT-CACTATAGGGTCTCGGGTCTTGCGCTA-3' and antisense primer 5'-TAATACGACTCACTATAGGGGACACAGGGAGCG-GTTTA-3' (underlined are the T7 promoter sequences). These primers were derived from the mouse Aqp3 sequence (Ma *et al.* 2000). The PCR was conducted for 35 cycles of 94 °C for 30 s, 62 °C for 30 s, and 72 °C for 30 s. The PCR product contained the open-reading frame of Aqp3 sequence. From this product, the dsRNA of Aqp3 was synthesized with the T7 RibomAX Express RNAi System (Promega), according to the manufacturer's instructions.

Microinjection of AQP1 or Aqp3 cRNA into mouse oocytes or Aqp3 dsRNA into mouse zygotes

About 20–40 oocytes at the germinal vesicle stage were placed in a 200 µl drop of PB1 medium covered with paraffin oil in a Petri dish (90×10 mm). Then, an oocyte was held with a holding pipette connected to a micromanipulator on an inverted microscope and injected with 2–5 pl water (control), AQP1 cRNA, or Aqp3 cRNA solution (1 pg/pl) with an injection needle connected to another micromanipulator. Then, the oocytes were cultured at 37 °C in a humidified CO₂ incubator (5% CO₂ in air) for 12–14 h in modified Eagle medium (11090-081; Gibco BRL) supplemented with 10% FCS (26140-079; Gibco BRL), 50 µg/ml sodium pyruvate, 2 mM glutamine, 60 µg/ml penicillin G, and 50 mg/ml streptomycin (maturation medium). Oocytes without the germinal vesicle and having a polar body after culture were considered matured and were used as water- (control), AQP1 cRNA- or Aqp3 cRNA-injected oocytes. As another control, we used non-injected oocytes. Only a limited number of matured oocytes were used in each experiment because the culture period for maturation needed to be limited to 12–14 h in order not to use matured but aged oocytes.

To prepare AQP3-suppressed morulae, an embryo at the one-cell stage was held with a holding pipette connected to a micromanipulator on an inverted microscope in PB1 medium and injected with 2–10 pl water (control) or Aqp3 dsRNA solution (1 g/l) with an injection needle connected to another micromanipulator. As an additional control, noninjected

embryos were used. The injected embryos with normal shape just after injection were cultured in M16 medium that was supplemented with 10 μ M EDTA, 1 mM glutamine, and 10 μ M β -mercaptoethanol (Pedro *et al.* 1997) in a humidified CO₂ incubator at 37 °C (5% CO₂ and 95% air) for 56–68 h, until they developed to the morula stage. Only compacted eight-cell embryos or early morulae were used in the experiments.

Confirmation of AQP-expression in oocytes

Evidence that a cell is expressing functional AQPs in its membrane is of two sorts. One is to demonstrate by immunofluorescence that the AQP protein has been synthesized in the cell and is preferentially located at or near the plasma membrane. In previous study, we confirmed that the injection of rat *Aqp3* cRNA made oocytes express AQP3 protein (Edashige *et al.* 2003). The other is that the treated cell possesses a Lp up to 10 $\times$ higher than that exhibited by a cell that does not express AQPs, and that the Ea of that Lp is about 4 kcal/mole as opposed to the usual 12 kcal/mole exhibited by a cell that lacks AQP channels. Since AQP3 is a glyceroaquaporin, an additional characteristic of its expression is that the permeability of the cell to EG or glycerol is substantially higher than that of a cell that lacks AQP3.

Detection of AQP3 and AQP1 by immunofluorescence

The expression of AQP3 in mouse oocytes has been confirmed immunologically in the papers of Edashige *et al.* (2003, 2007). To determine whether AQP1 is similarly expressed, we tested for the presence of the protein by immunofluorescent staining using a commercially available anti-human AQP1 rabbit antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA; sc-20810) that cross-reacts with human AQP1. This protocol is based on the method in papers (Barcroft *et al.* 2003, Edashige *et al.* 2006, 2007). After dezonation with acidic Tyrode's buffer at pH 2.5, noninjected and AQP1 cRNA-injected oocytes were fixed with a 2% paraformaldehyde solution in Dulbecco's Ca, Mg-free PBS (PBS (–), Gibco) at room temperature for 30 min, and washed with PBS (–) containing 5 mg/ml BSA. The samples were then incubated with PBS (–) containing 0.01% Triton X-100 (Invitrogen, HFH10), 0.1 M lysine (Sigma), and 1% non-immune goat serum (Sigma, G9023) at room temperature for 30 min. After rinsing with PBS (–) containing 5 mg/ml BSA, the oocytes were incubated with anti-human AQP1 rabbit antibody at 1:100 dilution in antibody dilution/wash buffer (ADB solution; 0.1 M lysine, 0.005% Triton X-100, 1% normal goat serum in PBS (–)) at 4 °C for 8 h. After washing with ADB solution, oocytes were incubated with diluted FITC-conjugated anti-rabbit immunoglobulin goat antibody (1:100 dilution, Santa Cruz Biotechnology, sc-2012, 200 mg/0.5 ml) in ADB solution at 25 °C for 2 h, washed with ADB solution, and observed under a fluorescence microscope for the location and intensity of the fluorescence.

Evidence of functioning AQPs from measurements of water and cryoprotectant permeability

To confirm the expression of AQP1 and AQP3, the permeability to water of each treated oocyte and embryo was determined by measuring the rate of its shrinkage after transfer from isotonic PB1 medium to PB1 medium that contained 0.43 M sucrose at 25 °C for 5 min (Pedro *et al.* 1997, Edashige *et al.* 2003, 2006, 2007). The osmolality of sucrose was calculated to be 0.50 Osm/kg from the published data on its colligative properties in aqueous solutions (Wolf *et al.* 1970, Kleinhans 1998, Kleinhans & Mazur 2007). The osmolality of PB1 medium (isotonic buffer: 0.30 Osm/kg) was measured with a freezing point depression osmometer (OM801; Vogel, Giessen, Germany). The total osmolality of the hypertonic sucrose/PB1 solution was 0.80 Osm/kg.

Acidic conditions are reported to close the pores of AQP3 expressed in *Xenopus* oocytes (Echevarria *et al.* 1994). To confirm this in mouse, the permeabilities of mouse morulae to water, glycerol, and EG were determined at pH 7.2, 6.0, 5.8, 5.5, and 5.3. After exposure to PB1 at those pHs for 5 min, the embryos were exposed to PB1 media including 0.43 M sucrose, 8% (v/v) EG, or 10% (v/v) glycerol at each pH. The permeabilities were determined from the recorded volume change as described previously (Pedro *et al.* 1997, 2005, Edashige *et al.* 2003, 2006, 2007).

Each oocyte or embryo was placed in a 100 μ l drop of PB1 medium and covered with paraffin oil in a Petri dish (90 $\times$ 10 mm) and was held with a holding pipette (outer diameter of 80–120 μ m) that was connected to a micro-manipulator on an inverted microscope. The temperature of the paraffin oil that covered the drops of PB1 containing sucrose or a cryoprotectant was taken as the temperature of the solution and was maintained at 25 $\pm$ 1 °C by controlling the temperature of the room. The oocyte or embryo held with the holding pipette was then covered with a covering pipette that had a larger inner diameter ($\sim$ 200 μ m) and that was connected to another micromanipulator. Then, by sliding the dish, the oocyte or embryo was introduced into a drop of measurement solution (PB1 medium that contained sucrose, EG, or glycerol). By removing the covering pipette, the oocyte or embryo was abruptly exposed to the new solution (Edashige *et al.* 2003, 2006, Pedro *et al.* 2005).

The hydraulic conductivity (Lp) and cryoprotectant permeability (Ps) were calculated from the measured rate of shrinkage (or swelling) of oocytes or embryos after transfer from isotonic PB1 medium to the anisotonic measurement solution at 25 °C for 5 min (PB1 medium containing sucrose) or 10 min (PB1 containing a permeable cryoprotectant). The microscopic image of the oocytes/embryos during exposure to the measurement solution was recorded by a time-lapse videotape recorder (ETV-820; Sony, Tokyo, Japan) every 0.5 s for 5 or 10 min. The cross-sectional area of oocytes/embryos was measured using an image analyzer (VM-50; Olympus, Tokyo, Japan). This was expressed as a relative cross-sectional area, *S*, by dividing it by the area of the same oocyte in isotonic PB1 medium. The relative volume was obtained from $V = S^{3/2}$. The values of Lp and Ps were considered to be the values that provided the best fit of the classical Lp equation and the solute permeability equation to the experimentally determined values

Table 5 Linkam cryostage cooling and warming ramps for oocytes frozen in 1.0 M ethylene glycol/PBS.

Ramp no.	Rate (°C/min)	Limit (°C)	Hold (s)	Capture interval (s) ^a	Comments
1	10	−5.0	0	30	Cooling
2	2	−8.0	0	10	Cooling and EIF ^b
3	2	−3.2	10	10	Warming and partial thawing
4	10	−7.0	0	10	Cooling
5	20	−70.0	0	10	Cooling and IIF ^b
6	10	−5.0	0	30	Warming and thawing
7	10	+20	60	20	Warming and thawing

^aTime interval in seconds between the storage of the successive images on the hard drive. ^bEIF and IIF refer to extracellular and intracellular ice formation respectively.

of cell volume versus the time in the hypertonic medium (Kleinhans 1998). The classical Lp equation is $dV/dt = LpARTN(1/V - 1/V_e)$, where V is the absolute volume of cell water, t is time, and A , R , T , and N are the area of the cell surface, the gas constant, the absolute temperature, and the osmoles of solute in the cell. V_e is the volume of cell water after equilibrium has been attained. The water volume equals the measured cell volume minus the volume of the cell solids and bound water.

The Lp of the normal mouse oocytes, which lack detectable AQPs, is between 0.4 and 0.8 $\mu\text{m}^2/\text{min per atm}$ (Leibo 1980, Edashige *et al.* 2003). If AQP1 and AQP3 are expressed in the plasma membrane, the Lp will be increased. If it was increased to more than 1.5 $\mu\text{m}^2/\text{min per atm}$, we considered that AQP1 and AQP3 had been expressed significantly.

Vitrifying oocytes/embryos and shipment

Oocytes expressing AQP1 or AQP3 were vitrified at Kochi University Japan. EG was diluted to 20 and 40% (v/v) with FS solution, which is PB1 medium containing 30% (w/v) Ficoll 70 (average molecular weight 70 000; GE Healthcare, Bio-Sciences AB, Uppsala, Sweden) plus 0.5 M sucrose, to make EFS20 and EFS40 respectively (Kasai *et al.* 1990, Zhu *et al.* 1993). EAFS10/10 consisted of 10% (v/v) EG and 10.7% (w/v) acetamide dissolved in a stock consisting of 30% (w/v) Ficoll 70 and 0.5 M sucrose in PB1. The final concentrations of sucrose and Ficoll 70 are 0.4 M and 24% (w/v) (Pedro *et al.* 1997, Seki & Mazur 2008) (The letters E, A, F, and S stand for EG, acetamide, Ficoll, and sucrose. The 10/10 refers to the volume % of EG and acetamide; 20 and 40 are the volume % of EG.).

The following solutions were successively aspirated into 1/4 ml straws: a 60 mm column of PB1 medium containing 0.5 M sucrose, a 20 mm air bubble, a 5 mm column of vitrification solution (EAFS10/10 for normal or water-injected oocytes and for AQP1-expressing oocytes; EFS40 for AQP3-expressing oocytes and for normal or water-injected morulae), a 5 mm air bubble, and a 12 mm column of vitrification solution containing the oocytes or embryos. Normal oocytes, water-injected oocytes, and AQP1-expressing MII oocytes were exposed to EAFS10/10 for 2 min at 25 °C. AQP3-expressing oocytes and normal morulae were exposed to EFS40 for 1 min at 25 °C. The morulae that had been injected with ds-Aqp3 RNA as zygotes were suspended in EFS30 for 2 min. During the exposure times, the oocytes and embryos were transferred into the previously prepared 12 mm column of appropriate

vitrification solution in each straw and the end of the straw opposite the polyvinyl alcohol plug was heat sealed. At the conclusion of the defined exposure time, the straw was placed for 3 min or more in liquid nitrogen (LN₂) vapor at −120 to −150 °C and then into LN₂. For shipment from Japan, the straws were transferred to a Taylor Wharton dry cryogenic shipper (Theodore, AL, USA) and sent to Knoxville by express mail. Upon receipt, the shipping container was checked to make certain it still contained LN₂.

For experiments in Tennessee, the vitrified oocytes or embryos in a straw were warmed rapidly in water at 25 °C and mixed with PB1 medium containing 0.5 M sucrose at 23–25 °C. Some 10 min later, the oocytes and embryos were transferred at 25 °C to PB1 lacking sucrose and then incubated in previously prepared droplets of M16 medium for some 2 h.

Experimental media and sample preparation

The experiments consisted of suspending the oocytes or embryos in a 1.0 M solution of EG in PBS, loading them on a Linkam cryostage, cooling them at 20 °C/min to −70 °C, and observing and recording the temperature at which they froze internally. Specifically, oocytes or embryos were transferred from a PB1 droplet to 1 ml Dulbecco PBS (pH 7.2) containing 1.0 M EG and 0.001% (w/v) Snomax (a commercial preparation of freeze-dried *Pseudomonas syringae*, the ice nucleating bacterium (York Snow, Victor, NY, USA). Snomax is included to minimize the supercooling of the suspending medium. Zeuthen & Klaerke (1999) have reported that an external pH below 6.0 causes the closing of AQP3 pores (but not AQP1 pores) in *Xenopus* both with respect to water and glycerol. Therefore, in one experimental group, we adjusted the pH of PBS to 5.7 with 1 M HCl.

Then, 15 min later, after adding oocytes or embryos to the experimental medium, a 1.5 μl droplet of the medium in each of the experimental groups was placed in the center of a 50 or 75 μm -thick spacer in a Linkam quartz sample cuvette, the oocytes/embryos pipetted in a minimum volume into that droplet, and a coverglass applied (The oocytes/embryos are 75 μm in diameter; nevertheless, the 50 μm spacer was used in most runs because the thinner layer of frozen medium yielded much better optics. With respect to IIF, the response of the oocytes/embryos was no different than that with the 75 μm spacer.). The sample cuvette was then inserted in a Linkam BCS 196 cryostage (Linkam Scientific Instruments, Waterfield, UK) and the freezing–thawing run initiated. The stage was

attached to a Zeiss microscope, and the sample observed with a 20× objective for a displayed magnification of 500×. The images were displayed at 40 frames/s on a monitor and captured on a computer hard drive at desired intervals as short as 1 image/10 s. In a few cases where changes occurred rapidly, the computer monitor was videotaped at 40 frames/s.

The Linkam cryostage, freezing protocols, and ramps

Using liquid nitrogen vapor for cooling and electrical resistors for heating, the Linkam cryostage with its associated Pax-it 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 5. The procedure was as follows: the oocytes/embryos were cooled rapidly to -5.0°C and cooled slowly to -8.0°C (Ramp 1 and 2). EIF occurred at a mean of -7.0°C . The sample was warmed (Ramp 3) to -3.2°C , which is just below the melting point of the medium. Most, but not at all, of the external ice melted. Re-cooling was then initiated in Ramp 4 after a 10 s 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 re-cooling began. If Ramp 3 was omitted, the EIF temperature was about 20°C higher than when the warming or an equivalent hold time were included (Mazur *et al.* 2005a).

Statistical analysis

Error figures in tables and error bars in graphs are S.E.M. (S.D. of the mean). Tests of significance were carried out by one-way ANOVA using GraphPad Software's InStat, V. 3.02 (La Jolla, CA, USA) followed by the Tukey–Kramer multiple comparison test. Usually, one to five oocytes or morulae were frozen in a single run and n is the total number subjected to given treatments.

Declaration of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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